FULL PAPER

The Use of Directing Groups Enables the Selective and Efficient Biocatalytic Oxidation of Unactivated Adamantyl C–H Bonds

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Abstract: Adamantane, 1- and 2-adamantanol and 2-adamantanone, were poor substrates for the cytochrome P450 enzyme CYP101B1. The CYP101B1 catalysed oxidation of 1-adamantyl methyl ketone, and methyl 2-(1-adamantyl acetate), were more active generating a majority of the 4-hydroxy metabolite. Substrate engineering using acetate and isobutyrate ester directing groups significantly increased the affinity, activity and coupling efficiency of CYP101B1 for the esters compared to the parent adamantanols, resulting in enhanced product formation rates (720 to 1350 nmol.(nmol-P450)$^{-1}$.min^{-1}). The majority of the turnovers were selective for C–H bond hydroxylation with 4-hydroxy-1-adamantyl isobutyrate and the 5-hydroxy-2-adamantyl esters being generated as the sole majority product, 97%, with high total turnover numbers, ranging from 4130 to 16500. In addition N-(1-adamantyl)acetamide, was oxidised by CYP101B1 whereas 1-adamantylamine, was not. Whole-cell biocatalytic reactions were used to generate the products in good yield. Overall the use of ester protecting groups and the modification of the amine to an amide enabled the more efficient and selective biocatalytic oxidation of adamantane frameworks.

Introduction

The application of cytochrome P450 (CYP) enzymes as biocatalysts for the regio- and stereo-selective insertion of an oxygen atom into the chemically inert carbon-hydrogen bonds of saturated hydrocarbons is of great interest due to the difficulties associated with these reactions.[1] Many monooxygenase enzymes have been investigated as potential biocatalysts for the oxidation of organic molecules with those from bacteria and fungi being the most promising.[2] The monooxygenase activity of CYP enzymes requires two electrons that are usually derived from NAD(P)H and these are delivered one at a time, when needed, to the CYP enzymes by electron transfer proteins.[3] The identification and application of highly active systems with known electron transfer partners coupled with a well-defined substrate range greatly facilitates biocatalytic C–H bond oxidation of organic molecules.[4] For example CYP102A1, from *Bacillus megaterium,* which is highly active for fatty acid substrates, has been modified via protein engineering to facilitate the selective oxidation of aliphatic and aromatic C–H bonds in many other substrates.[1e, 4b, 4c, 5] Class I CYP enzymes, such as CYP101A1 (P450cam), from a *Pseudomonas* sp., whose electron transfer systems consist of a flavin-dependent ferredoxin reductase and a ferredoxin, are also capable of high monooxygenase activities and the selective oxidation of hydrocarbons.[4f, 6] The wild-type enzymes and mutant forms of various class I CYPs have been reported to be biocatalysts for the oxidation of gaseous alkanes, hydrocarbons, terpenes and alkylbenzenes.[4a, 6b, 7]

Novosphingobium species are able to degrade a variety of hydrocarbons and therefore the oxygenase enzymes from this bacterium have potential applications as biocatalysts.[8] The bacterium *Novosphingobium aromaticivorans* DSM12444 contains many monooxygenase and dioxygenase enzyme coding genes.[8a] These include the P450 enzymes CYP101B1, CYP101C1, CYP101D1 and CYP101D2, which are related to CYP101A1. The CYP101 family of P450 enzymes are able to bind norisoprenoids, camphor and certain terpenoids with high affinity.[4f, 9] A class I electron transfer system from *N. aromaticivorans*, consisting of a flavin-dependent ferredoxin reductase, ArR, and a [2Fe-2S] ferredoxin, Arx, has been used to support the activity the CYP101B1, C1, D1 and D2 enzymes.[2c, 4f, 9a-c] Whole-cell systems containing these CYP enzymes coupled to, ArR and Arx, are capable of product formation on the gram-per-litre scale in shake flasks.[9a] CYP101B1 is a highly efficient biocatalyst for the C–H bond oxidation of norisoprenoids and monoterpenoid acetates.[9c, 10] The acetate side chain of the esters resembles the butenone side chain of norisoprenoids allowing substrate binding with high affinity which holds the substrate in place for efficient and selective oxidation. These oxidation reactions proceed with high catalytic activity, turnover number and coupling efficiency.[10] Therefore ester protecting groups, such as acetate which are simple to add to and remove from alcohols, and the amide equivalents of amines could act as chemical auxiliaries and directing groups to enable the improved oxidation of low affinity substrates as described by Griengl and others.[11]

Substituted tricyclic adamantane compounds are important chemicals which are used in the production of functional materials, polymers and pharmaceuticals.[12] Current chemical methods require harsh oxidants, are poorly selective and are prone to overoxidation.[12] A number of microbial strains are capable of slowly oxidising adamantane, **1**, and its derivatives and several CYP enzymes including CYP101A1 have been reported to hydroxylate adamantane and 2-adamantanone.[12-13] Here we investigate the biocatalytic oxidation of substrates with an adamantane skeleton by CYP101B1. We demonstrate that the enzyme is capable of acting as an efficient biocatalyst for the

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oxidation of acetate, acetamide and isobutyrate protected adamantyl derivatives.

Results

The oxidation adamantane, 2-adamantanone and adamantanols by CYP101B1

Tricyclic substrates, including adamantane, **1**, 1- and 2-adamantanol, **2** and **3**, and 2-adamantanone, **4**, were analysed with CYP101B1 (Fig. 1). The addition of 1-adamantanol, **2**, and 2-adamantanol, **3**, induced significant type I spin state shifts with CYP101B1 (55-60%). 2-Adamantanone, **4**, and adamantane, **1**, engendering lower shifts (20-30%, Table 1, Fig. S1). However 1-adamantanol, **2**, bound with lower affinity to CYP101B1 than 2-adamantanone, **4** (Table 1, Fig. S2). Low solubility and weak binding prevented the accurate measurement of the dissociation constants of 2-adamantanol, **3**, and adamantane, **1**.

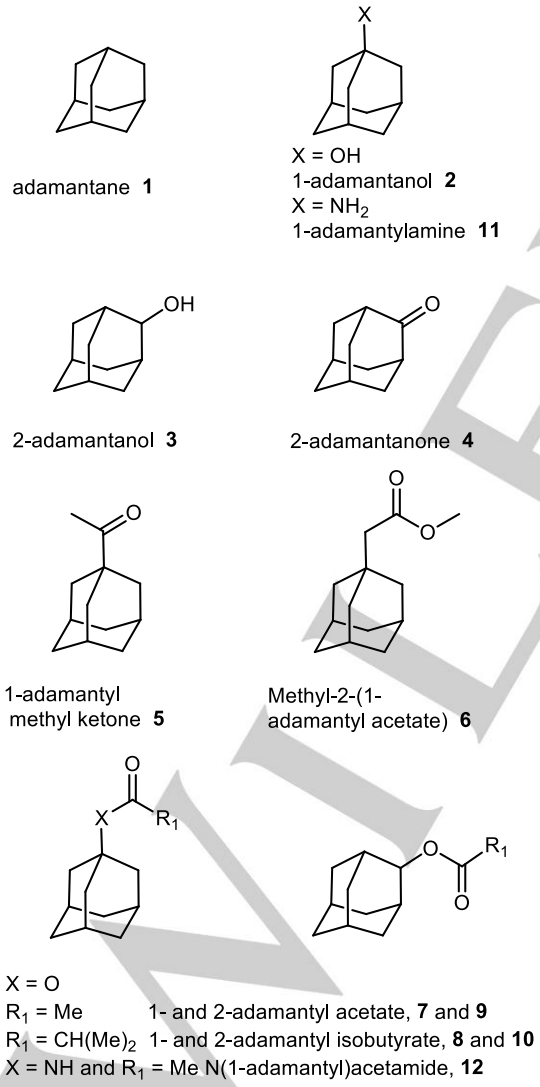


Figure 1. Substrates tested with CYP101B1 in this study.

The product formation rate and the coupling efficiency, which is a measure of the productive use of the NADH reducing equivalents, arising from the CYP101B1 catalysed turnover of adamantane, **1**, and the adamantanols, **2** and **3**, were paltry resulting in low levels of metabolite formation (Table 2). The product formation activity of 2-adamantanone, **4**, with CYP101B1 was the highest (PFR 103 nmol.(nmol-P450)$^{-1}$.min^{-1}; henceforth abbreviated to min^{-1}) being tenfold more active than the next best tricyclic compound (1-adamantanol, **2**). By way of comparison, the activity of CYP101B1 with 2-adamantanone was half that of the monoterpenoids, (1*R*)-(+)-camphor, 1,8-cineole and (+)-fenchone and ten-fold lower than those of norisoprenoids and monoterpenoid acetates.[14]

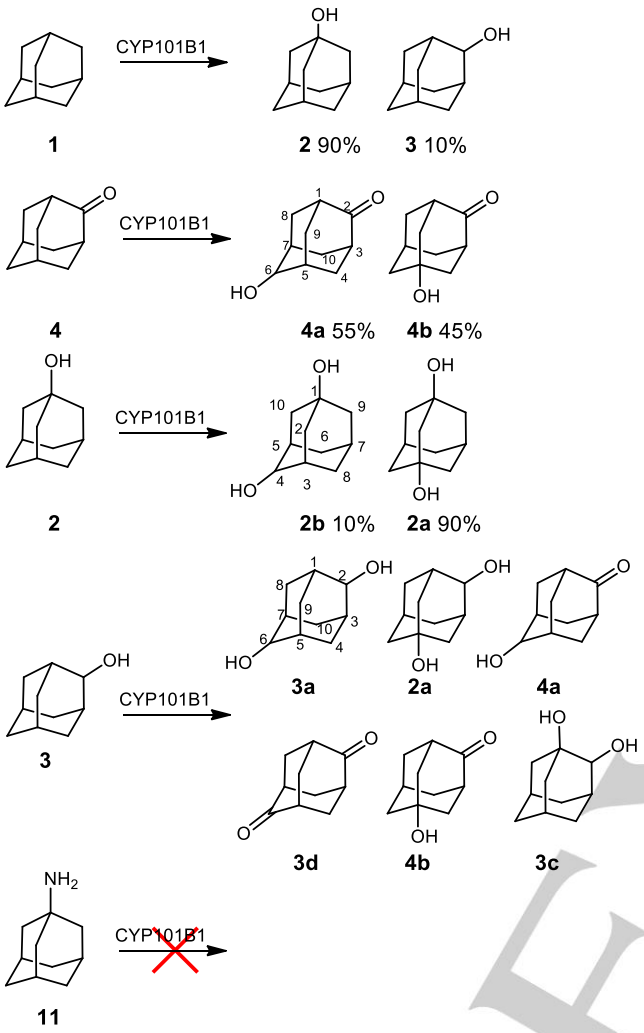
Table 1. Substrate binding, kinetic and coupling data for CYP101B1 with adamantane and related substrates. Steady state turnover activities were measured using a ArR:Arx:CYP101B1 concentration ratio of 1:10:1 (0.5 µM CYP enzyme, 50 mM Tris, pH 7.4).

	HS[a]	K_d[b] (µM)	N[c]	PFR[d]	C[e] %	TTN[f]
1	30%	-[g]	140 ± 10	0.2 ± 0.1	0.11	n.d.
2	60%	711 ± 26	415 ± 55	11 ± 4	3	n.d.
3	55%	-[g]	563 ± 10	11 ± 3	2	n.d.
4	35%	548 ± 15	669 ± 7	104 ± 9	16	n.d.
5	70%	32 ± 1.0	923 ± 31	473 ± 22	51	6690
6	95%	0.2 ± 0.03	1120 ± 4	704 ± 28	63	16500
7	90%	3.9 ±0.1	1300 ± 16	821 ± 27	63	6050
8	95%	1.6 ± 0.1	1360 ± 7	1350 ± 22	99	4130
9	90%	1.9 ± 0.1	1170 ± 11	877 ± 18	75	11500
10	90%	0.6 ± 0.04	1060 ± 6	722 ± 10	68	7090
11	<5%	-[g]	35 ± 0.3	n.p.	n.p	n.p.
12	50%	55 ± 5	955 ± 25	166 ± 9	17	780

[a] HS: the percentage of high spin state heme iron induced by substrate binding. [b] K_d: the dissociation constant, µM [c] N: NADH turnover rate. [d] PFR: product formation rate. The rates are reported as mean ± S.D. ($n \geq 3$) and given in nmol.(nmol-P450)$^{-1}$.min^{-1}. [e] C: Coupling efficiency, which is the percentage of NADH consumed in the reaction that led to the formation of products. [f] TTN: The total turnover numbers (TTN) were determined using assays set up with the same ratio of enzymes as those above but with 0.1 µM CYP enzyme, 2 mM substrate and 4 mM NADH (theoretical maximum value 20,000). [g] Not calculated due to low detector response to addition of substrate or low substrate solubility interfered with the titration. n.p. no detectable product. n.d. not determined.

Adamantane, **1**, was oxidised by CYP101B1 to form predominantly 1-adamantanol, **2** (90%) with 2-adamantanol, **3**, being observed as a side product (10%, Scheme 1). Due to the low levels of product formation these were identified via GC-coelution experiments with authentic chemical standards.

CYP101B1 oxidation of 2-adamantanone, **4**, generated two products (both *m/z* = 166.1, Fig. S3(a)) and these were synthesised using a whole-cell oxidation system.[9a] After separation by silica gel chromatography the hydroxylated metabolites were identified by their mass fragmentation pattern and NMR spectra as 5-hydroxy-2-adamantanone, **4a** (45%) and 6-hydroxy-2-adamantanone, **4b** (55%, Scheme 1, Fig. S3(a), Fig. S4, Fig. S5(a), Fig. S5(b)).[13b]



Scheme 1. The major products formed from CYP101B1 turnovers of adamantane, adamantanols and 2-adamantanone. No product was formed with 1-adamantylamine.

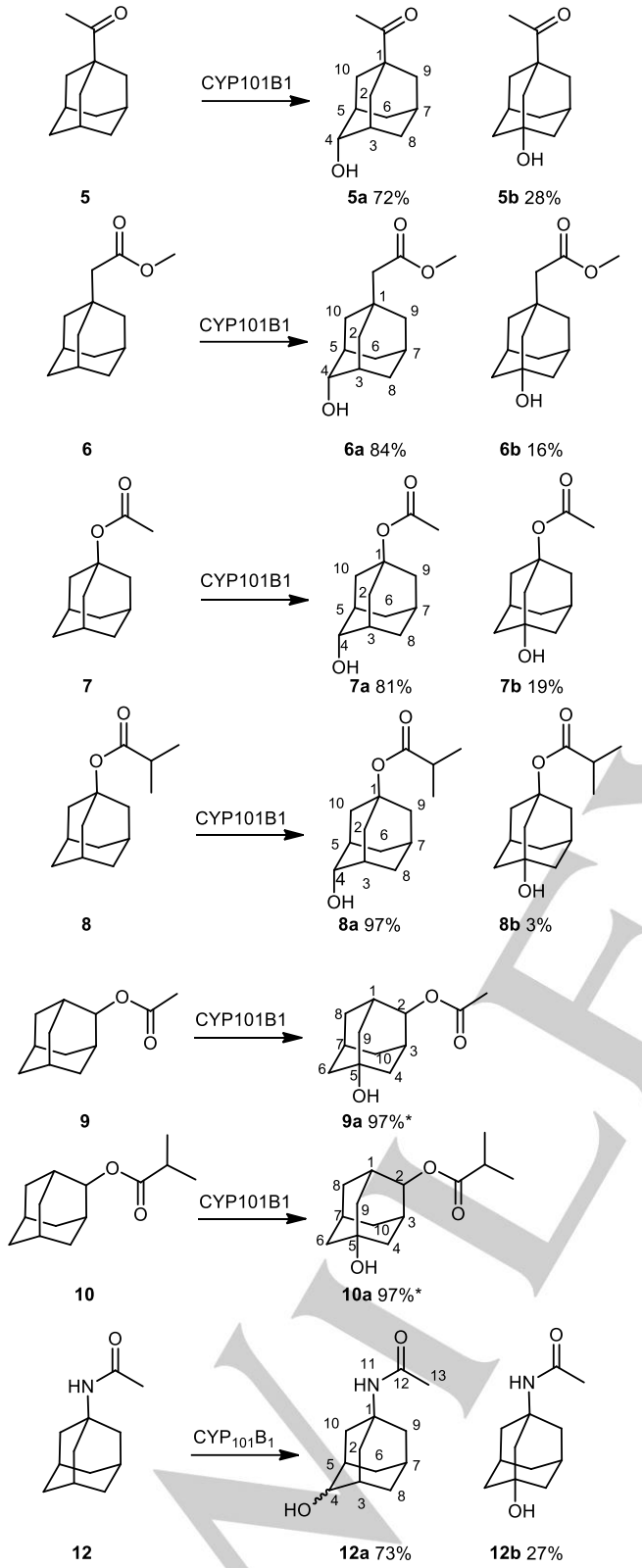
The oxidation of 2-adamantanol, **3**, was more complicated generating multiple products and the assessment of the product distribution was hampered by overlapping retention times. Whole-cell oxidation of 2-adamantanol produced six metabolites, two of which were identified as 6-hydroxy-2-adamantanone, **4a**, and 5-hydroxy-2-adamantanone, **4b**, by coelution with the products from the turnovers of 2-adamantanone, **4**, and their MS fragmentation patterns (*m/z* = 166.1, Fig. S4). 1,4-Adamantanediol, **2b**, was also identified after isolation and characterisation by NMR and MS (Fig. S4 and experimental section in supporting information). Another product was tentatively assigned as 2,6-adamantanediol, **3a**, based on its MS analysis and the subsequent formation of the further oxidation product 6-hydroxy-2-adamantanone, **4a** (Scheme 1, Fig. S3(a), Fig. S4). We also observed the formation of a small amount of a metabolite which had an MS fragmentation pattern characteristic of 1,2-adamantanediol, **3c** (Fig. S4).[15] By lengthening the duration of the whole-cell turnovers we observed the formation of 2,6-adamantandione, **3d** (*m/z* = 164.1, Fig. S4).

Oxidation of 1-adamantanol, **2**, generated a major product which was assigned as 1,3-adamantanediol, **2a**, from its mass spectrum (Scheme 1, Fig. S3(a), Fig. S4).[13c] An additional minor product which coeluted with 1,4-adamantendiol, **2b**, was also detected. The low levels of product formation prevented further more detailed characterisation.

The oxidation adamantane ketone, ester, amine and amide derivatives by CYP101B1

CYP101B1 binds norisoprenoid substrates which contain a butenone side chain with high affinity. The ketone moiety in these molecules has been shown to be important for tight binding.[10] In order to investigate if appending a ketone containing side chain to the tricyclic adamantane skeleton would improve substrate binding to CYP101B1 we tested 1-adamantyl methyl ketone, **5**, (Fig. 1). Addition of this substrate to CYP101B1 induced a shift in the spin-state to the high-spin form of 70%. While this was only slightly superior to the shift induced by 1-adamantanol, **2**, the binding affinity was an order of magnitude higher (K_d = 32 ± 1.0 µM, Table 1, Fig. S2). The NADH oxidation activity and the product formation rate of 473 ± 22 min^{-1} were also greater than that of 2-adamantanone, **4**. The 4-fold increase in the activity predominantly arose due to an improvement in the coupling efficiency from 16% to 51% (Table 1). Two products, in an approximate 3:1 ratio, were detected in the GC-MS analysis of the turnovers (Fig. S3(b)). These were both identified as monohydroxylated monooxygenase products from their MS data (m/z = 194.05, Fig. S4). The whole-cell oxidation system was used to generate both products in more substantial quantities and these were extracted and separated by silica chromatography (Fig. S3(b)). NMR spectroscopy was used to identify the major and minor products as *trans*-4-hydroxy-1-adamantyl methyl ketone, **5a**, and 3-hydroxy-1-adamantyl methyl ketone, **5b**, respectively (Scheme 2, Fig. S5(c), Fig. S5(d)).

Given the improvement observed in the binding affinity and activity of 1-adamantyl methyl ketone, **5**, over 2-adamantanone, **4**, and as monoterpenoid acetates have been reported to have a high binding affinity to CYP101B1 we tested adamantyl substrates which contain ester groups.[10] Methyl 2-(1-adamantyl acetate) **6**, induced an almost complete shift of CYP101B1 to the high spin form and bound with thirty-fold higher affinity than 1-adamantyl methyl ketone, **5** (Table 1, Fig. S1, Fig. S2). The NADH oxidation activity and coupling efficiency of the turnovers were also high (Table 1) resulting in a product formation rate of 704 ± 28 min^{-1}. The turnover was also more selective than that of 1-adamantyl methyl ketone, **5**, generating two products in the ratio 84:16 (Fig. S3(c)). Both had masses consistent with them being alcohols (*m/z* = 224.05, Fig. S4). After isolation the major product was identified using NMR as methyl 2-(*trans*-4-hydroxy-1-adamantyl) acetate, **6a** (Fig. S5(e)). The minor product was characterised as methyl 2-(3-hydroxy-1-adamantyl) acetate, **6b** (Scheme 2, Fig. S5(f)).



Scheme 2. The products formed from CYP101B1 turnovers of adamantane ester and ketone analogues. * there is a small, ≤ 3%, amount of an uncharacterised minor metabolite with a MS consistent with a hydroxylation product detected in these turnovers.

We next assessed if substrate engineering of 1- and 2-adamantanol, **2** and **3**, using an ester protecting group could improve the binding affinity and therefore the turnover activity. The acetate and isobutyrate esters of both adamantanols were synthesised, **7**-**10**, characterised and tested as substrates for CYP101B1 (see experimental section in supporting information, Fig. 1, Fig. S6).

Addition of both 1-adamantyl acetate, **7**, and 1-adamantyl isobutyrate, **8**, induced an almost complete shift of CYP101B1 to the high spin form (90%, Fig. S1). The affinity of CYP101B1 for both was high with that of the isobutyrate ester, 1.6 μM exceeding that of the acetate equivalent, 3.9 μM (Table 1, Fig. S2). The NADH oxidation rates of both were comparable, 1300 and 1360 min^{-1}, but the coupling efficiency of 1-adamantyl isobutyrate, **8**, was greater, 99%, than 1-adamantyl acetate, **7**, 63%. This resulted in a higher product formation activity for the isobutyrate ester, 1350 min^{-1} than for the acetate, 821 min^{-1} (Table 1).

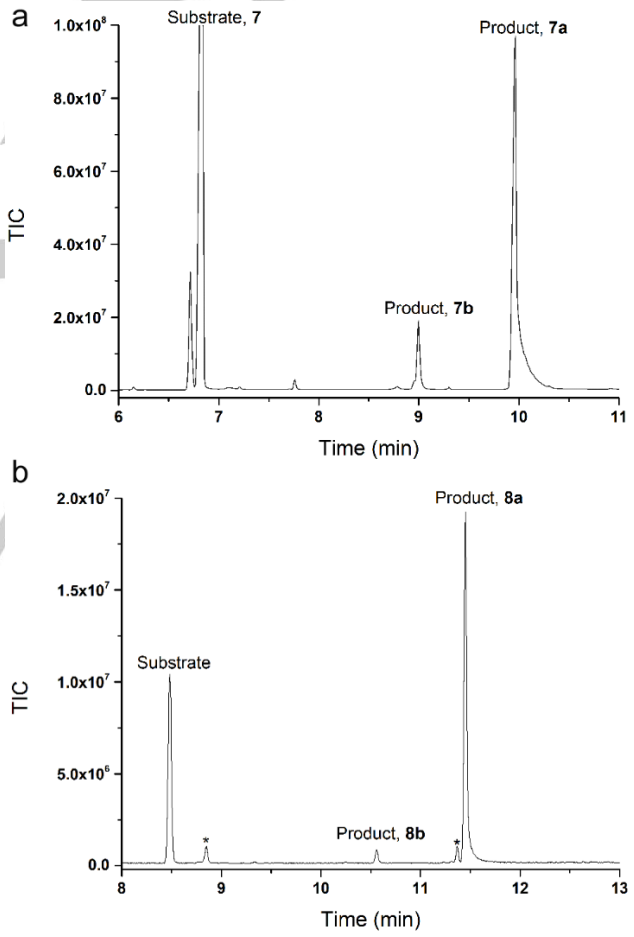


Figure 2 (a) The GC-MS analysis of the whole-cell turnover of 1-adamantyl acetate, **7**, by CYP101B1 analysed after 3 hours; 1-adamantyl acetate, **7** (RT 6.8 min) and the products; 3-hydroxy-1-adamantyl acetate, **7b** (RT 8.9 min) and *trans*-4-hydroxy-1-adamantyl acetate, **7a** (RT 9.9 min). **(b)** The GC-MS analysis of the *in vitro* turnover of 1-adamantyl isobutyrate, **8**, by CYP101B1; 1-adamantyl isobutyrate, **8** (RT 8.6 min) and the product; *trans*-4-hydroxy-1-adamantyl isobutyrate, **8a** (RT 11.5 min). The minor peak (3%) at 10.6 min has a MS and retention time consistent with it being 3-hydroxy-1-adamantyl isobutyrate, **8b**. Impurities are labelled (*), in figure 2 (a) the peak to the left of the substrate results is from 1-adamantanol from hydrolysis of the ester substrate (not present in other turnovers; Fig. S3d).

The turnover of 1-adamantyl acetate, **7**, generated two hydroxylated products (*m/z* = 210.0, Fig. S4) in an approximate 4:1 ratio (Scheme 2, Fig. 2(a), Fig.S3(d)). The major product was isolated and identified using NMR as *trans*-4-hydroxy-1-adamantyl acetate, **7a** (Fig. S5(g)). The minor product was not isolated as a pure compound but had NMR spectra consistent with 3-hydroxy-1-adamantyl acetate, **7b** (Scheme 2). CYP101B1 catalysed oxidation of 1-adamantyl isobutyrate, **8**, generated one major hydroxylated product, 97% (*m/z* = 238.05, Fig. 2(b), Fig. S3(e), Fig. S4) which was purified and characterised as *trans*-4-hydroxy-1-adamantyl isobutyrate, **8a** (Scheme 2, Fig.S5(h)). A small peak was detected via GC-MS which had a mass spectrum and retention time consistent with that of 3-hydroxy-1-adamantyl isobutyrate but was not isolated in sufficient yield from the whole-cell turnovers to be fully characterised (Fig. 2(b)).

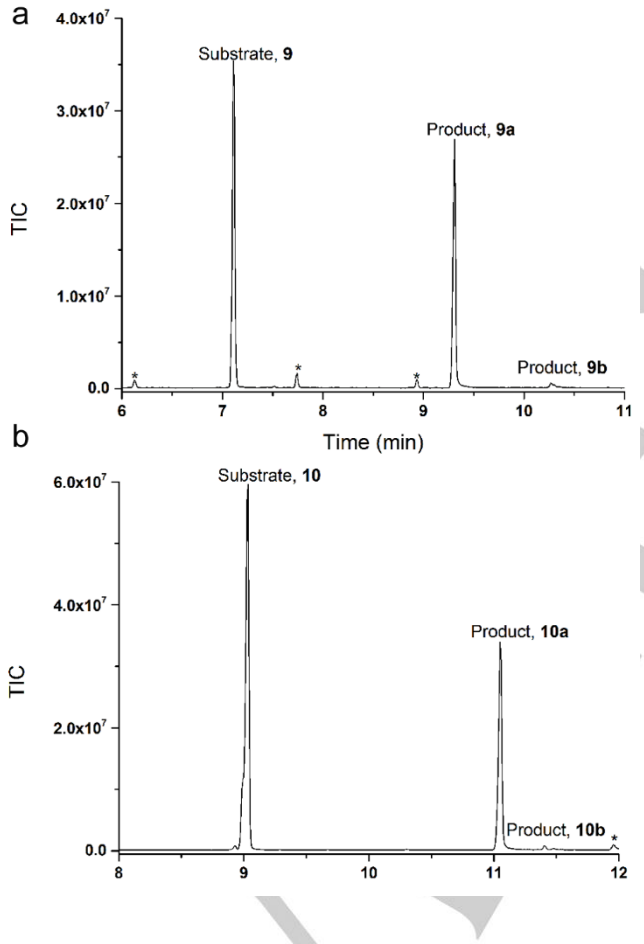


Figure 3. (a) The GC-MS analysis of the *in vitro* turnover of 2-adamantyl acetate, **9**, by CYP101B1; 2-adamantyl acetate, **9** (RT 7.1 min) and the product; 5-hydroxy-2-adamantyl acetate, **9a** (RT 9.4 min). **(b)** The GC-MS analysis of the *in vitro* turnover of 2-adamantyl isobutyrate, **10**, by CYP101B1; 2-adamantyl isobutyrate, **10** (RT 8.9 min) and the product; 5-hydroxy-2-adamantyl isobutyrate, **10a** (RT 11.1 min). Impurities are labelled (*). Both turnovers contained a minor product (**9b** and **10b**) at a higher retention time with an MS consistent with a hydroxylation product.

The addition of both 2-adamantyl acetate, **9**, and 2-adamantyl isobutyrate, **10**, to CYP101B1 induced a spin state of 90% high-spin (Fig. 1, Fig. S1). In line with the large spin state shift both substrates bound to the enzyme with high affinity. The dissociation constants were tighter than their 1-adamantyl ester counterparts (1.9 μM and 0.6 μM, Table 1, Fig. S2). The NADH oxidation rates and coupling efficiencies of both adamantyl esters were high (Table 1). The coupling efficiency of the turnover of 2-adamantyl acetate, **9**, was greater resulting in a product formation rate of 877 min^{-1} which was similar to that of 1-adamantyl acetate, **7** (Table 1).

GC and GC-MS analysis of the turnovers of both of these substrates showed the presence of a single major, hydroxylated product, ≥97 % (*m/z* = 210.1 and 238.15, respectively, Fig. 3, Fig. S3(f), S3(g), Fig. S4). After whole-cell oxidations and purification the products were established as 5-hydroxy-2-adamantyl acetate, **9a** (Scheme 2, Fig. S5(i)) and 5-hydroxy-2-adamantyl isobutyrate, **10a**, using their NMR spectra (Fig. S5(j)). The lower coupling and NADH oxidation rate of CYP101B1 with 2-adamantyl isobutyrate, **10**, resulted in a product formation rate of 722 min^{-1} (Table 1). The minor product in each turnover, <3%, was observed at a higher retention time and had mass spectra consistent with a hydroxylation product but these could not be characterised further.

Finally we tested the nitrogen containing compounds 1-adamantylamine, **11** (amantadine) and N-(1-adamantyl)acetamide, **12**, with CYP101B1 (Fig. 1). 1-Adamantylamine, **11**, did not induce any significant shift in the spin state of CYP101B1 enzyme (Fig. S1). The NADH oxidation activity was low and no product formation was detected (Table 1, Scheme 1, Fig. S3(i)). Converting the amine to the acetamide resulted in a significant increase in the spin state of CYP101B1 upon substrate binding (50%, Table 1). However, the binding affinity, as measured by the dissociation constant, was lower than those of the ester derivatives and 1-adamantyl methyl ketone, **5** (Table 1). The rate of NADH oxidation in the CYP101B1 system brought about by N-(1-adamantyl)acetamide, **12**, was high but the product formation activity was moderate, 166 min^{-1}, due to lower coupling efficiency of the reducing equivalents to metabolite formation (Table 1).

Two hydroxylated products (*m/z* = 209.1) were identified by GC-MS analysis in an approximate 3:1 ratio (Scheme 2, Fig. 4, Fig. S3). These were generated in greater quantities via a whole-cell oxidation system. The two products were isolated in a pure form after extraction and silica gel chromatography (the products are highly soluble in aqueous solution).[16] These were identified as 4-hydroxy-N-(1-adamantyl)acetamide, **12a**, and 3-hydroxy-N-(1-adamantyl)acetamide, **12b**, using their NMR spectra (Scheme 2, Fig. S5(k), Fig. S5(l)). Due to overlapping peaks we were unable to definitively assign the stereochemistry of the 4-hydroxy product (Fig. S5(k)).

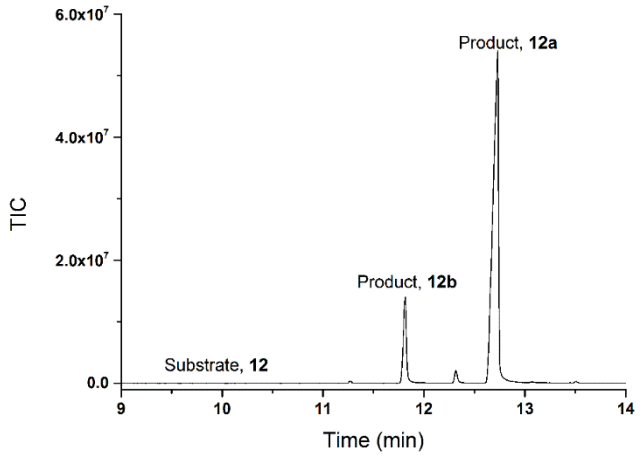


Figure 4. The GC-MS analysis of the whole-cell turnover of N-(1-adamantyl)acetamide, **12**, by CYP101B1. The substrate N-(1-adamantyl)acetamide, **12** (RT 9.75 min) and the products; 3-hydroxy-N-(1-adamantyl)acetatamide, **12b** (RT 11.8 min) and 4-hydroxy-N-(1-adamantyl)acetamide, **12a** (RT 12.8 min).

The turnover data (PFR and coupling efficiency) indicate that CYP101B1 is a good biocatalyst for synthetic applications with these adamantane ketone, ester and amide derivatives (Table 1). The standard *in vitro* turnover assays have turnover numbers limited by the amount of NADH added, which is lower than the substrate concentration. Therefore in order to measure the efficiency of the enzyme over a longer period of time we measured the total turnover number (TTN) with the best substrates using lower concentrations of the enzyme and an excess of reducing equivalents. *In vitro* the TTN of CYP101B1 with the esters and ketones, **5** to **10**, were high, ranging from 4130 to 16500 (Table 1). These were comparable to those obtained with norisoprenoids and monoterpenoid acetates (5240-8660) and show that the CYP101B1 enzyme is not rapidly deactivated by these substrates.[10] The TTN of the isobutyrate esters, **8** and **10**, were lower than those of the acetate esters, **7** and **9** (Table 1). The highest TTN was observed with methyl 2-(1-adamantyl acetate) **6**. The TTN of the N-(1-adamantyl)acetamide, **12**, was lower in line with the lower coupling efficiency observed with this substrate and would require further optimisation (Table 1).

The products generated using low cell density, shake flask. whole-cell oxidations (200 mL scale) were the same as those from the *in vitro* turnovers, Fig. 2-4, Fig. S3). The product yields and conversions from these oxidation reactions using low cell density cultures were also high. Despite the lower *in vitro* product formation rates and coupling efficiency of N-(1-adamantyl)acetamide, **12**, all of the added substrate (3 mM, 135 mg in 200 mL) was converted after 20 hours in the whole-cell turnovers. For the ester and ketone compounds the conversion of the substrate into product ranged from 40 – 80% of the added substrate (3-4 mM in 200 mL) in the shake flask reactions which corresponds to an excess of 4000 turnovers (based on a P450 concentration of 0.33 µM in the cell growths for the whole-cell turnovers).[9a] The yield of total purified products after silica chromatography ranged from 85 to 255 mg per litre for the esters and ketones. The conversions and yields could be significantly improved using higher density cell cultures in a fermentor with more control of the pH, nutrients combined with a substrate feeding/product removal regime.

Discussion

CYP101B1 is able to oxidise a broad range of substrates and optimal catalytic efficiency is achieved for norisoprenoids, monoterpenoid acetates and now adamantyl esters. All of these have a carbonyl group on a side chain attached to substituted ring systems. These substrates are hydroxylated with high activity, total turnover numbers and regioselectivity. The oxidation of adamantane derivatives is of great interest as they are building blocks for more complex drug molecules and polymers. Microbial oxidative biotransformations of adamantane, **1**, 2-adamantanone, **4**, adamantanols, **2** and **3**, and related substrates including the acetate esters are possible using different bacteria, including *Streptomyces,* and fungi including *Basidiomycota*, *Absidia cylindrospora* and *Beauveria (Sporotrichum*) *sulfurescens*.[12, 13c] 16-17] Certain P450 enzymes and their mutants have also been reported to oxidise adamantane based substrates including CYP101A1 and CYP101D2 though at lower activities than the turnover of the esters reported here.[13a, 13b, 13d]

The importance of the ketone containing side chain for efficient biocatalysis with CYP101B1 can be clearly seen by comparing the binding properties and activities of the adamantanols, **2** and **3**, and 2-adamantanone, **4**, with the adamantyl acetates and isobutyrates, **7**-**10**. The turnovers of 2-adamantanone, **4**, and 2-adamantanol, **3**, resulted in the formation of low levels of multiple products. Oxidation of adamantane, **1**, and 1-adamantanol, **2**, were selective but the activities were low. The oxidation of the esters proceeded more efficiently and was more selective. Modification of the ester substituent was also observed to have an effect on the binding and catalytic properties. For example methyl 2-(1-adamantyl acetate), **6**, bound with the highest affinity to CYP101B1 and the isobutyrate esters, **8** and **10**, bound more tightly than the equivalent acetates, **7** and **9**. The oxidation of 1-adamantyl isobutyrate, **8**, was also selective for the formation of the *trans*-4-hydroxy product, **8a**, whereas other substituents at the equivalent positions led to the production of the 3-hydroxy metabolite as a significant minor product (16 – 28%). It is of note that the selectivity of the isobutyrate ester, **8**, was higher than that of the acetate ester, **7**. Furthermore the selectivity of ester oxidation has been altered compared to 1-adamantanol, **2**, oxidation by CYP101B1 which favoured oxidation to 1,3-adamantanediol, **2a**. Therefore presence and the size of the ester group must affect the orientation of the substrate in the enzyme active site. The lower TTN of the isobutyrate esters despite the higher product formation activity may be in part due to lower solubility and requires further optimisation.

For the 2-adamantyl acetate and isobutyrate substrates, **9** and **10**, bond abstraction occurs predominantly at the more

reactive bridgehead position to produce the 5-hydroxy metabolite. For 2-adamantanone, **4**, and 2-adamantanol, **3**, significant levels of oxidation were also observed at the methylene groups at C6. Overall the ketone containing side chain appeared to hold the adamantyl tricyclic ring in such a position that only certain C–H bonds are attacked compared to the parent adamantanols which presumably bind in multiple orientations or are mobile in the enzyme active site. For the adamantyl substrates with ester and amide substituents at the bridgehead carbon (**5**-**8** and **12**) oxidation to the *trans*-4-hydroxy was always favoured over the more reactive tertiary position. In addition no product corresponding to a *cis*-4-hydroxy species was detected in any of the substrate turnovers.

1-Adamantylamine, **11**, and its derivatives e.g. rimantadine and adapromine are drug molecules which are used as anti-virals.[18] The N-(1-adamantyl)acetamide, **12**, framework is also found in many drugs. e.g. tromantadine.[19] The selective functionalisation of the acetamide derivative could generate drug metabolites for activity studies or could be used to selectively add functional groups for the development of new drugs with improved properties. The lower coupling efficiency and therefore product formation activity and total turnover numbers of the acetamide versus the ester requires further investigation but could be due an alternative binding mode as reflected in the lower spin state shift induced by the acetamide. Despite the lower turnover activity the substrate was efficiently converted using the whole-cell oxidation system and the reduced isolated yield of product could be improved using a different extraction method for the more water soluble products.[16]

The selective oxidation of the adamantyl esters with such high activity by CYP101B1 would be useful for synthetic chemistry. Acetate, acetamide, isobutyrate and other ester or amide groups could be used as directing groups to improve activity and tune the selectivity without protein engineering. The crystal structure of CYP101B1 has not yet been solved but using ester and amide groups we have been able to improve the activity and selectivity of oxidation with a range of adamantyl substrates. The *in vitro* activity and total turnovers achieved with CYP101B1 are significantly greater than those reported for the majority of other P450 systems. Large quantities of the products were generated (up to 3 mM conversion of all of the added substrate, ~680 mg L^{-1}) using the whole-cell *E. coli* oxidation system at low cell densities in shake flask without optimisation. This is encouraging for the scale-up of enzyme activity in the future.

Conclusions

CYP101B1 is able to efficiently oxidise adamantane derivatives including adamantyl acetates and isobutyrates, **7**-**10**, 1-adamantyl methyl ketone, **5**, and methyl 2-(1-adamantyl acetate) **6**. It was also able to oxidise N-(1-adamantyl)acetamide, **12,** whose structural framework is a compnonent of a class of drug molecules. The oxidation of adamantane, **1**, 2-adamantanone, **4** and adamantanols, **2** and **3**, was less efficient and was unselective and 1-adamantylamine, **11**, was not a substrate for the enzyme. The modification of alcohols and amines by the addition of an ester or amide directing group was a simple method for improving the activity and selectivity of the oxidations. Modification of the size of the ester resulted in increased binding affinity and improved the selectivity. As a result we were able to selectively oxidise unactivated C–H bonds at product formation rates of up to 1360 min^{-1} and coupling efficiencies approaching 99%. The isobutyrate ester of both adamantanols resulted in increased binding affinity compared to the acetate. In the case of 1-adamantyl isobutyrate, **8**, this resulted in increased activity, coupling efficiency and selectivity. When incorporated into a whole-cell oxidation system, with the physiological electron transfer partners, ArR and Arx, CYP101B1 was capable of generating the oxidation products in higher yields. The efficient and selective turnovers catalysed by CYP101B1 will enable the development of biocatalytic routes to functionalise adamantane and other hydrocarbon derivatives.

Supporting information summary paragraph
The Experimental Section, NMR data and spectra, substrate binding analysis, GC-MS and GC chromatograms and mass spectra are available in the Supporting Information.

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Keywords: C–H bond oxidation • cytochrome P450 monooxygenases • enzyme catalysis • hydroxylation • regioselectivity

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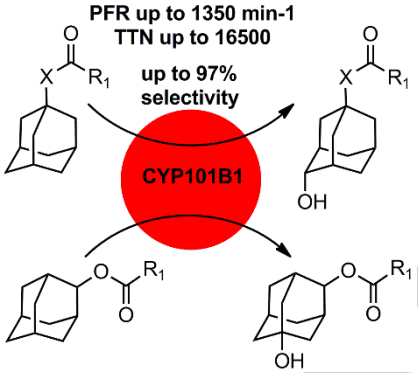
Entry for the Table of Contents

FULL PAPER

The selective biocatalytic oxidation of the unactivated C–H bonds in adamantanols and an amine analogue was achieved by modification of the substrate using ester and amide directing groups. This approach improved the binding affinity, activity and selectivity of product formation enabling the efficient generation of the hydroxylated metabolites using a monooxygase enzyme.



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The use of directing groups enables the selective and efficient P450 biocatalytic oxidation of unactivated adamantyl C–H bonds