Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2545–2548

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Studies on Phytanoyl-CoA 2-Hydroxylase and Synthesis of Phytanoyl-Coenzyme A

Nadia J. Kershaw[a,†], Mridul Mukherji[a,†] Colin H. MacKinnon,[a] Timothy D. W. Claridge,[a] Barbara Odell,[a] Anthony S. Wierzbicki,[b] Matthew D. Lloyd[a,*] and Christopher J. Schofield[a,*]

[a]*The Oxford Centre for Molecular Sciences and The Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, UK*
[b]*Department of Chemical Pathology, King's, Guy's and St. Thomas' Medical School, St Thomas' Hospital Campus, Lambeth Palace Road, London SE1 7EH, UK*

Received 25 April 2001; accepted 11 July 2001

Abstract—Phytanoyl-CoA 2-hydroxylase (PAHX), an iron(II) and 2-oxoglutarate-dependent oxygenase, catalyses an essential step in the mammalian metabolism of β-methylated fatty acids. Phytanoyl-CoA was synthesised and used to develop in vitro assays for PAHX. The product of the reaction was confirmed as 2-hydroxyphytanoyl-CoA by NMR and mass spectrometric analyses. In accord with in vivo analyses, hydroxylation of both 3*R* and 3*S* epimers of the substrate was catalysed by PAHX. Both pro- and mature- forms of PAHX were fully active.

Phytanic acid (**1**) in the human diet is derived via microbial cleavage of chlorophyll followed by reduction/oxidation of the resultant phytol side chain. The presence of the 3-methyl group in **1** prevents its normal degradation by the fatty acid β-oxidation pathway. Phytanic acid (**1**) is normally present in small amounts in human tissues, but accumulates in patients with Refsum's disease. Some of the symptoms of Refsum's disease can be ameliorated by treatment but others including blindness, anosmia and deafness are irreversible.[1]

It is possible that some metabolism of phytanic acid (**1**) occurs by an ω-oxidation pathway. However, the major pathway for its oxidation is in liver peroxisomes via a preliminary α-oxidation pathway in which one carbon atom is removed to give pristanic acid (**2**), which is then metabolised via the normal β-oxidation pathway (Scheme 1).[2,3] The sequence of events in the preliminary pathway has recently been elucidated as follows: (i) phytanic acid (**1**) is converted to phytanoyl-CoA (**3**) probably by a non-specific ligase that may be associated with the peroxisomal membrane;[4] (ii) phytanoyl-CoA (**3**) is then oxidised to give 2-hydroxyphytanoyl-CoA (**4**) in a step catalysed by phytanoyl-CoA 2-hydroxylase (PAHX);[3,5] (iii) the TPP-dependent enzyme 2-hydroxyphytanoyl-CoA lyase catalyses the unusual conversion of (**4**) to pristanal (**5**) and formyl-CoA;[6] (iv) finally, pristanal (**5**) is oxidised, in an NAD^+-dependent reaction, to give pristanic acid (**2**).[7]

Comparison of the sequence and predicted structures for PAHX suggests it is a member of the super-family of 2-oxoglutarate-dependent oxygenases.[8–10] Conserved motifs present in PAHX include the iron binding HXD motif encompassing residues His-175 and Asp-177 in the human PAHX sequence. Recently, Mihalik et al. reported the cloning and expression in *Escherichia coli* of the gene (*pahx*) for PAHX.[3] PAHX, with its peroxisomal targeting sequence attached, was produced in *E. coli* as a fusion protein (using the pMALc expression vector). This enzyme was shown to catalyse the conversion of **3** to **4** in the presence of ferrous ions and 2-oxoglutarate by an HPLC assay using radioactive phytanoyl-CoA (**3**)[3] prepared by enzyme-catalysed condensation of phytanic acid (**1**) and coenzyme A. Here, we report the chemical synthesis of phytanoyl-CoA (**3**) and its use to study the reaction of recombinant wild-type pro- and mature-PAHX.

*Corresponding authors. Tel.: +44-1865-275625; fax: +44-1865-275674; e-mail: matthew.lloyd@chem.ox.ac.uk; christopher.schofield@chem.ox.ac.uk

†Nadia J. Kershaw and Mridul Mukherji contributed equally to this work.

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00494-2

Chemistry

Phytanic acid (**1**) was synthesised from an *E*/*Z* mixture of phytol (**6**) according to a modification of the published procedure.[11] Hydrogenation of the double bond to (3*RS*)-phytanol (**7**) was achieved using Rh/alumina in methanol. This procedure reduced the proportion of hydrogenolysis of the allylic alcohol obtained using PtO_2 in ethanol.[11] In contrast to the reported use of Jones reagent, oxidation of (3*RS*)-phytanol (**7**) to phytanic acid (**1**) was achieved by initial reaction with TPAP/NMO[12,13] to give a crude mixture of aldehyde and acid products (approx. 2:1), which was immediately treated with potassium permanganate in aqueous acetone to effect oxidation of residual aldehyde.

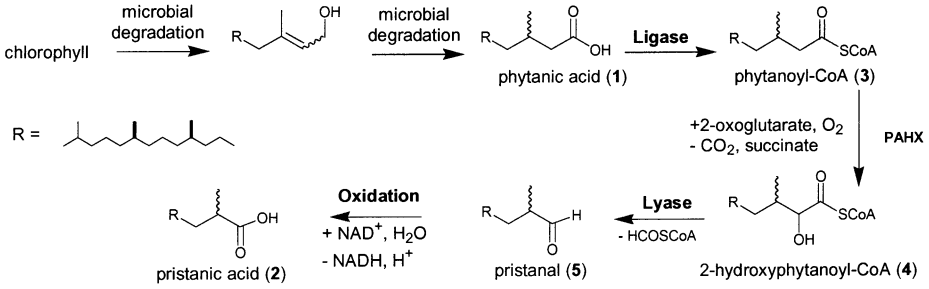
Preliminary attempts at the coupling of phytanic acid (**1**) with the lithium salt of CoASH using the *N*-hydroxysuccinimide ester of phytanic acid prepared using DCCI/NHS[14] led to poor yields. This may have reflected difficulties in purifying the product via reported procedures for fatty acid CoA derivatives[15–17] probably due to the amphiphilic nature of the product and, possibly, steric hindrance in the coupling reaction. Subsequently, carbonyl diimidazole was successfully used to directly couple phytanic acid (**1**) and CoASH.[18] The crude product was solubilised with the aid of β-cyclodextrin and purified by reverse-phase HPLC (Scheme 2).[19]

Incubation of Phytanoyl-CoA with PAHX

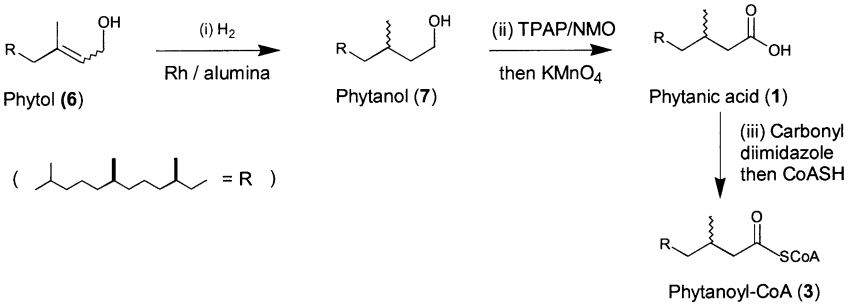
The *pahx* gene was cloned from a human liver cell cDNA library and expressed in *E. coli*. Pro-PAHX (i.e., with an N-terminal PTS-2 signal sequence) was produced as an N-terminal MBP-fusion,[3] whilst mature PAHX was prepared without a fusion 'tag'. Both forms of the enzyme were purified to >95% purity (by SDS-PAGE analysis).[20]

Initial assays measured 2-oxoglutarate oxidation[21] in order to test with highly purified PAHX if phytanic acid (**1**) was a substrate and to confirm the conversion of **3**. These demonstrated that 2-oxoglutarate conversion was enhanced in the presence of phytanoyl-CoA (**3**), but not phytanic acid (**1**), indicating that only the former is a substrate. Phytanoyl-CoA (**3**) was then incubated with both pro- and mature-PAHX.[22] We used a modification of the method of Mihalik et al. to assay for conversion of epimeric phytanoyl-CoA (**3**) to the hydroxylated product.[22] Optimised conditions included ATP[23] as well as tris(carboxyethyl)phosphine in place of dithiothreitol.[24] Reverse-phase HPLC analysis of the reaction mixtures revealed the presence of a new peak, absent from negative controls, with a retention volume of 16 mL compared to that of 18 mL for the starting material. The intensity of the new peak increased with increasing enzyme concentration and incubation times.

Multiple incubations were used to produce enough material for characterisation by ^{1}H NMR and electrospray ionisation mass spectrometry (ESI MS).[25] Product was again isolated by reverse-phase HPLC, Negative ion ESI MS analysis was consistent with formation of 2-hydroxyphytanoyl-CoA (**4**): (*m*/*z*: 1076 $[M-H]^-$). Analyses by 1-D and 2-D ^{1}H NMR (500 MHz) confirmed the product as 2-hydroxyphytanoyl-CoA (**4**). Selected NMR data: Starting material, phytanoyl-CoA



Scheme 1. Metabolism of phytanic acid (**1**).



Scheme 2. Synthesis of phytanoyl-CoA (**3**). Reagents: (i) rhodium on alumina, MeOH (60%); (ii) tetrapropylammonium perruthenate (TPAP) and *N*-methylmorpholine *N*-oxide (NMO) in CH_3CN then $KMnO_4$ in acetone/H_2O (80%); (iii) carbonyl diimidazole, THF, then coenzyme A (CoASH) in THF/H_2O (2:1) (50% after HPLC purification).

(**3**): δ 2.58 and 2.39 (2×1H, CH_2C(O)SCoA); product, 2-hydroxyphytanoyl-CoA (**4**): δ 4.12, 1H, C*H*(OH)-C(O)SCoA (Fig. 1). Note that the ^{1}H NMR signals for the 3*R*/3*S* epimers of the starting material and those for the two diastereoisomeric products were coincident.

Under appropriate conditions, that is with high enzyme:substrate ratios, >95% conversion of the 3-epimeric phytanoyl-CoA (**3**) to products could be achieved, demonstrating both epimers to be substrates for PAHX. Previous in vivo studies using rat liver are consistent with this observation,[26] implying both 3*R*- and 3*S*-methylhexadecanoic acid are equally well metabolised. The earlier work also elegantly demonstrated that the 3*R* epimer is converted to the 2*S*,3*R* *threo* product and the 3*S* epimer to the 2*R*,3*S* *threo* product. The observation that both epimers of the natural substrate are substrates indicates that PAHX activity is due to a single enzyme that is responsible for the metabolism of both stereoisomers. Further, it seems that the individual epimers are converted to the products with the same relative stereochemistry. With the obvious exception of racemases/epimerases, most enzymes, including non-haem oxygenases are highly stereoselective.[27] It will be interesting to study how PAHX binds its epimeric susbtrate. It is difficult to envisage how a 'rigid' three point attachment of CoA, β-methyl and hydrocarbon side-chain can lead to the observed selectivity. One possibility is that a two point attachment occurs, most probably involving selective binding of the β-methyl group, but only one of the CoA and hydrocarbon side-chain moieties.

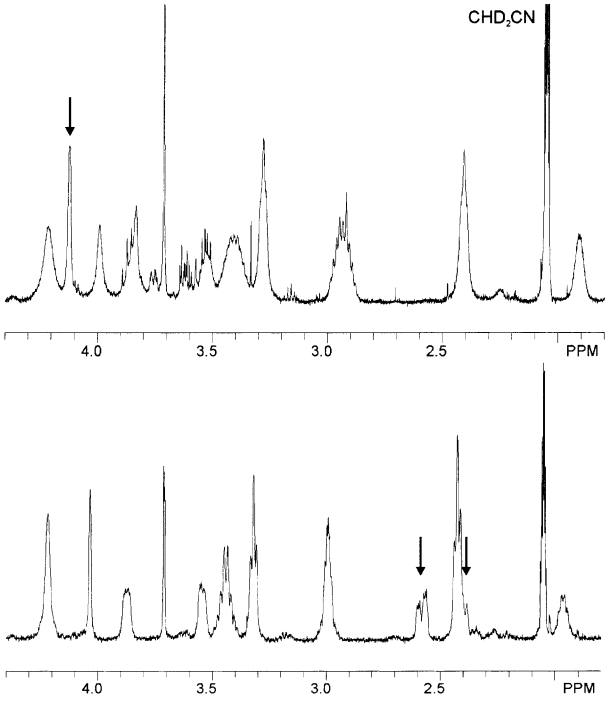


Figure 1. Partial ^{1}H NMR spectra (500 MHz, CD_3CN–D_2O, recorded using a 3 mm microprobe) of phytanoyl-CoA (**3**) (lower trace) and 2-hydroxyphytanoyl-CoA (**4**) (upper trace). Arrows indicate the resonance changes discussed in the text. The broad lines observed result from the poor solubility and probable aggregation of these materials due to the presence of both hydrophobic and hydrophilic moieties.

Phytanic acid (**1**) in the mammalian diet is present as a mixture of epimers and it seems PAHX has evolved to accept both substrates with approximately equal efficiency. The results for PAHX also suggest that the next two reactions in the β-oxidation pathway are non-stereoselective, that is the lyase will accept both (2*S*,3*R* and 2*R*,3*S*) stereoisomers of 2-hydroxyphytanoyl-CoA (**4**) and the oxidoreductase will accept both 2*R* and 2*S* pristanal to give 2*R*/2*S* pristanic acid (**2**). However, only the 2*S* stereoisomer of pristanic acid (**2**) is processed via the α-oxidation pathway,[26] and thus the results are consistent with the presence of a racemase. The recent characterisation of an α-methylacyl-CoA racemase deficiency[28] supports this hypothesis.

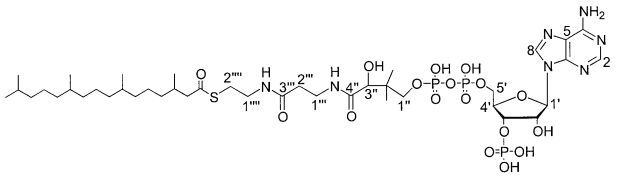
Acknowledgements

We thank Dr. R. T. Aplin for mass spectrometric analyses and Dr. V. Lee for helpful discussions and also the BBSRC, MRC, EPSRC, Felix foundation (scholarship to MM), E.U. Biotechnology project and the Wellcome Trust for financial support of the work.

References and Notes

1. Wanders, R. J. A.; Jakobs, C.; Skjelda, O. M. Refsum's disease. In *The Metabolic and Molecular Basis of Inherited Disease*; Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Childs, B., Kinzler, K. W., Vogelstein, B., Eds.; McGraw Hill: New York, 2001; pp 3303–3321.
2. Herndon, J. H.; Steinberg, D.; Uhlendorf, B. W.; Fales, H. M. *J. Clin. Invest*. **1969**, *48*, 1017.
3. Mihalik, S. J.; Morrell, J. C.; Kim, D.; Sacksteder, K. A.; Watkins, P. A.; Gould, S. J. *Nat. Genet*. **1997**, *17*, 185 and references therein.
4. Steinberg, S. J.; Wang, S. J.; Kim, D. G.; Mihalik, S. J.; Watkins, P. A. *Biochem. Biophys. Res. Commun*. **1999**, *257*, 615.
5. Jansen, G. A.; Ferdinandusse, S.; Ijlst, L.; Muijsers, A. O.; Skjeldal, O. H.; Stokke, O.; Jakobs, C.; Besley, G. T. N.; Wraith, J. E.; Wanders, R. J. A. *Nat. Genet*. **1997**, *17*, 190.
6. Foulon, V.; Antonenkov, V. D.; Croes, K.; Waelkens, E.; Mannaerts, G. P.; Van Veldhoven, P. P.; Casteels, M. *Proc. Natl. Acad. Sci. U.S.A*. **1999**, *96*, 10039.
7. Verhoeven, N. M.; Schor, D. S. M.; ten Brink, H. J.; Wanders, R. J. A.; Jakobs, C. *Biochem. Biophys. Res. Commun*. **1997**, *237*, 33.
8. Lloyd, M. D.; Lee, H.-J.; Harlos, K.; Zhang, Z.-H.; Baldwin, J. E.; Schofield, C. J.; Charnock, J. M.; Garner, C. D.; Hara, T.; Terwisscha van Scheltinga, A. C.; Valegard, K.; Viklund, J. A. C.; Hajdu, J.; Andersson, I.; Danielsson, A.; Bhikhabhai, R. *J. Mol. Biol*. **1999**, *287*, 943.
9. Valegård, K.; Terwisscha van Scheltinga, A. C.; Lloyd, M. D.; Hara, T.; Ramaswamy, S.; Perrakis, A.; Thompson, A.; Lee, H.-J.; Baldwin, J. E.; Schofield, C. J.; Hajdu, J.; Andersson, I. *Nature* **1998**, *394*, 805.
10. Zhang, Z.-H.; Ren, J.; Stammers, J. K.; Baldwin, J. E.; Harlos, K.; Schofield, C. J. *Nature Struct. Biol*. **2000**, *7*, 127.
11. Steinberg, D.; Avigan, J.; Mize, C. E.; Baxter, J. H.; Cammermeyer, J.; Fales, H. M.; Highet, P. F. *J. Lipid Res*. **1966**, *7*, 684.
12. Ley, S. V.; Norman, J.; Griffith, W. P.; Marsden, S. P. *Synthesis* **1994**, 639.
13. Xu, Z. M.; Johannes, C. W.; Houri, A. F.; La, D. S.; Cogan, D. A.; Hofilena, G. E.; Hoveyda, A. H. *J. Am. Chem. Soc*. **1997**, *119*, 10302.

14. Al-Arif, A.; Blecher, M. *J. Lipid Res*. **1969**, *10*, 344.
15. Seubert, W. *Biochem. Prep*. **1960**, *7*, 80.
16. Stadtman E. R. In *Methods in Enzymology*; Colonwick, S. P., Kaplan, N. O., Eds.; Academic: New York, 1957; Vol. 3, p 931.
17. Pullman, M. E. *Anal. Biochem*. **1973**, *54*, 188.
18. Kawaguchi, A.; Yoshimura, T.; Okuda, S. *J. Biochem*. **1981**, *89*, 337.
19. To a stirred solution of carbonyl diimidazole (20 mg, 120 μmol) in THF (2 mL) was added phytanic acid (36 mg, 100 μmol) in THF (2 mL). After 2 h the solvent was removed in vacuo and the residue dissolved in 2:1 THF–water (1 mL). A solution of the lithium salt of coenzyme A (80 mg, 100 μmol) in 2:1 THF–water (2.5 mL) was added and the reaction stirred under argon for 18 h. THF was removed in vacuo and the residue diluted with water (2 mL). The pH was adjusted to pH 3–4 by addition of Dowex-50 resin then filtered. The filtrate was lyophilised and the white solid triturated with EtOAc (10 mL) to remove any unreacted phytanic acid. The crude product was collected by filtration, dissolved in 5 mM Tris–HCl, pH 8, containing 5 mM ammonium bicarbonate in 5% (v/v) acetonitrile, and solubilised with β-cyclodextrin, with sonication for 5 min. This solution was purified by HPLC using a 10×250 mm Hypersil ODS reverse phase column. Samples were eluted with a linear gradient from 10 mM ammonium bicarbonate in 10% (v/v) acetonitrile to 50% (v/v) acetonitrile at a flow rate of 4 mL/min over 10 min. This was followed by an isocratic gradient for 10 min, at 4 mL/min, and a linear gradient from 50% (v/v) acetonitrile to 10 mM ammonium bicarbonate in 10% (v/v) acetonitrile over 2 min to return to the initial conditions. Absorbance of samples was monitored by ultraviolet absorbance of the adenine group of CoASH at 254 nm. The fraction eluting at 14 min was evaporated in vacuo to remove acetonitrile and lyophilised to give phytanoyl-CoA (60 mg, 50%) as a white solid.



^{1}H NMR (500 MHz), CD_3CN–D_2O: 0.75 (3H, s, 2″-CCH_3), 0.85–0.95 (18H, m, 5×CH_3, 2″-CCH_3), 1.55 (1H, m, C*H*(CH_3)$_2$), 1.95 (1H, m, C*H*(CH_2C(O)S), 2.41 (3H, m, 2‴-CH_2, 1 of CH_2C(O)S), 2.58 (1H, m, 1 of CH_2C(O)S), 2.98 (2H, m, 2⁗-CH_2), 3.30 (2H, m, 1⁗-CH_2), 3.40 (2H, m, 1‴-CH_2), 3.54 (1H, m, 1″-CH_2), 3.85 (1H, m, 1″-CH_2), 4.02 (1H, s, 3″-CH), 4.24 (2H, m, 5′-CH_2), 4.55 (1H, m, 4′-CH), 4.82 (2H, m, 2′ and 3′-CH), 6.12 (1H, d, 1′-CH), 8.23 (1H, s, 8-CH), 8.52 (1H, s, 2-CH). A 2-D COSY spectrum was consistent with the connectivity. *m*/*z* (−ve ESI-MS): 1060 [M−H]$^-$.

20. Mukherji, M.; Kershaw, N. J.; Chien, W.; Clifton, I. J.; Schofield, C. J.; Wierzbicki, A. S.; Lloyd, M. D. *Hum. Mol. Gen*. **2001**, in press.
21. Lee, H.-J.; Lloyd, M. D.; Harlos, K.; Schofield, C. J. *Biochem. Biophys. Res. Commun*. **2000**, *267*, 445 and references therein.
22. Mihalik, S. J.; Rainville, A. M.; Watkins, P. A. *Eur. J. Biochem*. **1995**, *232*, 545.
23. Croes, K.; Foulon, V.; Casteels, M.; Van Veldhoven, P. P.; Mannaerts, G. P. *J. Lipid Res*. **2000**, *41*, 629.
24. Mukherji, M.; Kershaw, N. J.; MacKinnon, C. H.; Clifton, I. J.; Schofield, C. J.; Wierzbicki, A. S.; Lloyd, M. D. *J. Chem. Soc., Chem. Commun*. **2001**, 972.
25. Incubations for product isolation were performed as described[20] using 50×100 μL incubations containing 6.4 nmol/min of PAHX for 60 min. HPLC analysis suggested a >95% conversion to product. Product was isolated by reverse phase HPLC (ODS Hypersil, 250×4.6 mm). Samples were eluted using a flow rate of 1 mL/min and a linear gradient of 30–15 mM NH_4OAc and 35–75% (v/v) acetonitrile over 10 min, reverting to 30 mM NH_4OAc and 35% (v/v) acetonitrile over 2 min and re-equilibrating under these conditions for a further 8 min. Product eluted with a retention volume of 16 mL. A ^{1}H NMR (COSY) spectrum was consistent with the proposed connectivity.
26. Croes, K.; Casteels, M.; Dieuaide-Noubhani, M.; Mannaerts, G. P.; Van Veldhoven, P. P. *J. Lipid Res*. **1999**, *40*, 601.
27. Prescott, A. G.; Lloyd, M. D. *Nat. Prod. Rep*. **2000**, *17*, 367.
28. Ferdinandusse, S.; Denis, S.; Clayton, P. T.; Graham, A.; Rees, J. E.; Allen, J. T.; McLean, B. N.; Brown, A. Y.; Vreken, P.; Waterham, H. R.; Wanders, R. J. A. *Nature Genet*. **2000**, *24*, 188.