A Journal of the Gesellschaft Deutscher Chemiker
Angewandte
GDCh
International Edition
Chemie
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Accepted Article

Title: CYP505E3 – a novel self-sufficient ω-7 in-chain hydroxylase

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.202001055
Angew. Chem. 10.1002/ange.202001055

Link to VoR: http://dx.doi.org/10.1002/anie.202001055
http://dx.doi.org/10.1002/ange.202001055

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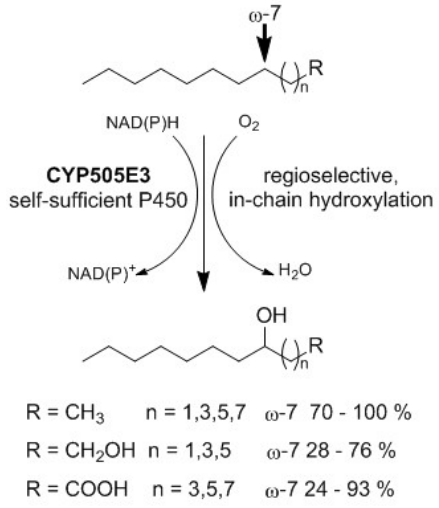
CYP505E3 – a novel self-sufficient ω-7 in-chain hydroxylase

M.J. Maseme,[a, b] A. Pennec,[a, b] J. van Marwijk,[a, b] D.J. Opperman,[a, b] and M.S. Smit,[a, b*]

Abstract: A self-sufficient cytochrome P450 monooxygenase, CYP505E3, identified in *Aspergillus terreus* catalyzes regioselective in-chain hydroxylation of alkanes, fatty alcohols and fatty acids at the ω-7 position. It thus hydroxylates C10 to C16 alkanes with > 70 % regioselectivity at the eighth carbon from one methyl terminus, also displaying remarkably high activity towards decane (TTN ≈ 8 000) and dodecane (TTN ≈ 2 000). CYP505E3 converts dodecanoic acid to 5-hydroxydodecanoic acid (24 % regioselectivity), which at low pH lactonises to δ-dodecalactone. With 1-dodecanol as substrate it displays 55% regioselectivity towards the ω-7 position to yield 1,5-dodecanediol, which was converted to δ-dodecalactone by horse liver alcohol dehydrogenase (HLADH).

Regioselective activation of C-H bonds remains a problem for which neither chemists nor biochemists have found the perfect solution. Cytochrome P450 monooxygenases are the enzymes most extensively studied for this purpose. P450s are known to hydroxylate linear alkanes, alcohols and fatty acids of different chain lengths. The most extensively studied alkane and fatty acid hydroxylating P450s such as the CYP153s and CYP102s mainly hydroxylate at the terminal or sub-terminal carbons[1–3]. However, there are several P450s which show in-chain hydroxylation of medium chain fatty acids such as decanoic acid and dodecanoic acid, although hydroxylation is rarely more in-chain than the ω-6 position[3]. One exception in this regard is CYP116B46 from *Tepidiphilus thermophiles*[4] which gives C5 hydroxylation of decanoic and dodecanoic acid. It displays in the case of decanoic acid exquisite regioselectivity (99 %) and enantioselectivity to produce the "non-natural" (S)-δ-decalactone with > 90 % ee. In the case of dodecanoic acid C5 regioselectivity was 76 %. However, these substrates are most likely not the natural substrates of CYP116B46, since uncoupling with decanoic acid was 11.5 % and TTNs were 242 with decanoic acid and 27 with dodecanoic acid. Terminal hydroxylation is the main focus of reports on *n*-alkane and fatty alcohol hydroxylation, with very few reports on the latter[5,6], despite the potential that not only diterminal diols, but also internal alkane diols hold for the synthesis of polymers[5] and even lactones and lactams[7]. Recently, Sakai et al. (2018) reported that CYP505D6, a self-sufficient P450 from the white-rot fungus *Phanerochaete chrysosporium* preferably hydroxylated 1-dodecanol and dodecanoic acid at the sub-terminal positions (ω-1 to ω-3), but also yielded the in-chain hydroxylated products (ω-4 to ω-7) as minor products[8]. However, to date, no catalyst have been described for the regioselective in-chain oxyfunctionalization of *n*-alkanes, where no directing functional group is available.

Here we report the novel ω-7 in-chain hydroxylase activity of a self-sufficient cytochrome P450 monooxygenase, CYP505E3, which we have identified in *Aspergillus terreus*[9][10]. CYP505E3 is the first P450 that gives regioselective in-chain ω-7 hydroxylation of *n*-alkanes with chain lengths ranging from C10 to C16, enabling the one step biocatalytic synthesis of rare alcohols such as 5-dodecanol and 7-tetradecanol. Additionally, CYP505E3's regioselectivity for ω-7 hydroxylation also extends to fatty acids and fatty alcohols (Scheme 1). This ω-7 hydroxylase activity towards tetradecanoic acid, dodecanoic acid and 1-dodecanol also makes it an attractive starting point for the development of biocatalytic processes for production of δ-dodecalactone. δ-Dodecalactone is a high value flavour compound responsible for fruity and buttery notes[11] as well as anti-fungal properties[12]. It occurs naturally in fruits and dairy products in low concentrations[13][14].



Scheme 1. Regioselective in-chain hydroxylation of alkanes, fatty alcohols and fatty acids by CYP505E3.

CYP505E3 and the glucose dehydrogenase (GDH) from *Bacillus megaterium* were expressed in *Escherichia coli* BL-21 Gold (DE3) and the enzymes were recovered from the cells as cell-free extracts (CFEs). The P450 concentration in the CFEs derived from 0.2 g wet cell weight per ml was typically ca. 6 µM (Figure S1) and the final measured CYP505E3 concentration in the aqueous biotransformation reaction mixtures (BRMs) ≈ 0.7 – 1.5 µM.

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CYP505E3 containing CFEs showed activity towards the C10 to C16 alkanes (Figures S2 – S9). The reactions with decane, yielded 3-decanol as the major product (71 %). Dominant alcohol products were also observed in the extracts from the dodecane, tetradecane and hexadecane reactions. When the mass spectra of these main products were analysed (Figure 1) and the derived structures closely examined, a unique regioselectivity of CYP505E3 for C10 to C16 alkanes became apparent. It appeared that CYP505E3 preferentially hydroxylated these alkanes on the 8th carbon i.e. at what could be described as a ω-7 position (Figure 2A and 2B, Table S2). Preliminary docking experiments with decane and dodecane using a homology model of CYP505E3, placed these n-alkanes in positions which agree with the observed ω-7 hydroxylase activity. It shows the "ω-end" penetrating deep into a hydrophobic pocket parallel to helix I, placing the ω-7 carbon in close proximity to the oxygen of compound I (Figure 3).

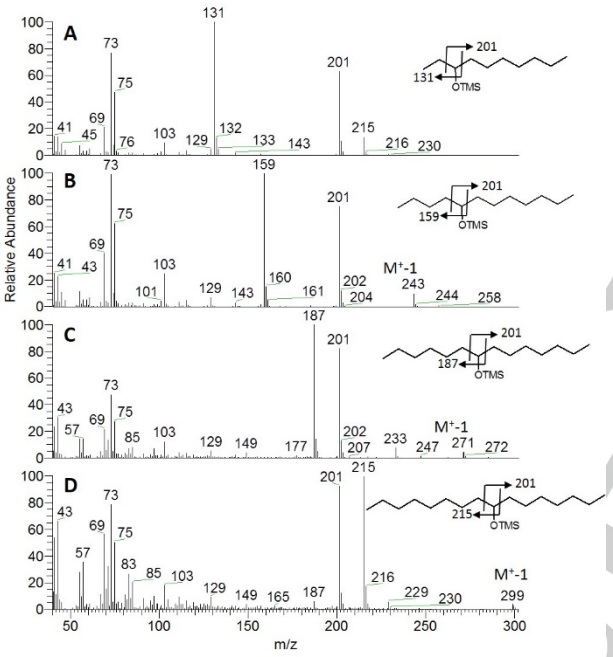

Figure 1. Mass spectra of major products from GC-MS analysis of trimethylsilylated samples from reactions of CYP505E3 with decane (A), dodecane (B), tetradecane (C) and hexadecane (D). BRM (1 mL) contained: CYP505E3 in cfe (225 µL from 200 g_{wcw} L^{-1} cells, final [CYP] 0.9 µM), GDH in cfe (25 µL from 200 g_{wcw} L^{-1} cells), 0.1 mM NAD^+, 748 µL MOPS buffer (200 mM, pH 8, 100 mM glucose, 100 mM glycerol) with 250 µl alkane added.

The alkane hydroxylase activity observed with CYP505E3 is not only remarkable for its regioselectivity, but also for the high activity observed with decane (TTN ≈ 8 000) and dodecane (TTN ≈ 2 000) (Table S2) even without any attempt at optimization. In a study where CYP153s and CYP102A1 mutants with high reported activities towards alkanes were compared on C6 to C10 alkanes under similar unoptimized conditions in CFEs, the highest TTNs obtained with decane were around 2 500[15] (Table S3). The preferred substrates of these CYPs were n-hexane and n-octane with TTNs of up to 10 000 obtained with the CYP102A1 R47L-Y51F mutant on hexane and around 4 000 with CYP153A6 and some CYP102A1 mutants on n-octane.

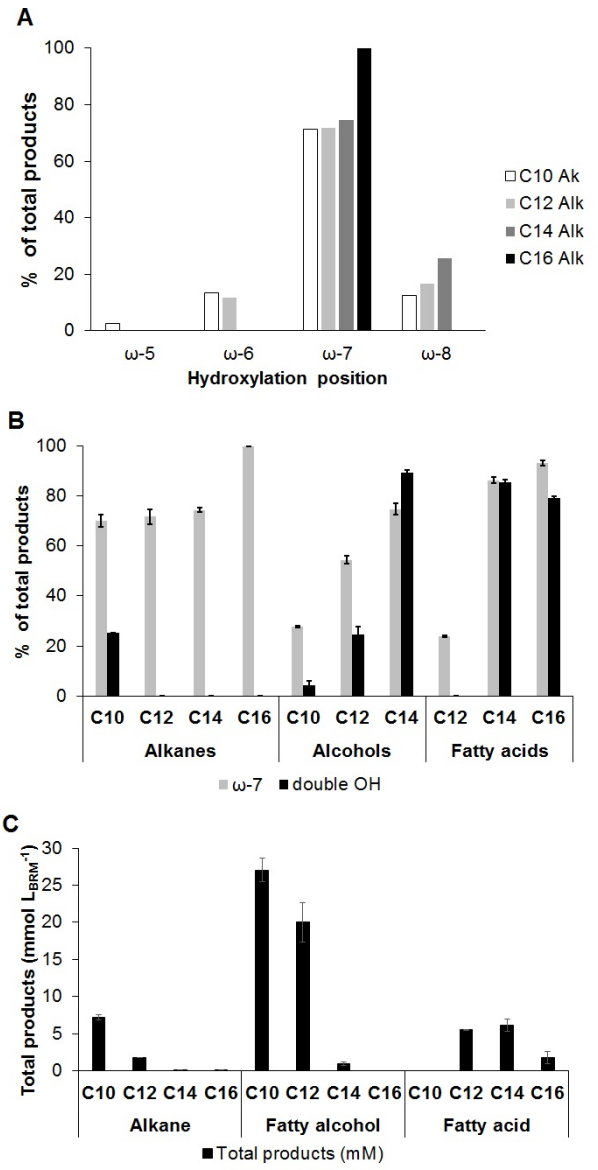

Figure 2. Regioselectivity (A and B) and substrate preference (C) of CYP505E3 towards alkanes, fatty alcohols and fatty acids. BRM (1 mL) contained: CYP505E3 in cfe (225 µL from 200 g_{wcw} L^{-1} cells, final [CYP] 0.9 µM), GDH in cfe (25 µL from 200 g_{wcw} L^{-1} cells), 0.1 mM NAD^+, 748 µL MOPS buffer (200 mM, pH 8, 100 mM glucose, 100 mM glycerol). Regioselectivity calculations were based on assumption that initial hydroxylation of double hydroxylated products was at the in-chain position (ω-6, ω-7 or ω-8).

CYP505E3's unique ω-7 regioselectivity was also observed with the C12 to C16 fatty acids and the C10 to C14 alcohols, when these were tested as substrates (Figure 2B, Figures S10 – S23, Table S2), although the products of ω-7 hydroxylation were not in all cases the major products. No activity was observed with the C10 fatty acid and the C16 alcohol. In the case of dodecanoic acid the products of ω-7 hydroxylation, 5-hydroxydodecanoic acid and δ-dodecalactone (formed by lactonization of the 5-hydroxy acid at low pH), comprised 24 % of the total products. Activity towards the fatty acids was similar to the activity towards alkanes

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with 5.5 mmol L_{BRM}^{-1} total products formed from 10 mmol L_{BRM}^{-1} dodecanoic acid added (TTN ≈ 6 000) (Figure 1C, Table S2). The C14 and C16 fatty acids were double hydroxylated to yield dihydroxy acids hydroxylated at the ω-7 position as well as a sub-terminal position (ω-1, ω-2 or ω-3).

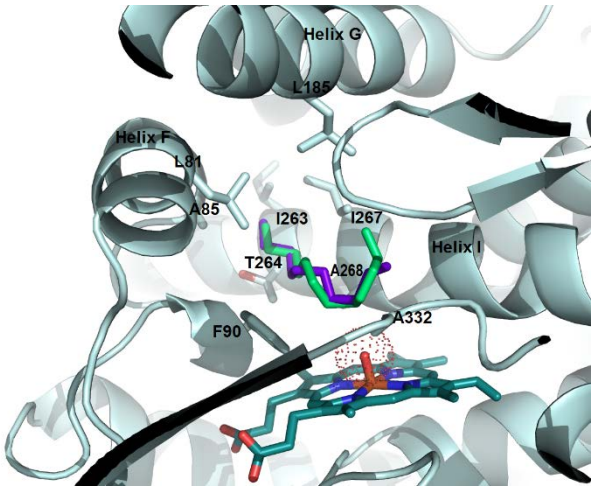


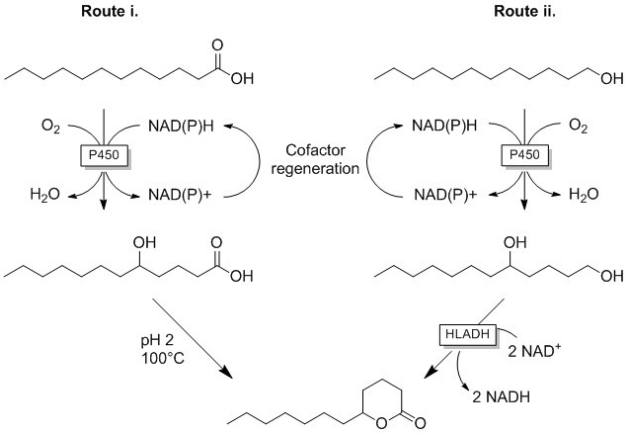
Figure 3. The lowest energy poses of decane (purple) and dodecane (green) obtained from docking experiments carried out with a homology model of CYP505E3. Residues lining the binding pocket are shown as sticks and the oxygen of compound I as a dotted sphere. Substrate docking in the CYP505E3 homology model was performed with YASARA version 14.12.2 using VINA AutoDockLGA and the AMBER03 force field. Image was created using PyMOL software.

1-Decanol was the preferred substrate of CYP505E3 with 27 mmol L_{BRM}^{-1} total products formed (TTN ≈ 30 000) (Figure 2, Table S2). Although 1,3-decanediol, the product of ω-7 hydroxylation was a major product (28 % of diol products), regioselectivity was not as strong as with 1-dodecanol, since equal amounts of 1,7-decanediol (ω-3 hydroxylation) was also produced. In the case of 1-dodecanol, the substrate with the second highest activity (20 mmol L_{BRM}^{-1} total products, TTN ≈ 22 000) the product from ω-7 hydroxylation, 1,5-dodecanediol, was the major product (ca. 39 % of total diol products) with double hydroxylated triol products forming 25 % of total products (Figure 2, Figure S13, Table S2). When both diol and triol products were considered regioselectivity for ω-7 hydroxylation was 55 %. With tetradecanol as substrate hydroxylation at the ω-7 position was still preferred, but the major products were double hydroxylated triols.

The possibility that 1,5-dodecane diol might also be converted to δ-dodecalactone did not escape our attention. Oxidation of alkanediols by horse liver alcohol dehydrogenase (HLADH) for synthesis of lactones is an established enzymatic reaction[16–19]. More specifically, HLADH has been shown to oxidize 1,5-pentanediol, 1,4-decanediol and 1,5-decanediol with preference for 1,5-diols[17,18]. Conversion of 1-dodecanol to δ-dodecalactone was tested by replacing GDH with purified HLADH. GC-MS analysis of extracts from this reaction showed that the 1,5-dodecanediol and a small amount of 1,4-diol (from ω-8 hydroxylation) were completely converted to δ- and γ-dodecalactones, respectively, while the other diols remained (Figure S24). In this case no triols were observed and δ-dodecalactone comprised 55 % of the total products, confirming earlier estimates of the regioselectivity.

In order to test 1-dodecanol conversion on a larger scale, a time course experiment was run in three 100 mL shake flasks using 10 mL BRM. Two of the flasks contained 180 mmol L_{BRM}^{-1} 1-dodecanol and the third 60 mmol L_{BRM}^{-1}. In flasks with 180 mmol L_{BRM}^{-1} 1-dodecanol the final total diols reached a concentration of ca. 20 mmol L_{BRM}^{-1}, while the triol concentrations fortuitously remained at less than 10 % (Figure S25.). With 60 mmol L_{BRM}^{-1} 1-dodecanol the production of diols fell to 12 mmol L_{BRM}^{-1}, but the percentage conversion improved from 11% to 20% (Table S4). Addition of extra enzymes and co-factor to this latter reaction brought the final total diol concentration to 15 mmol L_{BRM}^{-1}. After completion of the reactions the remaining BRMs were combined, extracted and the 1,5-diol separated by flash chromatography to yield 50 mg of > 90 % pure 1,5-diol (Figure S26). ^{1}H- and ^{13}C-NMR spectra were congruent with 1,5-dodecane diol (Figure S27). Chiral analysis of the δ-dodecalactone produced by HLADH oxidation of the purified 1,5-diol showed that the R-enantiomer, the major enantiomer in natural δ-dodecalactone, was produced in an enantiomeric excess of 29% (Figure S28). Given the relatively low enantioselectivity observed with 1-dodecanol, a substrate with a directing hydroxyl group, chiral separation of other products was not pursued.

We have thus demonstrated two routes by which CYP505E3 can be used for the synthesis of δ-dodecalactone (i) via hydroxylation of dodecanoic acid and lactonisation of the 5-hydroxy fatty acid and (ii) via hydroxylation of 1-dodecanol followed by oxidation and lactonisation by an alcohol dehydrogenase (Scheme 2). Both these routes require extensive optimisation most likely involving both protein and process engineering. The latter could include using whole cells of *E. coli* or perhaps a yeast, screening for other alcohol dehydrogenases[20] or oxidases[21] for oxidation of the 1,5-diol as well as *in situ* product removal to avoid over hydroxylation. Hydroxylation of tetradecanoic acid followed by one round of β-oxidation and lactonisation could in principle also yield δ-dodecalactone, but would require expression of CYP505E3 in an organism, probably a yeast, with partially disrupted β-oxidation[22].



Scheme 2. Routes demonstrated for the production of δ-dodecalactone from dodecanoic acid (Route i) and 1-dodecanol (Route ii) using CYP505E3.

Here we have reported the first catalyst for the regioselective in-chain hydroxylation of *n*-alkanes. Given the high TTNs obtained and already high regioselectivity, CYP505E3 is a promising catalysts for further development of in-chain hydroxylases with improved or adjusted regioselectivity and substrate scope to enable synthesis of unique "natural" alcohols from "natural" biosynthesized *n*-alkanes[23]. This could for instance be done by directed evolution through iterative saturation mutagenesis of selected residues as reported for P450pyr (CYP153A7)[24]. Additionally, CYP505E3 can be employed for the synthesis of valuable diols and lactones. Alternative biocatalytic processes for the synthesis of C10 and C12 δ-lactones are highly sought after[4,22] given that expensive natural substrates, variable supply and environmental concerns[25] present challenges to processes currently used for industrial production of "natural" lactones from unsaturated massoia δ-lactones[26].

Acknowledgements

Financial support by the University of the Free State, the South African Department of Science and Technology/National Research Foundation Centre of Excellence in Catalysis is gratefully acknowledged. We also thank Sarel Marais for technical assistance with GC analyses and Dr. Matthias Esterhuysen for insightful discussions. The BmGDH gene was kindly provided by Dr. Dirk Holtmann (Dechema, Germany). Natural δ-dodecalactone was a kind gift from Givaudan, USA.

Keywords: alkane • CH-activation • cytochrome P450 • in-chain hydroxylation • lactone

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10.1002/anie.202001055

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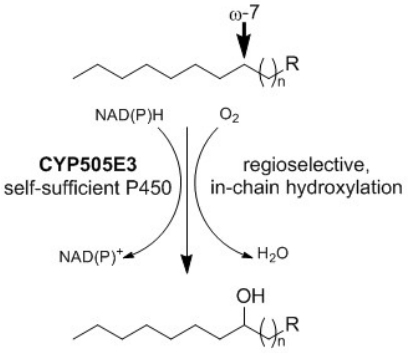
Regioselective in-chain C-H activation: CYP505E3, a self-sufficient cytochrome P450 monooxygenase from the fungus *Aspergillus terreus,* catalyzes regioselective in-chain hydroxylation of alkanes, fatty alcohols and fatty acids at the ω-7 position. It can thus be used for the biocatalytic production of δ-dodecalactone from dodecanoic acid and 1-dodecanol.



R = CH_3 n = 1,3,5,7 ω-7 70 - 100 %
R = CH_2OH n = 1,3,5 ω-7 28 - 76 %
R = COOH n = 3,5,7 ω-7 24 - 93 %

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