ORGANIC LETTERS

2003
Vol. 5, No. 18
3341−3344

Products of Cytochrome P450$_{BioI}$ (CYP107H1)-Catalyzed Oxidation of Fatty Acids

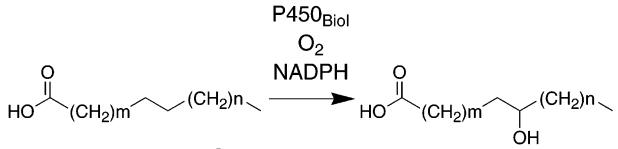
Max J. Cryle, Nick J. Matovic, and James J. De Voss*

Department of Chemistry, University of Queensland, St. Lucia, Brisbane 4072, Australia

devoss@chemistry.uq.edu.au

Received July 7, 2003

ABSTRACT



C_{14}: m = 8-10; n = 2-0.
C_{16}: m = 8-10; n = 4-0.

Oxidation of tetradecanoic and hexadecanoic acids by cytochrome P450$_{BioI}$ (CYP107H1) produces mainly the 11-, 12-, and 13-hydroxy C_{14} fatty acids and the 11- to 15-hydroxy C_{16} fatty acids, respectively. In contrast to previous reports, terminal hydroxylation is not observed. The enantiospecificity of fatty acid hydroxylation by P450$_{BioI}$ was also determined, and the enzyme was shown to be moderately selective for production of the (*R*)-alcohols.

The cytochromes P450 (P450s) comprise a superfamily of oxidative hemoproteins that catalyze a fascinating array of oxidative transformations, including oxygen insertion into unactivated C−H bonds and C−C bond cleavage reactions.[1,2] P450$_{BioI}$ (CYP107H1) is encoded by a gene (*bioI*) located within the biotin biosynthetic operon of *Bacillus subtilis*.[3] Analysis of bacterial mutants suggested that P450$_{BioI}$ played a role in the production of pimelic acid (heptanedioic acid), the biosynthetic origin of the majority of carbons in biotin.[3] P450$_{BioI}$ has previously been overexpressed in *E. coli*, purified, and characterized.[4,5] It was isolated both as a pure protein and as a complex with an acyl-acyl carrier protein (ACP); turnover of the complex was shown to produce a pimeloyl-ACP equivalent as the sole product.[4] It was also demonstrated that fatty acids could bind to the purified P450$_{BioI}$ and were oxidized to both dicarboxylic acids and molecules that had GC/MS behavior consistent with hydroxy fatty acids. Thus, P450$_{BioI}$ appeared to catalyze a unique C−C bond cleavage to produce a pimelic acid equivalent for use in biotin biosynthesis as well as a simple hydroxylation of free fatty acids. However, others similarly characterized the oxidation of free fatty acids and tentatively identified the product of tetradecanoic acid oxidation as 14-hydroxytetradecanoic acid, the product of ω-oxidation.[5] This would suggest a very different role for P450$_{BioI}$ in biotin biosynthesis, in which it produced an ω-functionalized fatty acid that was eventually converted into pimelic acid via chain-shortening reactions. We therefore undertook careful identification of the products from P450$_{BioI}$-mediated oxidation of tetradecanoic and hexadecanoic acids.

Initial analysis of the MS fragmentation (GC/MS) patterns of the esterified products obtained from incubation of free fatty acids with a catalytically active P450$_{BioI}$ system[4] indicated that the major products of enzymatic turnover were hydroxy fatty acids. Various diacids, e.g., pimelic acid, were also identified within the turnover mixtures, consistent with

(1) Ortiz de Montellano, P. R. In *Cytochrome P450*: *Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995; p 245.
(2) Ortiz de Montellano, P. R.; De Voss, J. J. *Nat. Prod. Rep.* **2002**, *19*, 477.
(3) Bower, S.; Perkins, J. B.; Yocum, R. R.; Howitt, C. L.; Rahaim, P.; Pero, J. *J. Bacteriol.* **1996**, *178*, 4122.
(4) Stok, J. E.; De Voss, J. J. *Arch. Biochem. Biophys.* **2000**, *384*, 351.
(5) Green, A. J.; Rivers, S. L.; Cheesman, M.; Reid, G. A.; Quaroni, L. G.; Macdonald, I. D. G.; Chapman, S. K.; Munro, A. W. *J. Biol. Inoorg. Chem.* **2001**, *6*, 523.

10.1021/ol035254e CCC: $25.00
Published on Web 08/15/2003

the previously postulated role for $P450_{BioI}$ in biotin biosynthesis.[4]

The fragmentation patterns of monohydroxy fatty acid esters have been investigated previously,[6−8] and several characteristic fragmentations have been reported. The fragments that are indicative of the position of hydroxylation correspond to ones arising from cleavage α to the hydroxyl group (Figure 1, **A**) and from α-cleavage and loss of

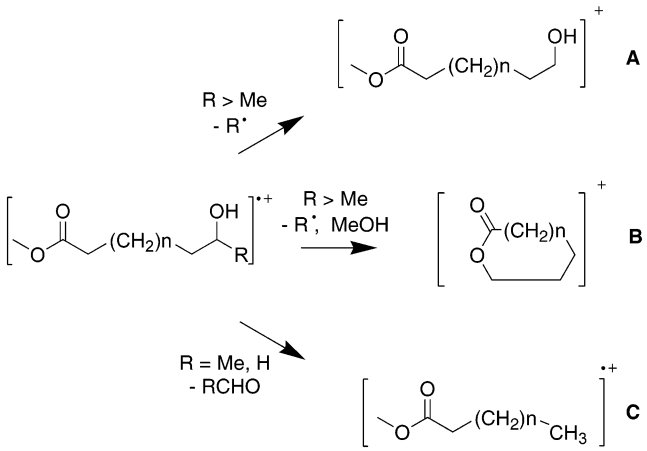
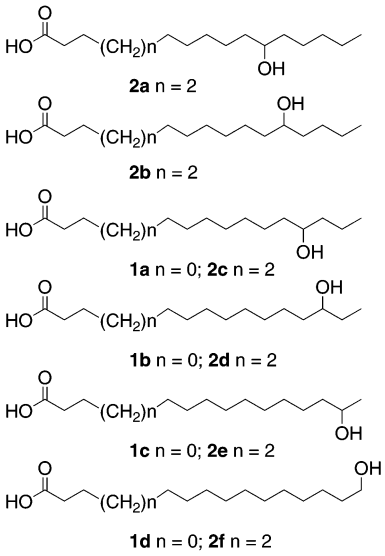


Figure 1. Characteristic mass spectral fragments of monohydroxy fatty acid methyl esters.

methanol (Figure 1, **B**). A plausible mechanism for the formation of this latter peak is lactonization with the resultant loss of methanol, followed by radical scission of the carbon chain α to the lactone ring oxygen. As previously reported, the *ω*- and *ω*-1-hydroxy fatty acid esters follow different major fragmentation paths resulting from specific hydrogen transfer (Figure 1, **C**).[6−8]

Utilizing these characteristic fragmentation patterns of hydroxy fatty acid esters, we undertook GC/MS analysis of the $P450_{BioI}$-catalyzed oxidation of tetradecanoic and hexadecanoic acids.

11-Hydroxytetradecanoic acid **1a** and 12-hydroxytetradecanoic acid **1b** were tentatively identified as the major products from tetradecanoic acid oxidation (major fragments of the methyl esters at *m*/*z* 215 (**A**)/183 (**B**) for **1a** and 229 (**A**)/197 (**B**) for **1b**). 11- and 12-hydroxyhexadecanoic acids (**2a** and **2b**, respectively) were also identified from hexadecanoic acid oxidation. However, analysis of the (better resolving) trifluoroacetate esters of the esterified products of $P450_{BioI}$ oxidation indicated that there were three hydroxy fatty acids produced from tetradecanoic acid oxidation and five from hexadecanoic acid. Unfortunately, the mass spectra of the TFA esters lacked the characteristic fragmentation required to identify the position of hydroxylation. However, we predicted that the position of oxidation in the unidentified products was likely to be closer to the methyl terminus of the fatty acid chain than in the 11- and 12-hydroxy isomers. This was based upon experience of the change in retention times relative to the position of the alcohol moiety in the fatty acid chain.



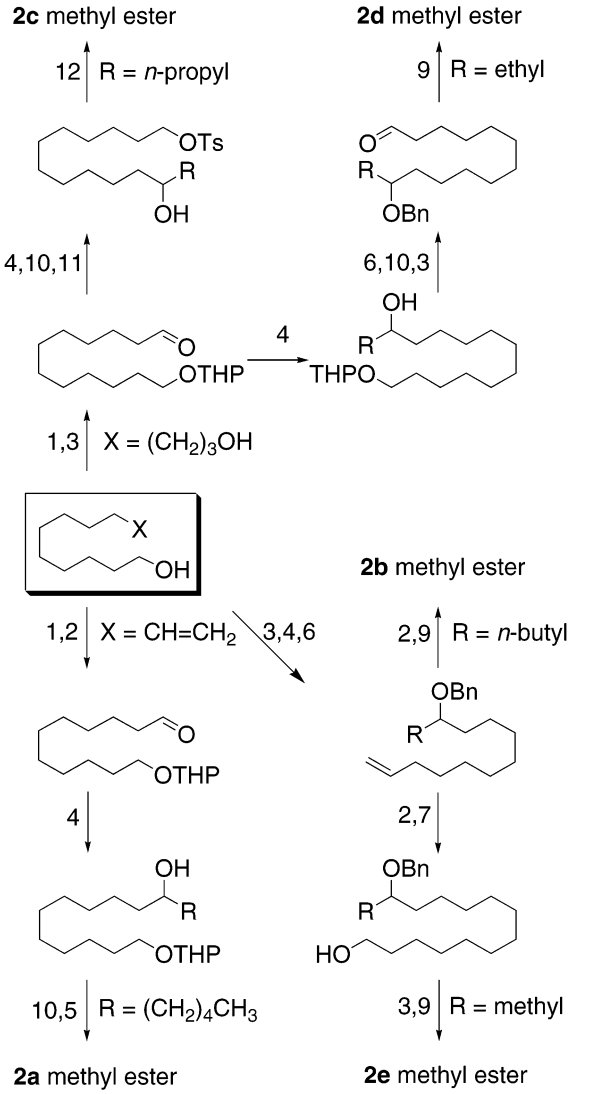
From our data, however, we were not able to exclude the possibility that *ω*-functionalization of the fatty acid was occurring. This would provide an additional path to pimelic acid for biotin biosynthesis and a possibly different biological role for $P450_{BioI}$ (*ω*-hydroxylation versus C−C bond cleavage). Thus, it was necessary to unambiguously identify all of the hydroxy fatty acids formed by $P450_{BioI}$ oxidation, and synthesis of a suite of hydroxy fatty acid standards was undertaken (Scheme 1).

The methyl esters of the required compounds (**1a**−**d** and **2a**−**e**) were accessed conveniently through standard transformations of the commercially available, terminally difunctionalized compounds, undec-10-en-1-ol and 1,12-dodecandiol. Use of these precursors allowed rapid assembly of all the required compounds via similar methodology (methyl esters of **2a**−**e** (Scheme 1), methyl esters of **1a**−**d** (see Supporting Information)). With the standards in hand, we were able to confirm the predicted trends in GC/MS behavior with respect to both retention time and MS fragmentation relative to the position of the hydroxyl on the fatty acid alkyl chain. GC/MS comparison of the synthetic standards and their TFA esters with the products of $P450_{BioI}$-catalyzed oxidation confirmed the presence of **1a**−**c** and **2a**−**e** within the enzymatic turnover mixtures of the C_{14} and C_{16} fatty acids, respectively.

Importantly, no product was observed within the detection limits of the GC/MS (<1% of total product) corresponding to the methyl ester of authentic 14-hydroxytetradecanoic acid **1d** in the tetradecanoic acid oxidations. There was also no unidentified peak in the hexadecanoic acid oxidations that may have been the methyl ester of 16-hydroxyhexadecanoic acid **2f**. These would be the products of *ω*-hydroxylation, and therefore $P450_{BioI}$ does not act as an *ω*-hydroxylase. Rather, it is only capable of in-chain hydroxylation at positions prior to the terminal carbon, as well as C−C bond cleavage to yield pimelic acid equivalents.

(6) Kawamura, K.; Gagosian, R. B. *J. Chrom.* **1988**, *438*, 309.
(7) Kerwin, J. L.; Torvik, J. J. *Anal. Biochem.* **1996**, *237*, 56.
(8) Wilson, R.; Smith, R.; Wilson, P.; Shepherd, M. J.; Riemersma, R. A. *Anal. Biochem.* **1997**, *248*, 76.

Scheme 1[a]



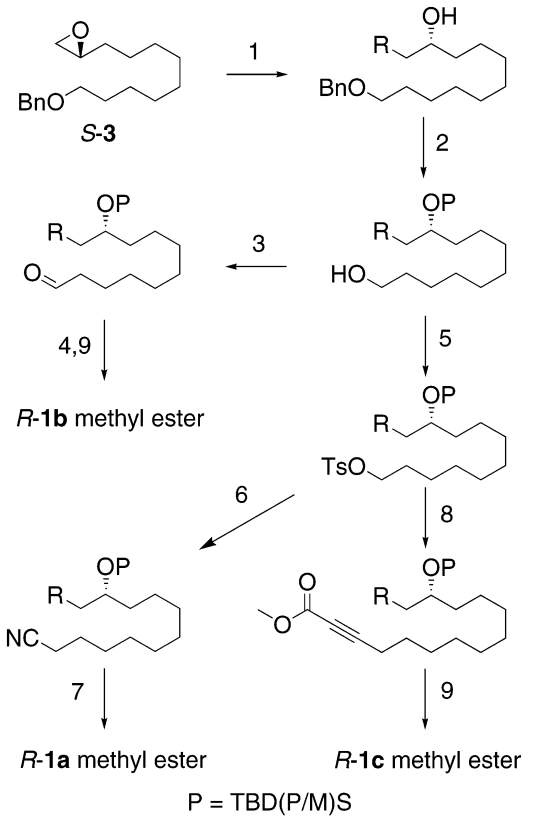
[a] Reagents and conditions: (1) Dihydropyran, H^+, CH_2Cl_2, 68%. (2) (a) BH_3·DMS, CH_2Cl_2; H_2O_2, Δ. (b) PCC, NaOAc, CH_2Cl_2, 67%. (3) PCC, CH_2Cl_2, 90%. (4) RMgBr, Et_2O, −40 °C, 70%. (5) (a) $CrO_3-H_2SO_4$, acetone. (b) CH_2N_2, Et_2O. (c) $NaBH_4$, MeOH, 0 °C, 45%. (6) NaH, BnBr, n-$Bu_4N^+I^-$, THF, 94%. (7) (a) $Ph_3P{=}CHCO_2Me$, CH_2Cl_2, Δ. (b) LAH, THF, 0 °C, 63%. (8) O_3, CH_2Cl_2, −78 °C, DMS, 100%. (9) (a) $Ph_3P{=}CHCO_2Me$, CH_2Cl_2, Δ. (b) H_2, Pd−C, hexane, 64%. (10) H^+, MeOH, 94%. (11) TsCl, pyridine, 0 °C, 67%. (12) (a) NaCN, DMF, Δ. (b) HCl−MeOH, 57%.

Table 1. Relative Products and Stereochemistry of Fatty Acid Hydroxylation by $P450_{BioI}$[a]

fatty acid	site of hydroxylation % (ee (*R*)-isomer)					
	ω-5	ω-4	ω-3	ω-2	ω-1	w
C_{14}	0	0	**1a**	**1b**	**1c**	**1d**
(±1%)			49 (59)	30 (72)	21 (75)	0
C_{16}	**2a**	**2b**	**2c**	**2d**	**2e**	**2f**
(±2%)	12 (40)	20 (40)	24 (55)	29 (75)	15 (nd)	0

[a] Percentage of products as determined by GC/MS analysis of the TFAI-derivatized methyl esters of C14 and C16 fatty acid oxidation. Enantiomeric excess as determined by chiral HPLC of the benzoyl fatty acid methyl esters.

Scheme 2[a]



[a] Reagents and conditions: (1) LAH, THF, 0 °C, 90%, or RMgX, THF, −10 °C, 79%. (2) (a) TBD(P/M)SCl, imidazole, MeCN. (b) H_2, Pd−C, hexane, 80%. (3) $(COCl)_2$, DMSO, CH_2Cl_2, TEA, −78 °C, 75%. (4) $Ph_3P{=}CHCO_2Me$, CH_2Cl_2, Δ, 94%. (5) TsCl, DABCO, CH_2Cl_2, 92%. (6) NaCN, DMF, Δ, 80%. (7) (a) HCl-MeOH, 59%. (8) Propiolic acid, n-BuLi, HMPA, THF, 0 °C, 71%. (9) (a) H_2, Pd−C, hexanes. (b) TBAF, THF, 0 °C, 74%.

The distribution of the hydroxy fatty acid products (Table 1) was slightly different for the two fatty acids examined. Hexadecanoic acid was metabolized relatively nonselectively with moderate levels of all products from ω-5- to ω-1-hydroxylation observed. Tetradecanoic acid was oxidized principally at the ω-3-position, but significant levels of ω-2- and ω-1-hydroxylation were also seen. $P450_{BM3}$, from *Bacillus megaterium*, also hydroxylates fatty acids and gives a range of ω-3- to ω-1-oxidized products with differences in the product distribution between the C_{14} and C_{16} fatty acids.[9−11] However, unlike $P450_{BioI}$, $P450_{BM3}$ turnover is highly efficient and produces no ω-5- or ω-4-oxidation with hexadecanoic acid.

We also wished to determine the enantioselectivity of the hydroxylation reactions catalyzed by $P450_{BioI}$. Scalemic

(9) Miura, Y.; Fulco, A. J. *Biochim. Biophys. Acta* **1975**, *388*, 305.

(10) Narhi, L. O.; Fulco, A. J. *J. Biol. Chem.* **1986**, *261*, 7160.

(11) Boddupalli, S. S.; Estabrook, R. W.; Peterson, J. A. *J. Biol. Chem.* **1990**, *265*, 4233.

standard samples of **1a**−**c** were synthesized from enantiomerically enriched (*R*)-10,11-epoxy-1-benzyloxyundecane **3**. This useful precursor was available via resolution of the racemic epoxide using Jacobsens's catalyst as previously reported by Kitching.[12] The epoxide (*S*)-**3** obtained via this procedure had an ee of 90% as determined by enantioselective HPLC (Chiracel OD). Conversion of (*R*)-**3** to the methyl esters of (*R*)-**1a**−**c** was again achieved by standard methods (Scheme 2). The methyl esters of **1a**−**c** thus obtained were converted into the corresponding benzoates **4a**−**c** to facilitate both detection and separation by enantioselective HPLC (Chiracel OD). Such analysis demonstrated that the conversion of **3** into the methyl esters of **1a**−**c** had occurred without loss of stereochemical purity. The conditions developed for this HPLC analysis allowed simultaneous quantification of the enantiomers of **4a**−**c**, albeit with incomplete resolution of (*R*)-**4a** and (*R*)-**4b**. It was found that the (*R*)-isomer of **4a**−**c** eluted before the (*S*)-isomer, and a scalemic sample of the benzoate of **2c** indicated that this appears to be true for the C_{16} series as well. Enantioselective HPLC analysis of the benzoate esters **4a**−**c** obtained from derivatization of the products of $P450_{BioI}$-catalyzed oxidation of tetradecanoic acid indicated that there was a moderate preference for formation of the (*R*)-isomer in all cases (Table 1). When the same analysis was carried out on the products (**2a**−**e**) of $P450_{BioI}$-catalyzed hexadecanoic acid oxidation, the complexity of the product mixture made accurate analysis of the ee for hydroxylation difficult. However, the same preference for production of the (*R*)-isomers was apparent. Interestingly, the enantioselectivity of oxidation in both cases appeared to decrease, as oxidation occurred further from the methyl terminus of the fatty acid. This same loss of enantioselectivity has previously been observed with the $P450_{BM3}$-catalyzed oxidation of hexadecanoic acid, where ω-1- and ω-2-oxidation is highly selective (R:S = 98:2) in comparison to ω-3-oxidation (R:S = 72:28).[13,14,15]

In summary, the major products of $P450_{BioI}$-catalyzed fatty acid oxidation have been identified as the hydroxy fatty acids **1a**−**c** from tetradecanoic acid and **2a**−**e** from hexadecanoic acid. ω-Hydroxylation does not occur, as has been previously suggested,[5] indicating that $P450_{BioI}$ cannot provide pimelic acid for biotin biosynthesis via this route. A C−C bond cleavage reaction, as suggested by our previous work, is clearly an attractive alternative for the production of a pimelic acid equivalent.[4] The enantioselectivity of the hydroxylation reactions catalyzed by $P450_{BioI}$ has also been established and shown to vary from moderate to poor. Investigations into the mechanism of formation of pimelic acid by $P450_{BioI}$-catalyzed oxidation will be reported in following communications.

Acknowledgment. The authors are grateful for an Australian Postgraduate Research Award to M.J.C.

Supporting Information Available: Synthesis and characterization of the methyl esters of compounds **1a**−**d** and **2a**−**e**; GC separation of derivatized C14 and C16 standards; and GC separation of derivatized C14 and C16 turnovers. This material is available free of charge via the Internet at http://pubs.acs.org.

OL035254E

(12) Chow, S.; Kitching, W. *Tetrahedron*: *Asymmetry* **2002**, *13*, 779.

(13) Truan, G.; Komandla, M. R.; Falck, J. R.; Peterson, J. A. *Arch. Biochem. Biophys.* **1999**, *366*, 192.

(14) Capdevila, J. H.; Wei, S.; Helvig, C.; Falck, J. R.; Belosludtsev, Y.; Truan, G.; Graham-Lorence, S. E.; Peterson, J. A. *J. Biol. Chem.* **1996**, *271*, 22663.

(15) Cowart, L. A.; Falck, J. R.; Capdevila, J. H. *Arch. Biochem. Biophys.* **2001**, *387*, 117.