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**(54) Title:** MUTANT MONO-OXYGENASE CYTOCHROME P-450$_{cam}$

**(57) Abstract**

A mutant of the mono-oxygenase cytochrome P-450$_{cam}$ in which the tyrosine residue at position 96 and/or the cysteine residue at positon 334 is replaced by the residue of any amino acid except phenylalanine.

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# Mutant mono-oxygenase cytochrome P-450cam

The present invention relates to a mutant of the mono-oxygenase cytochrome P-450$_{cam}$ and method of oxidising certain organic compounds with the mutant.

Mono-oxygenases catalyse the selective oxidation of non-functionalised hydrocarbons using oxygen[1], and are therefore of great interest for potential use in organic synthesis. However, progress in this area has been hampered by the difficulty in isolating sufficient quantities of enzyme and the associated electron-transfer proteins. Despite the availability of amino acid sequences of more than 150 different cytochrome P-450 mono-oxygenases, to date structural data of only three are available[2,3,4], and few have been successfully over-expressed in bacterial systems[5].

One cytochrome P-450 mono-oxygenase, which is soluble and can be expressed in sufficient quantities, is the highly specific P-450$_{cam}$ from P.putida which catalyses the regio- and stereo-selective hydroxylation of camphor (1) to 5-exo-hydroxycamphor[6]. The high resolution crystal structure of P-450$_{cam}$ has been determined[2], and since the mechanism of action of this bacterial enzyme is believed to be very similar to that of its mammalian counterparts, it has been used as a framework on which models of mammalian enzymes are based.

The nucleotide sequence and corresponding amino acid sequence of P-450$_{cam}$ have been described[5]. The location of an active site of the enzyme is known and structure-function relationships have been investigated[13,14]. Mutants of P-450$_{cam}$ have been described, at the 101 and 185 and 247 positions[15], and at the 87 position[16]. A mutant in which tyrosine 96 has been changed to phenyl alanine-96 has been described[12,17,18]. But in all these cases the papers report effects of the mutations on the mechanisms of known oxidation reactions. There is no teaching or suggestion that mutation might be used to provide biocatalysts for oxidation of different substrates.

In an attempt to find new biocatalysts, we have initiated a project which aims to redesign the active site of P-450$_{cam}$, such that it is able to carry out specific oxidations of organic molecules which are not substrates for the wild-type protein. Our initial aim was to incorporate an "aromatic pocket" into the P-450$_{cam}$ active site, which would encourage the binding of substrates containing aromatic side-chains.

In addition, a surface residue remote from the active site was identified (cysteine-334) with effects on protein handling and stability. The cysteine is responsible for unwanted dimerisation of the protein during purification and an alanine residue was therefore substituted for the cysteine in order to improve both of these properties.

The three dimensional structure of P-450$_{cam}$ shows the active site to provide close van der Waals contact with the hydrophobic groups of camphor as shown in Figure 1. Three aromatic residues (Y96, F87 and F98) are grouped together and line the substrate binding pocket, with a hydrogen bond between tyrosine 96 and the camphor carbonyl oxygen maintaining the substrate in the correct orientation to ensure the regio-and stereo-specificity of the reaction. Replacement of any of these aromatic residues with a smaller, hydrophobic non-aromatic side-chain could provide the desired "aromatic pocket".

Molecular modelling was used to investigate the likely effects of point mutations to the three aromatic residues. The program GRID[7] was used to calculate an energy of interaction between an aromatic probe and possible mutants of cytochrome P-450$_{cam}$ where these residues were changed to alanine (F87A, Y96A and F98A). The results were then examined graphically using the molecular modelling package Quanta[8].

The mutant F98A appeared to have the strongest binding interaction within the active site cavity accessible to the aromatic probe, with that of Y96A being slightly smaller, and that of F87A being substantially less. It was decided in the first instance to mutate tyrosine 96 to alanine as it is more central to the binding pocket, whereas phenylalanine 98 is in a groove to one side. Also, removal of tyrosine 96 should

decrease the specificity of the enzyme towards camphor due to the loss of hydrogen bonding to the substrate.

According to one aspect of the present invention a mutant of the mono-oxygenase cytochrome P-$450_{cam}$ is provided in which the tyrosine residue at position 96 and/or the cysteine residue at position 334 is replaced by the residue of any amino acid except phenylalanine.

According to another aspect of the present invention a mutant of the mono-oxygenase cytochrome P-$450_{cam}$ is provided in which the tyrosine residue at position 96 and/or the cysteine residue at position 334 is replaced by another amino acid residue, which mutant has the property of catalysing the oxidation of any one of the following:- polycyclic aromatic hydrocarbons, linear or branched alkanes, diphenyl and biphenyl compounds including halogenated variants of such compounds and halogenated hydrocarbons.

According to yet another aspect of the present invention a method is provided of oxidising a compound selected from a polycyclic aromatic hydrocarbon, a linear or branched alkane, a diphenyl or biphenyl compound including a halogenated variant of such a compound or a halogenated hydrocarbon, the method comprising contacting the selected one of the compounds under oxidising conditions with mono-oxygenase cytochrome P-$450_{cam}$ in which the tyrosine residue at position 96 and/or the

cysteine residue at position 334 is replaced by another amino acid residue.

Preferably the amino acid is selected from any one of the following:- alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine and valine except that in the case of the tyrosine residue at position 96, the amino acid is not tyrosine and in the case of the cysteine residue at position 334, the amino acid is not cysteine.

The amino acid which replaces tyrosine at position 96 is conveniently one of the small hydrophobic amino acids, e.g. alanine, glycine, valine, leucine or isoleucine, with alanine being preferred as exemplified below.

Alternatively the amino acid replacing tyrosine at position 96 may be one of the charged amino acids, e.g. a negatively charged acid such as aspartic acid or glutamic acid for hydrogen bonding to a positively charged substrate; or a positively charged compound such a lysine, arginine or histidine for hydrogen bonding to a negatively charged substrate which are not members of the camphor family.

The mutation at position 96 is believed to be the key which enables the mutant enzymes to catalyse the oxidation of a relatively wide range of organic substrates. Other amino

acids adjacent to the active site of the enzyme may also be mutated in order to change the shape and specificity of the active site. These other amino acids include those at positions 87, 98, 185, 244, 247, 295 and 297. It is envisaged that the amino acid at one or more of these positions may be replaced by: a small hydrophobic amino acid so as to enlarge the active site; or a large hydrophobic amino acid so as to reduce the size of the active site; or by an amino acid having an aromatic ring to bond to a corresponding aromatic ring of a substrate.

Regarding the oxidising reactions, the conditions are described in the literature references attached. The enzyme system typically includes putidaredoxin and putidaredoxin reductase together with NADH as co-factors in addition to the mutant enzyme. Various classes of organic compounds are envisaged:-

i) The organic compound is an aromatic compound, either a hydrocarbon or a compound used under conditions in which it does not inactivate or denature the enzyme. Since the mutation has been effected with a view to creating an aromatic-binding pocket in the surface of the enzyme, the mutant enzyme is capable of catalysing oxidation of a wide variety of aromatic compounds. Oxidation of example aromatic and polyaromatic compounds is demonstrated in the experimental section below and is believed very

surprising given that the wild-type enzyme catalyses the oxidisation of only members of the camphor family.

ii) The organic compound may be a hydrocarbon, e.g. aliphatic or alicyclic, carrying a functional group. An aromatic protective group is added to the functional group prior to the oxidation reaction and removed from the functional group after the oxidation reaction. A suitable aromatic group is a phenyl group. The aromatic protection group is expecteed to hold the substrate in place. Thus the protecting group serves two purposes: firstly it makes the substrate more hydrophobic and hence increases binding to the hydrophobic enzyme pocket; secondly it holds the substrate in place at the active site. Thus, with the correct aromatic protection group, both regio- and stereo-selective hydroxylation of the substrate may be achieved. The example of cyclohexylbenzene is described in the experimental section below.

Examples of monofunctionalised hydrocarbons are cyclohexyl, cyclopentyl and alkyl derivatives (Scheme 1). The oxidation products of these compounds are valuable starting materials for organic synthesis, particularly when produced in a homochiral form. A range of aromatic protecting groups are envisaged, e.g. benzyl or naphtyl ethers and benzoyl or naphthoyl esters and amides (Scheme 1). Of interest are also benzoxazole groups as carboxyl protecting groups and N-benyl oxazolidine groups as

aldehyde protecting groups. Both can be easily cleaved after the enzymatic oxidation and have previously been described in the literature for the microbial oxidations of aldehydes and acids.

iii) The organic compound is a C5 to C12 aliphatic or alicyclic hydrocarbon. Oxidation of cyclohexane and linear hydrocarbons is demonstrated in the experimental section below and once again it is believed quite surprising given that the wild-type enzyme catalyses the oxidation of only members of the camphor family.

iv) The organic compound is a halogenated aliphatic or alicyclic hydrocarbon. Oxidation of lindane (hexachlorocyclohexane) is also described below.

Reference is directed to the accompanying drawings in which Figure 2 is a gas chromatograph of diphenylmethane (A) and hydroxylated product formed following incubation with $P\text{-}450_{cam}$ Y96A mutant.

Based on the above considerations, mutant proteins were constructed which contained alanine, lysine, valine, or phenylalanine instead of tyrosine at position 96 (Y96). Additional mutants were constructed in which these active site replacements were combined with the surface mutation of cysteine at position 334 to alanine. Lastly several active site mutations and the surface mutation were combined in one

protein to constitute a multiple mutant enzyme. The genes encoding cytochrome P-450$_{cam}$, and its natural electron-transfer partners puridaredoxin and putidaredoxin reductase, were amplified from the total cellular DNA of P. Putida using the polymerise chain reaction (PCR). The expression vector/E. coli host combinations employed were pRH1091[9] in strain JM109 for P-450$_{cam}$, pUC 118 in strain JM109 for putidaredoxin, and pGLW11 in strain DH5$^{\alpha c}$ for putidaredoxin reductase. Oligonucleotide-directed site-specific mutagenesis was carried out using an M13mp19 subclone by the method of Zoller and Smith[10], and mutant selection was by the method of Kunkel[11].

The mutant Y96A was shown to catalyse the hydroxylation of camphor (1), although compared to the wild-type enzyme the reaction was less selective, similar to that reported for the mutant Y96F[12]. This decrease in selectivity can be attributed to the loss of the hydrogen bond between Y96 and camphor. The properties of wild-type and Y96A proteins were further investigated with a variety of binding and activity assays.

Binding of potential substrates was investigated by spectroscopic methods. The wild-types enzyme in the absence of substrate is in the 6-co-ordinated, low-spin form with a weakly bound water occupying the sixth co-ordination site, and shows a characteristic Sorét maximum at 391 nm. Binding of the substrate analogues adamantanone (2), adamantane (3) and norbornane (4) also fully converted the haem to the high-spin

form. However, diphenylmethane (5) did not give a shift in the absorption spectrum.

The Y96A mutant, while giving the same results for compounds (3) and (4), was not fully converted to the high-spin form even when (1) and (2) were added in excess. Most interestingly however, and in contrast to the wild-type, Y96A showed partial conversion to the haem to the high-speed form with diphenylmethane, indicating binding of this compound to the mutant protein.

As expected, the dissociation constants ($K_{app}$) for camphor and adamantanone are increased in Y96A. On the other hand, the $K_{app}$ values for the hydrophobic substrates adamantane and norbornane are reduced, indicating that the enzyme pocket has become more selective for hydrophobic substrates. The greatest change in binding was obtained with diphenylmethane, which bound poorly to wild-type protein, but showed greatly enhanced affinity for the Y96A mutant (Table 1).

Once binding of diphenylmethane by the Y96A protein had been established, catalytic substrate turnover was investigated. The mutant protein was reconstituted with putidaredoxin and putidaredoxin reductase. Diphenylmethane (5) was added and the mixture was incubated with NADH and oxygen.

A solution containing 10 μM putidaredoxin, 2 μM putidaredoxin reductase, 1 μM cytochrome $P\text{-}450_{cam}$ mono-oyxgenase (wild-type

or mutant) and 1 mM diphenylmethane in 100 mM KCl, 20 mM $KH_2PO_4$pH7.4 was preincubated at 25°C in a shaker for 5 min. The enzymatic reaction was initiated by firstly adding NADH to a total concentration of 2 mM. Further four aliquots of NADH (to increase the NADH concentration by 1 mM each time) were added in intervals of 5 min and the reaction quenched after 30 min by adding 0.5 ml chloroform. The chloroform layer was analysed by gas chromatography.

Organic extracts of the crude incubation mixture were analysed by gas chromatography. Only one major new peak was detected by GC (see Figure 2), which had the same retention time as an authentic sample of para-hydroxydiphenylmethane (6). The other aromatic hydroxylation products, the ortho and meta isomers, had different retention times. Further confirmation of the identity of the product as structure (6) was provided by mass spectrometry, which gave the correct mass peak at 184.

Using the above experimental techniques, the inventors have investigated a considerable number of organic compounds as substrates for both the wild-type P-$450_{cam}$ enzyme and also the mutant version Y96A. Further work has included mutants designated Y96V; Y96L; Y96F; C334A; the combined mutant F87A, Y96G, F193A and the combined active site and surface mutants of Y96A, C334A; Y96V, C334A; Y96L, C334A; Y96F, C334A; F87A, Y96G, F193A, C334A.

The results for Y96A are set out in Table 2, in which structurally related molecules are grouped together. Those substrates where oxidation has been demonstrated by means of NADH turnover are marked with a + sign.

Spin high/low: numbers shows the percentage of P-450 ($OD_{417}$ 0.2-0.4) converted from the low- to high-spin equilibrium state in the presence of 200 μM test compound, in phosphate buffer (40 mM phosphate, 68 mM potassium, pH 7.4). Spin state equilibrium is assessed with a UV/vis spectrophotometer: low spin at $OD_{417}$ and high spin at $OD_{392}$ nd; not done.

Vs DTT: numbers show the percentage displacement of DTT (200 μM) bound to P-450 by competition with test compounds (200 μM) in phosphate buffer. DTT binding to P-450 results in absorbance peaks at $OD_{374}$ and $OD_{461}$, so displacement is measured with a UV/vis spectrophotometer.

Examples are included in Table 2(a) to 2(h) for each class of compounds identified in points i) to iv) above.

Reaction products for some substrate compounds have been purified by high performance liquid chromatography and identified by mass spectroscopy, nuclear magnetic resonance, and/or co-elution. Table 3 details the NADH consumption for oxidation of small linear, branched and cyclic hydrocarbons by the mutant Y96A, C334A. Table 4(a) to 4(h) details the

product distributions for mutant and substrate combinations where this has been elucidated to date.

**Scheme 1:**

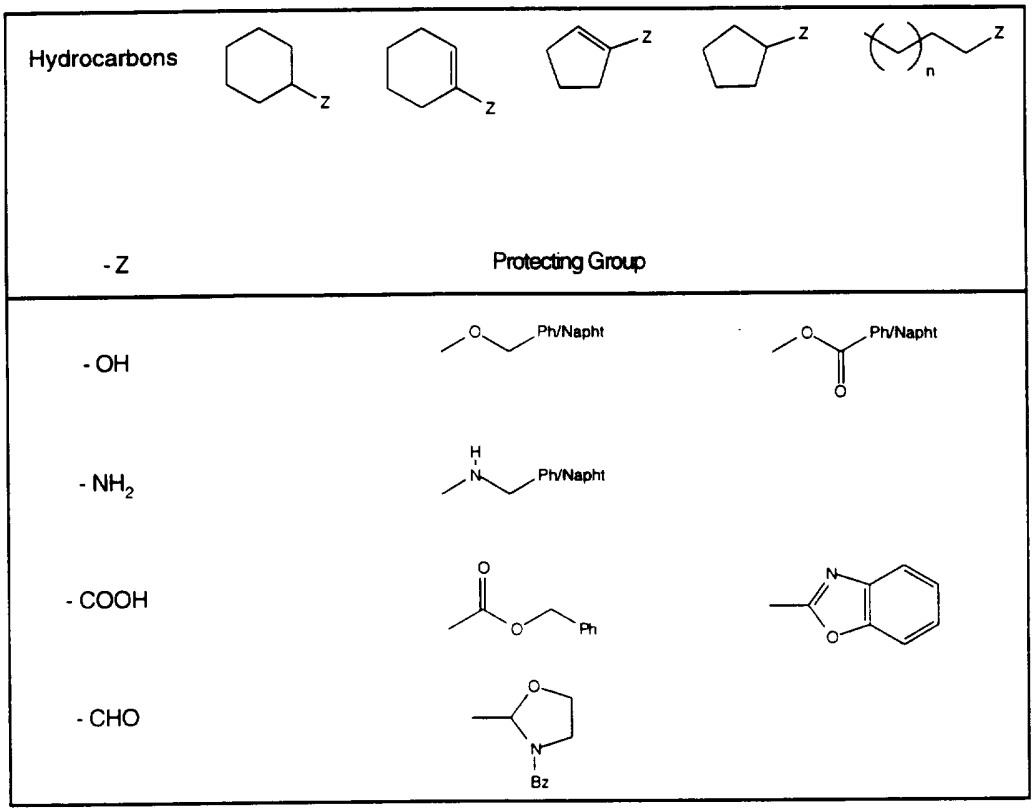
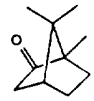
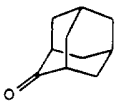
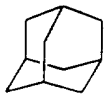
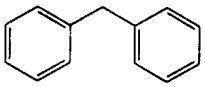

Hydrocarbons
Z
n
- Z
Protecting Group
- OH
Ph/Napht
O
- $NH_2$
H
N
- COOH
Ph
N
- CHO
Bz

**Table 1:**

| | | $K_{app}$ (μM)[a] | |
|---|---|---|---|
| | | **WT** | **Y96A** |
|  | **1** | 6.3 | 12 |
|  | **2** | 12 | 28 |
|  | **3** | 8.4 | 1.4 |
|  | **4** | 330 | 92 |
|  | **5** | >1500[b] | 73 |

[a] Values are the average of two independent measurements using the method of Sligar (S.G. Sligar, *Biochemistry*, **1976**, *15*, 5399 - 5406). The value of $K_{app}$ is strongly dependent on the concentration of $K^+$ in the buffer. At $[K^+]>150$ mM, $K_{app}$ for camphor is 0.6 μM for both wildtype and Y96A. Data in this table were determined at $[K^+] = 70$ mM in phosphate buffer, pH 7.4, in order to avoid salting out of substrates at higher ion concentrations.

[b] Saturation not reached.

**Table 2(a)**

**P450cam-substrate interactions**

| | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| **Subgroup: 1-ring** | | | | | | | | |
| Benzene | - | - | - | - | | | | |
| Toluene | - | - | 30 | 30 | | | | |
| Ethylbenzene | - | - | 40 | 40 | | | | |
| Styrene | - | - | 30 | 30 | | | | |
| Cyclohexene | - | 5 | 40 | 40 | | | | |
| 1,3-Cyclohexadiene | nd | nd | nd | nd | | | | |
| 1,4-Cyclohexadiene | - | 5 | 15 | 20 | | | | |
| Cyclohexane | - | - | 60 | 60 | | | + | |
| Hexane | - | - | 70 | 60 | | | + | |
| Methylcyclohexane | 50 | 50 | 100 | 70 | | | | |
| (S)-(+)-Carvone | 10 | 60 | 10 | 80 | | | | |

Table 2(b)

P450cam-substrate interactions

| | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| Subgroup: 2-ring, Naphthalene | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| Naphthalene | - | - | 15 | 20 | | | | |
| 1-Ethylnaphthalene | - | - | 5 | 20 | | | | |
| 2-Ethylnaphthalene | - | - | 10 | 20 | | | | |
| 2-Naphthylacetate | - | 5 | - | 5 | | | | |
| 1-Naphthylacetate | - | 5 | - | 5 | | | | |
| 1-Naphthylpropionate | - | 20 | 0 | 20 | | | | |
| 1-Naphthylbutyrate | - | 5 | - | 5 | | | | |
| Naphthylphenylketone | - | 5 | - | 5 | | | | |
| 1,2-Dihydronaphthalene | 5 | 20 | 30 | 90 | | | | |
| 1,2,3,4-Tetrahydro naphthalene | 5 | 10 | 40 | 40 | | | | |

**Table 2(c)**

**P450cam-substrate interactions**

| Subgroup: 2-ring, DPM | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| Diphenylmethane | - | 5 | 45 | nd | | | + | + |
| Diphenylether | 10 | 5 | 20 | 50 | | | | |
| Benzophenone | - | 20 | - | 20 | | | | |
| Cyclohexylphenylketone- | 30 | | 60 | nd | | | | |
| Phenylbenzoate | - | 5 | - | - | | | | |
| N-Phenylbenzylamine | 5 | | 45 | nd | | | | |
| Bibenzyl | - | - | 55 | 55 | | | | |
| cis-Stilbene | - | 20 | 40 | 50 | | | | |
| Biphenyl | - | 20 | - | 90 | | | | |
| Cyclohexylbenzene | 20 | 20 | 80 | nd | | | | |
| *trans*-Stilbene | - | - | - | - | | | | |
| Benzylether | - | 5 | 55 | nd | | | | |

**Table 2(d)**

**P450cam-substrate interactions**

**Subgroup: 3-ring**

| | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| Anthracene | | | | | | | | |
| Phenanthrene | - | - | 20 | 20 | | | + | |
| Fluorene | - | - | - | 50 | | | | |
| 2-Fluorencarboxaldehyde | - | - | - | 50 | | | | |
| 9-Fluorenone | - | 20 | - | 5 | | | | |
| Anthrone | - | 5 | - | 5 | | | | |
| Anthraquinone | | | | | | | | |
| 2-Ethylanthraquinone | | | | | | | | |

Table 2(e)

| P450cam-substrate interactions | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| Subgroup: 4,5-ring | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| Chrysene | - | - | - | - | | | | |
| 1,2-Benzanthracene | - | - | - | - | | | | |
| Fluoranthene | - | 5 | 20 | 10 | | | | |
| Pyrene* | - | - | - | - | | | | |
| Perylene* | - | - | - | - | | | | |

Table 2(f)

| P450cam-substrate interactions | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| **Subgroup: Cyclic Alkanes** | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  *cis*-Decahydronaphthalene | nd | nd | nd | nd | | | | |
|  *trans*-Decahydronaphthalene | 20 | 10 | 90 | 70 | | | | |
|  Cyclohexane | - | - | 60 | 60 | | | + | |
|  Methylcyclohexane | 50 | 50 | 100 | 70 | | | | |

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Table 2(g)

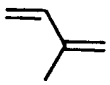
| P450cam-substrate interactions | | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|---|
| **Subgroup: n-Alkanes** | | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| | n-Pentane | - | 5 | 55 | 40 | | | + | |
| | n-Hexane | - | - | 60 | 40 | | | + | |
| | n-Heptane | 5 | 5 | 60 | 40 | | | + | |
| | n-Octane | - | 5 | 80 | 45 | | | + | |
| | n-Nonane | - | - | 70 | 45 | | | + | |
| | n-Decane | nd | nd | nd | nd | | | | |
| | n-Undecane | nd | nd | 20 | 20 | | | | |
| | n-Dodecane | nd | nd | 5 | 5 | | | | |
| $CH_3(CH_2)_{14}CH_3$ | n-Hexadecane | - | - | - | - | | | | |
| $CH_3(CH_2)_{15}CH_3$ | n-Heptadecane | - | - | - | - | | | | |
| $CH_3(CH_2)_{11}OSO_3,Na$ | SDS | - | 20 | - | 60 | | | | |
| $CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$ | Oleic acid* | - | 10? | - | 20? | | | | |
| $[(CH_3)_2CH(CH_2)_3CH(CH_3)(CH_2)_3CH(CH_3)CH_2CH_2\text{-}]_2$ | Squalane | - | - | - | 20 | | | | |
|  | Isoprene | - | - | 10 | 10 | | | | |

Table 2(h)

| P450cam-substrate interactions | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| **Subgroup: Camphor-like** | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| (1R)-(-)-Camphorquinone | 80 | 80 | 80 | 80 | | | | |
| (1R)-(-)-Fenchone | 40 | 70 | 50 | 80 | | | | |
| Dicyclopentadiene | 50 | 80 | 90 | 90 | | | | |

Table 3

# Turnover of Small Alkanes by P450cam Mutants

All mutants listed below also contain the C334A mutation.

Turnover rate measured as NADH consumption rate (nmole NADH/nmole P450cam/s).

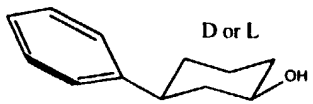
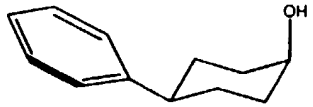
| Alkane substrate: Main chain length | Name | Wild type | Y96A |
|---|---|---|---|
| C4 | *n*-butane | - | - |
| C4 | 2-methyl butane | background | 4.6 |
| C4 | 2,3-dimethyl butane | background | 16.8 |
| C4 | 2,2-dimethyl butane | background | 14.0 |
| C5 | *n*-pentane | background | 5.8 |
| C5 | 2-methyl pentane | 3.8 | 11.7 |
| C5 | 3-methyl pentane | 1.3 | 14.2 |
| C5 | 2,4-dimethyl pentane | 0.2 | 12.6 |
| C5 | 2,2-dimethyl pentane | 5.2 | 12.8 |
| C5 | 2,2,4-trimethyl pentane | 0.9 | 5.3 |
| C5 | 3-ethyl pentane | background | 16.2 |
| C6 | *n*-hexane | background | 6.0 |
| C6 | 2-methyl hexane | background | 10.6 |
| C7 | *n*-heptane | 2.7 | 4.4 |
| C7 | 2-methyl heptane | background | 2.1 |
| C7 | 4-methyl heptane | 1.4 | 10.2 |
| C8 | *n*-octane | background | 5.8 |
| C7 | cycloheptane | 4.4 | 42.5 |

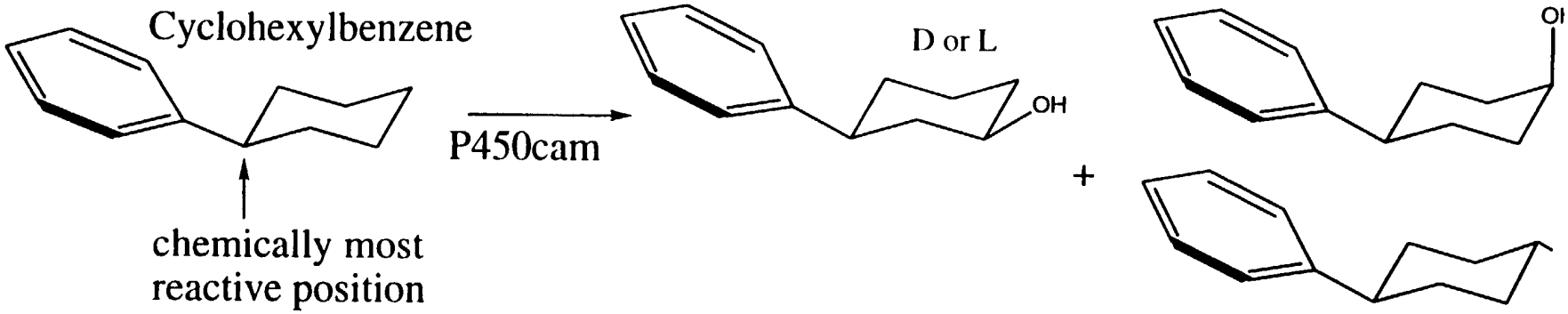
Product structures and distributions following oxidation of substrates with P450cam active site mutants.

"background" - typical background NADH oxidation rate is 0.07 nmole NADH (nmole P450cam)$^{-1}$ sec$^{-1}$

Table 4(a)

**Product structure and distributions following oxidation of substrates with P450cam active site mutants. All mutants shown below also contain tl C334A mutation.**

| Cyclohexylbenzene Products | | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V |
|---|---|---|---|---|---|---|
|  | 3-ol | 43 | 20 | 54 | 38 | 28 |
| " " L or D | 3-ol | 20 | 20 | 27 | 23 | 39 |
|  | Trans -4-ol | 25 | 15 | 6 | 23 | 10 |
|  | Cis- 4-ol | 12 | 45 | 13 | 16 | 23 |
| Total products(area/$10^5$) | | 0.8 | 7.4 | 1.1 | 10.4 | 12.5 |



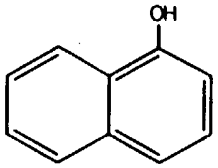
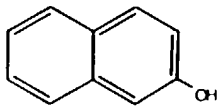

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Table 4(b)

| Phenylcyclohexene | Products (%) for mutants: | | |
|---|---|---|---|
| Products | WT | 25Y96A | |
| 3-one (A) | 24 | 25 | |
| 3-ol (B) | 76 | 75 | |
| Total products(area/$10^6$) | 42 | 36 | |

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Table 4(c)

| Naphthalene Products | | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|---|
|  | 1-ol | 100 | 100 | 100 | 100 | 100 | 100 |
|  | 2-ol | 0 | 0 | 0 | 0 | 0 | 0 |
| Total products (area/$10^5$) | | (0.016) | 1.1 | 2.4 | 0.7 | 1.4 | 0.1 |

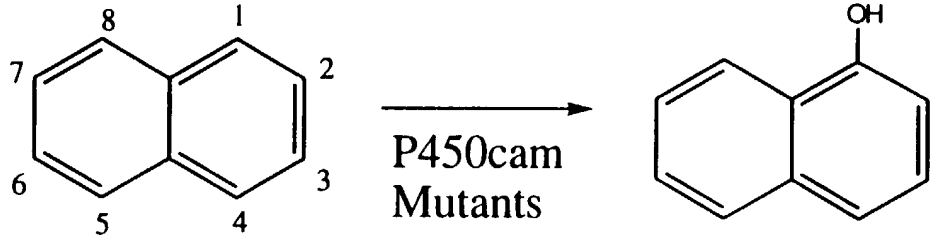
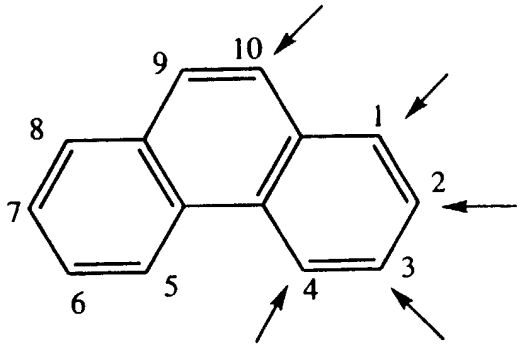


Table 4(d)

| Phenanthrene Products | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|
| A | 38 | 49 | 41 | 35.5 | 41 | 27 |
| B | 15 | 23 | 31 | 41 | 38 | 41 |
| C | 12 | 13 | 5 | 9 | 11 | 3 |
| D | 35 | 15 | 23 | 14.5 | 10 | 29 |
| Total products (area/$10^6$) | 0.075 | 7.0 | 4.5 | 2.8 | 1.6 | 0.065 |

Phenanthrene

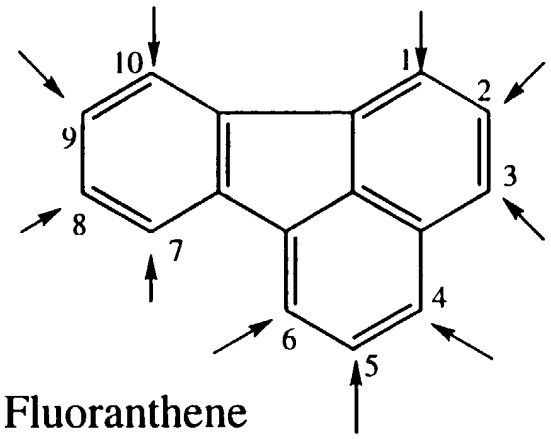



P450cam mutants → 4 hydroxylated products

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Table 4(e)

| Fluoranthene Products | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|
| A | 0 | 84 | - | - | - | 0 |
| B | 0 | 16 | - | - | - | 100 |
| Total products (area/$10^6$) | 0 | 2.7 | - | - | - | 0.2 |




Fluoranthene

P450cam mutants → 2 hydroxylated products



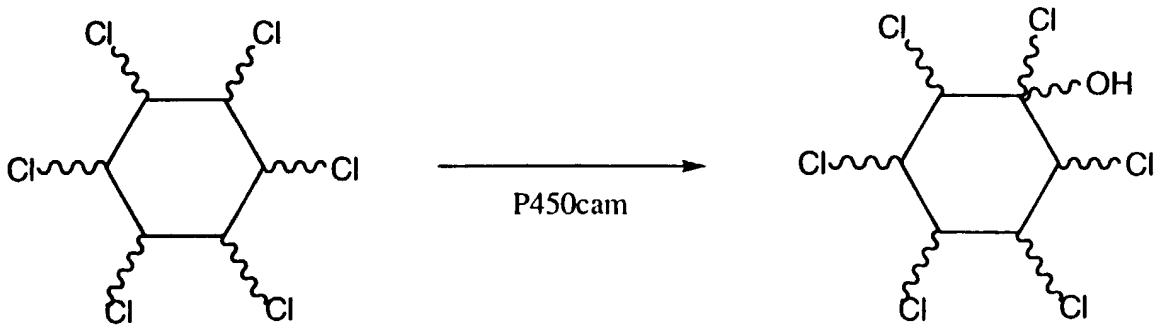
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Table 4(f)

| Pyrene Products | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|
| A | 0 | 40 | 43 | 23 | 30 | 33 |
| B | 0 | 43.6 | 29 | 64.5 | 55 | 40 |
| C | 0 | 5 | 12.5 | 7.9 | 12 | 20 |
| D | 0 | 11.4 | 15.5 | 4.6 | 3 | 7 |
| Total products (area/$10^6$) | 0 | 1.2 | 1.5 | 1.5 | 1.6 | 0.02 |

Table 4(g)

| Lindane Products (hexachlorocyclohexane) | Products (%) for mutants WT | Y96A |
|---|---|---|
| A | 100 | 100 |
| Turnover rate nmole NADH (nmoleP450)$^{-1}$s$^{-1}$ | 7.5 | 43.5 |




Hexachlorocyclohexane

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Table 4(h)

| Hexane Products | Products (%) for mutants: Y96F | Y96A |
|---|---|---|
| 2-hexanone | 10 | 15 |
| 3-hexanone | 16 | 28 |
| 2-hexanol | 24 | 26 |
| 3-hexanol | 50 | 32 |
| Relative activity (WT = 1) | 18.2 | 25.5 |

| 2-Methyl hexane Products | Products (%) for mutants: Y96F | Y96A |
|---|---|---|
| 2-methyl-2-hexanol | 72 | 74 |
| 5-methyl-2-hexanone | 16 | 14 |
| 2-methyl-3-hexanol | 7 | 4 |
| 5-methyl-2-hexanol | 5 | 8 |
| Relative activity (WT = 1) | 2.3 | 2.6 |

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**CLAIMS**

1. A mutant of the mono-oxygenase cytochrome P-450$_{cam}$ in which the tyrosine residue at position 96 and/or the cysteine residue at position 334 is replaced by the residue of any amino acid except phenylalanine.

2. A mutant of the mono-oxygenase cytochrome P-450$_{cam}$ in which the tyrosine residue at position 96 and/or the cysteine residue at position 334 is replaced by another amino acid residue, which mutant has the property of catalysing the oxidation of any one of the following:- polycyclic aromatic hydrocarbons, linear or branched alkanes, diphenyl and biphenyl compounds including halogenated variants of such compounds and halogenated hydrocarbons.

3. A mutant as claimed in claim 1 or claim 2 in which the amino acid is selected from any one of the following:- alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine and valine except that in the case of the tyrosine residue at position 96, the amino acid is not tyrosine and in the case of the cysteine residue at position 334, the amino acid is not cysteine.

4. A mutant as claimed in any of claims 1 to 3 in which the amino acid residue at one or more of the positions 87, 98, 185, 244, 247, 295 and 297 is replaced by another amino acid residue.

5. A method of oxidising a compound selected from a polycyclic aromatic hydrocarbon, a linear or branched alkane, a diphenyl or biphenyl compound including a halogenated variant of such a compound or a halogenated hydrocarbon, the method comprising contacting the selected one of the compounds under oxidising conditions with mono-oxygenase cytochrome $P\text{-}450_{cam}$ in which the tyrosine residue at position 96 and/or the cysteine residue at position 334 is replaced by another amino acid residue.

6. A method as claimed in claim 5 in which the amino acid is selected from any one of the following:- alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine and valine except that in the case of the tyrosine residue at position 96, the amino acid is not tyrosine and in the case of the cysteine residue at position 334, the amino acid is not cysteine.

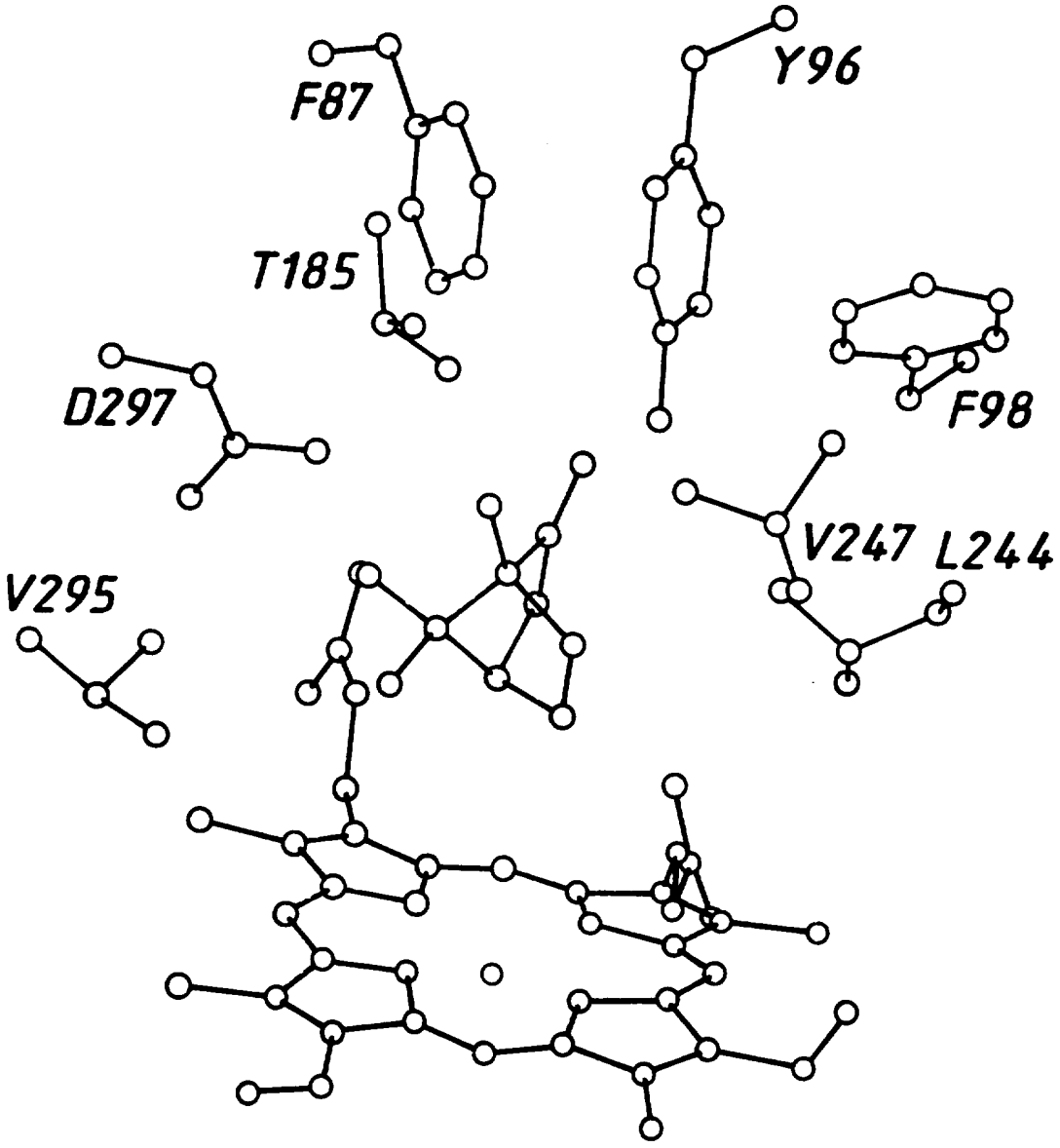
7. A method as claimed in claim 5 or claim 6 in which the amino acid residue at one or more of the positions 87,

98, 185, 244, 247, 295 and 297 is replaced by another amino acid residue.

8. A mutant of the mono-oxygenase cytochrome $P\text{-}450_{cam}$ substantially as hereinbefore described with reference to the accompanying drawings and/or examples.

9. A method of oxidising a compound selected from a polycyclic aromatic hydrocarbon, a linear or branched alkane, a diphenyl or biphenyl compound including a halogenated variant of such a compound or a halogenated hydrocarbon substantially as hereinbefore described with reference to the accompanying drawings and/or examples.

FIG.1.



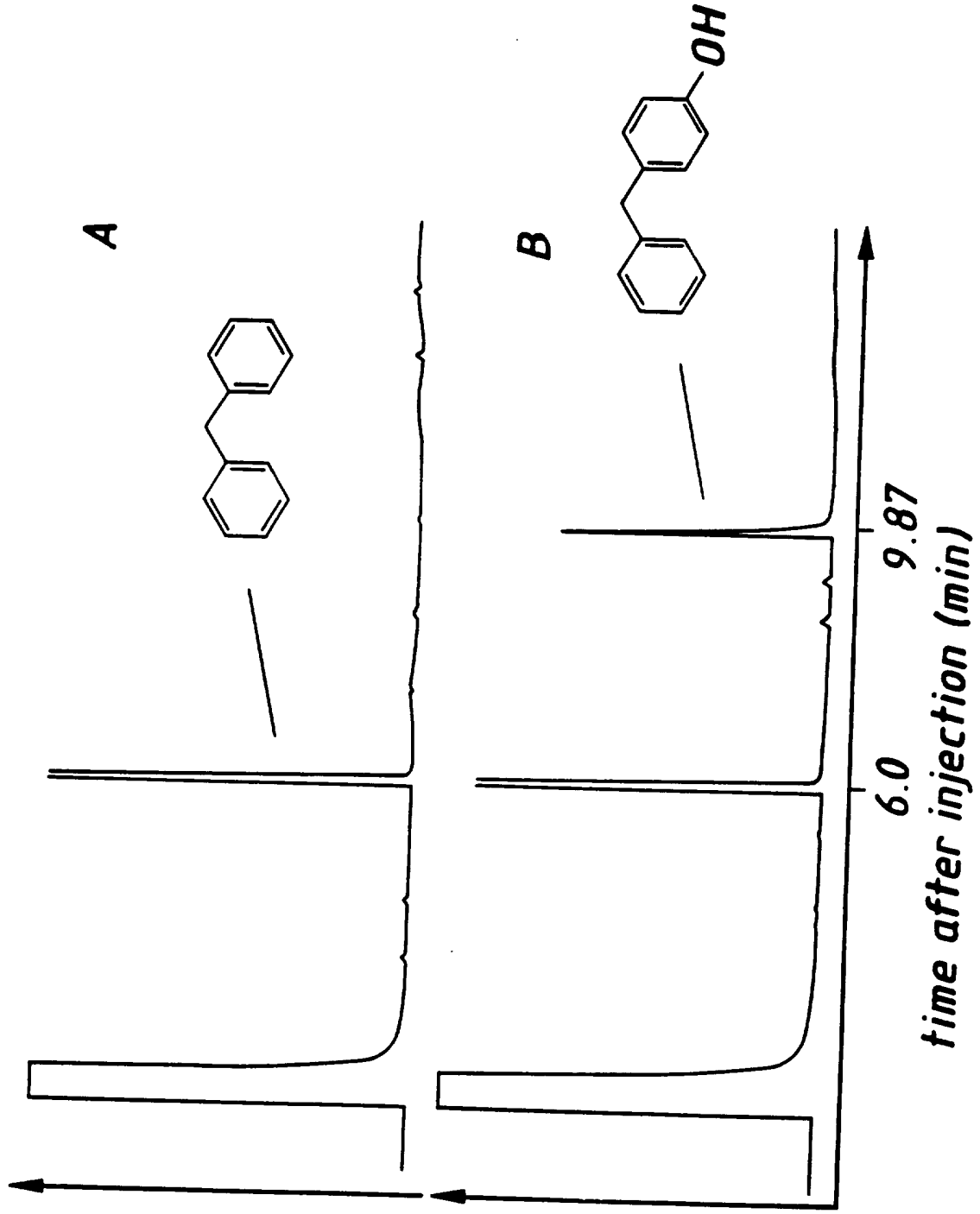



FIG. 2.

# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 95/02588

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N9/02 C12P7/22 C12P7/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 35, 15 December 1988 pages 18842-18849, W.M. ATKINS ET AL. 'The role of active site hydrogen bonding in cytochrome P-450 cam as revealed by site-directed mutagenesis.' cited in the application | 2,8 |
| Y | see the whole document --- | 1,3-7 |
| X | THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 10, 5 April 1990 pages 5361-5363, C. DI PRIMO ET AL. 'Mutagenesis of a single hydrogen bond in cytochrome p450 alters cation binding and heme solvation.' cited in the application | 2,8 |
| Y | see the whole document --- | 1,3,4 |
| | -/-- | |

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2

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 27 March 1996 | 22.04.96 |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. ( + 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax ( + 31-70) 340-3016 | Authorized officer<br>Hix, R |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | BIOCHEMISTRY,<br>vol. 29, no. 5, 6 February 1990<br>pages 1271-1275,<br>W. M. ATKINS ET AL. 'Tyrosine-96 as a natural spectroscopic probe of the cytrochrome P-450cam active site'<br>cited in the application | 2,8 |
| Y | see the whole document<br>--- | 1,3,4 |
| Y | THE JOURNAL OF BIOLOGICAL CHEMISTRY,<br>vol. 268, no. 1, 5 January 1993<br>pages 269-275,<br>S. F. TUCK ET AL. 'Active sites of the cytochrome p450cam {CYP101} F87W and F87A mutants.'<br>cited in the application<br>see the whole document<br>--- | 1,3-7 |
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2

# INTERNATIONAL SEARCH REPORT

.formation on patent family members

Interr nal Application No
PCT/GB 95/02588

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
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| WO-A-9516041 | 15-06-95 | AU-B- 1108295 | 27-06-95 |