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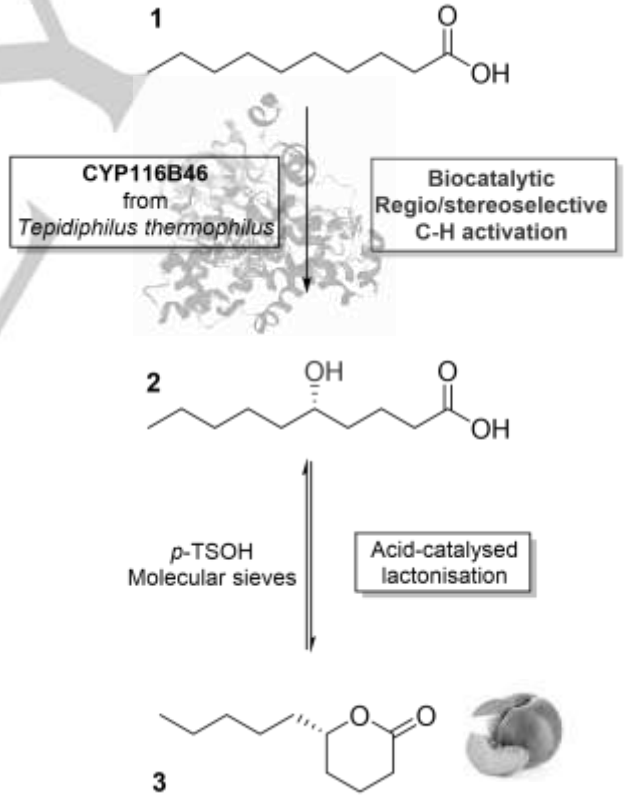
Regio- and Enantio-selective Chemo-enzymatic C-H-lactonization of Decanoic Acid to (*S*)-δ-Decalactone.

Jack Manning[a], Michele Tavanti[a], Joanne L. Porter[a], Nico Kress[a], Sam P. De Visser[b], Nicholas J. Turner[a] and Sabine L. Flitsch[a]*

Abstract: The conversion of saturated fatty acids to high value chiral hydroxy-acids and lactones poses a number of synthetic challenges: the activation of unreactive C-H bonds and the need for regio- and stereoselectivity. Here we report the first example of a wild type cytochrome P450 monooxygenase (CYP116B46 from *Tepidiphilus thermophilus*) capable of enantio- and regioselective C5 hydroxylation of decanoic acid **1** to (*S*)-5-hydroxydecanoic acid **2**. Subsequent lactonization yielded (*S*)-δ-decalactone **3**, a high value fragrance compound, with > 90 % *ee*. Docking studies provide a rationale for the high regio- and enantioselectivity of the reaction.

Hydroxy fatty acids (HFAs) are a group of naturally derived compounds that are ubiquitous in Nature. They find a wide range of diverse applications in many high value chemical industries such as flavourings, fragrances, food supplements and pharmaceuticals.[1–4] The relationship between hydroxyl and carboxylic acid functional groups often has a marked effect in their properties. For example, α-hydroxyacids are used in many cosmetics and have been shown to possess anti-ageing properties whereas short chain β-hydroxyacids have been shown to display antimicrobial properties.[5,6] Hence, there is a need for selective synthetic methods towards HFAs, ideally by direct oxidation of readily available fatty acids. The chemical oxyfunctionalisation of non-activated C-H bonds in fatty acids is one of the most demanding chemical reactions, because of the additional need for regio-selective targeting of one of many methylene groups and demands on enantioselectivity on highly flexible substrates.[7] In this regard, biocatalysis using P450 monooxygenases (P450s, CYPs) represents an attractive option for selective C-H bond activation with the added advantage of mild reaction conditions. Consequently, fatty acid hydroxylation catalysed by P450s has been widely investigated, but often suffers from poor regioselectivity and limitation to terminal positions.[8–12] For example, P450-BM3 has been reported to hydroxylate unsaturated fatty acids (C10:0-C18:0) at positions ω-1, ω-2 and ω-3.[13,14] Some elegant studies using genome mining and protein engineering have sought to address this issue and resulted in new product profiles.[15–18] Nevertheless, the regioselective 'mid-chain' C5 and C6 hydroxylation (γ- and δ-positions, respectively) of fatty acids, and additional stereoselectivity, is still highly challenging.[19] Yet, γ- and δ-hydroxylated fatty acids are important intermediates for the production of the corresponding lactones *via* intramolecular esterification. These lactones find a variety of commercial uses within flavourings and fragrances,[20] in particular δ-decalactone (**3**), a common fragrance compound, with the (*S*)-enantiomer presenting a significantly more intense creamy peach note compared to the (*R*)-isomer, which is found preferentially in Nature.[21,22] A number of stereoselective biocatalytic methods have been reported for these lactones including use of Baeyer–Villiger monooxygenases,[23] alcohol dehydrogenases and biocatalytic cascades, respectively.[24,25] However, all reported synthetic methods start from functionalized materials including 11-hydroxy palmitic acid, coriolic acid and Massoia lactone.[26–29]



Scheme 1. A) Biocatalytic synthesis of δ-decalactone **(3)** by regio- and stereoselective hydroxylation of decanoic acid **(1)** with CYP116B46 followed by *para*-toluenesulfonic acid catalysed lactonization to *S*-δ-decalactone**.**

The extraction of Massoia lactone from *Cryptocaria massoia* poses environmental concerns,[30] whereas the chemical synthesis requires transition metal catalysts, peracids and organic

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Table 1. Initial screen of fatty acids **1,4** and **5** against a panel of self-sufficient cytochrome P450 monooxygenases.

Substrate[A]	Regio-selectivity [%]	Enzyme							
		AT	AX	TB	TT	JT	Hal1	RhF	BM3
Octanoic acid (**4**)	conversion[B] [%]	89	65	18	46	>99	94	31	n.d.
	C7	37	33	39	67	43	57	41	0
	C6	60	40	61	18	46	39	56	0
	C5	2	27	0	14	11	4	3	0
Decanoic acid (**1**)	conversion [%]	49	42	30	83	63	60	n.d.	>99
	C9	25	48	79	0	28	35	0	66
	C8	20	17	21	0	0	65	0	21
	C7	15	14	0	0	0	0	0	13
	C6	16	0	0	10	21	0	0	0
	C5	24	22	0	**90**	51	0	0	0
Dodecanoic acid **(5)**	conversion [%]	90	65	78	68	>99	68	63	>99
	C11	72	48	90	0	9	74	88	51
	C10	12	8	10	0	5	26	12	40
	C9	0	0	0	0	0	0	0	9
	C6	17	10	0	24	12	0	0	0
	C5	0	34	0	76	74	0	0	0

[A]. 400 µl reactions performed with cell-free extracts containing 10 µM enzyme, 0.4 mM substrate, 1 mg mL^{-1} $NADP^+$, 1 mg mL^{-1} glucose dehydrogenase (CDX-901), 10 mg mL^{-1} D-glucose, 100 mM sodium phosphate buffer (pH 8), 30˚C, 24h. [B]. Substrate conversion was determined based on substrate depletion measured by comparison with reactions carried out with an empty vector control. [n.d.] not detected.

solvents.[31] Therefore, the direct P450-catalysed γ- and δ-hydroxylation of simple bioavailable fatty acids such as **1** represents a highly attractive alternative to access lactones such as **3**, in particular when targeting single enantiomers (Scheme 1). Here we report the screening and identification of P450 monooxygenases capable of generating hydroxy derivatives of fatty acids at mid-chain positions. To our surprise, one of the enzymes (P450-TT) was found to catalyse the production of (*S*)-5-hydroxydecanoic acid (**2**) with high regio- and enantio-selectivity, leading to the fragrance lactone (**3**) by subsequent chemical lactonization.

In the first instance, a panel of recently reported class VII P450s was screened with saturated fatty acids of chain lengths 8, 10 and 12 carbon atoms (**1, 4**, **5** respectively) (Table 1).[32–34] Class VII P450s (such as CYP116B) are self-sufficient monooxygenases, which had not previously been investigated with fatty acids in such a systematic manner. The better-studied P450-BM3 (member of the other self-sufficient class VIII P450s) was included in the assembled P450 panel for comparison, due to its well established high activity towards medium chain length fatty acids.[13,35] Initial screening reactions were performed with 0.4 mM substrate concentration and cell-free extracts containing 10 µM enzyme as determined by CO difference spectroscopy. A cofactor recycling system consisting of glucose and glucose dehydrogenase was used to regenerate the NADPH cofactor. All P450s showed activity towards fatty acid substrates, with P450-AT and P450-JT showing particularly good conversions up to quantitative levels across the panel of fatty acids (Table 1).

Octanoic acid (**4**) was mainly converted to the C6 and C7 hydroxy fatty acids with P450-TT and P450-Hal1 showing slight preference to hydroxylation at C7, whereas P450-AT, P450-TB and P450-RhF generated the C6 hydroxylated isomer as the main product. Interestingly, P450-AX, TT and JT were capable of performing C-H activation at the C5 position more efficiently than the other enzymes tested, although the C7 and C6 hydroxylated isomers remained the major products. When presented with decanoic acid (**1**), P450-TB and Hal1 formed only the C9 and C8 hydroxy fatty acids. P450-TB hydroxylated decanoic acid predominantly at the C9 position (79%), whereas the opposite was observed for P450-Hal1 (65% C8 hydroxylation). P450-BM3 preferentially formed the C9 hydroxylated product (66%), with minor C8 and C7 hydroxy fatty acids, in accordance with literature reports.[13] A variety of hydroxylated products were observed when P450-AT and P450-AX were reacted with decanoic acid: P450-AT did not show any preferred position for hydroxylation, whereas P450-AX preferentially formed the C9 hydroxylated product (48%).

Of particular interest and surprise was the observation that some of the enzymes tested are capable of forming the mid-chain C6 and C5 hydroxylated products. Hydroxylation of decanoic acid by P450-AX generated 5-hydroxydecanoic acid amongst the other regioisomers (22%), whereas P450-AT formed both C6 and C5 hydroxylated products (16% and 24% products, respectively). P450-JT predominantly targeted the C5 position (51%), whereas P450-TT displayed excellent regioselectivity for the C5 position (90%), with only minor (10%) C6 hydroxylated product formed. Indeed, this observed regioselectivity for wild-type P450-TT is significantly higher than reported previously for engineered P450-BM3 variants (42% and 19% for C5 and C6 regioisomers respectively).[19] Using dodecanoic acid (**5**) as substrate, P450-TB, Hal1 and RhF again exclusively formed C11 and C10 hydroxy fatty acids and P450-BM3 preferentially targeted the C11 position (51%). P450-AT formed a mixture of products, including C6 hydroxylated dodecanoic acid (17%). Again, P450-TT and P450-JT displayed the best regioselectivity for C-H bonds in the middle of the fatty acid chain, forming more than 70% C5 hydroxylated product.

We were especially interested in the excellent regioselectivity for the challenging C5 hydroxylation of decanoic acid to 5-hydroxy decanoic acid displayed by P450-TT, because this reactivity creates precursor **2** for the valuable δ-decalactone (**3**). Using the recently solved crystal structure of P450-TT,[36] we performed molecular docking simulation of decanoic acid into the heme domain of P450-TT (Figure 1). The energetically most favoured simulation creates a reasonable binding pose for promoting

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substrate hydroxylation.[37] This simulation suggests that a 'U-shaped' conformation of decanoic acid above the heme prosthetic group favours the regioselective C5 hydroxylation, which is in accordance with previous reports of P450-mediated hydroxylation of fatty acids in the centre of the fatty acid chain.[38] Furthermore, the formation of the (*S*)-hydroxy fatty acid enantiomer is favoured by this model. The substrate carboxy group seems to be positioned by polar interactions to residues T202 and E277 (Figure 1), while multiple hydrophobic interactions promote the U-shape binding of the carbon chain. In particular, residues L92, A270 and V273 (Figure 1) appear to sandwich the substrate's tail. Given that the enzyme pocket does not contain an obvious counterion to the carboxylate, as observed in P450-BM3, substrate docking was performed both with the protonated as well as in its deprotonated substrate (Figure S1). Although both simulations resulted in a similar U-shaped conformation, it is interesting to note that only the binding pose of the protonated substrate places the pro-*S* C-5 hydrogen closest to the iron centre in agreement with the experimentally observed selectivity.

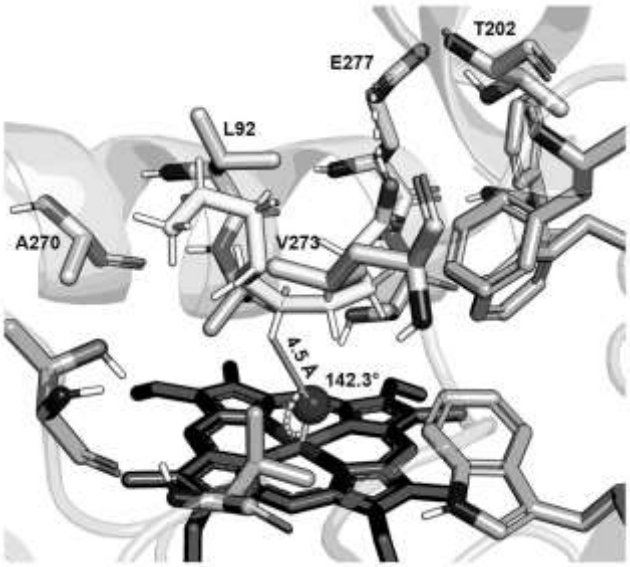

Figure 1. Docking structure (VINA approach as implemented in the YASARA software) of decanoic acid (white) in P450-TT (PDB 6GII). The figure shows the distance (dotted line) between the hydrogen at the C-5 pro-*S* hydrogen and the compound I oxygen (black ball). of the heme prosthetic group (black) and the potential reaction trajectory (angle).

Next, the biotransformation of P450-TT and decanoic acid was studied in more detail to achieve a set of conditions optimal for the formation of **3**. The best conversion value (68%) was obtained when 10 µM enzyme was incubated with 2 mM substrate at 40°C (Figure S3c). Spontaneous lactonization of 5-hydroxy decanoic acid to the corresponding lactone was indeed observed after GC analysis, however this accounted for only ~6.5% of substrate converted (Figure S12). Some slight increase in substrate conversion was observed when 30% decane was added to the biotransformation (Table S3). Using the optimized conditions, the reaction between decanoic acid and P450-TT was then performed on a preparative scale to confirm the product identity and determine product stereochemistry, since the stereoisomers are not commercially available. Preparative scale reactions were carried out at larger volumes (10 and 100 mL) with different substrate loadings (5 and 20 mM, Table S4). The identity of isolated product **3** was confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy (see Supporting Information). The product was analysed by chiral normal-phase HPLC (Figures 2 and S14) which confirmed formation of the (*S*)-δ-decalactone with over 90% enantiomeric excess in broad agreement with the molecular docking predictions (Figure 1). Absolute configuration of the lactone product was assigned using previously reported methods (see supporting information).

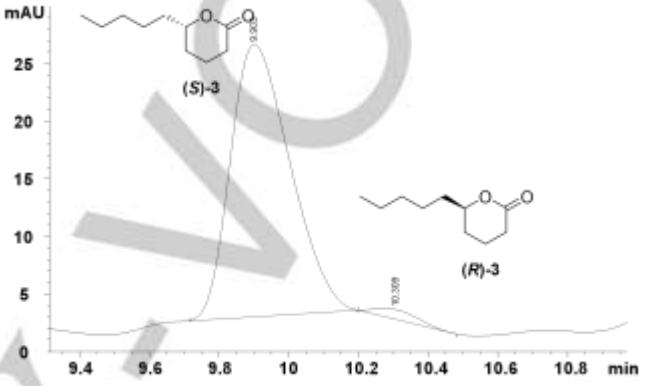

Figure 2. Chiral normal-phase HPLC trace for the isolated δ-decalactone **3** after preparative scale biotransformation of decanoic acid **1**

In summary, our work provides evidence that class VII P450s are useful catalysts for generating a variety of hydroxy-carboxylic acids directly from the parent acids. Both terminal and 'mid-chain' C-H activation was observed, with the latter leading to important products such as the valuable fragrance compound (*S*)-δ-decalactone **3** with remarkable regio- and stereo-selectivity. The wild-type enzymes identified here represent a valuable addition to the protein scaffolds that can be employed as starting points for further protein engineering to generate optimised biocatalysts for chiral lactone synthesis.

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Keywords: Biocatalysis • Cytochrome P450 • Regioselectivity • Fatty acids • Lactones

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