Human carnitine biosynthesis proceeds via (2S,3S)-3-hydroxy-\(N^e\)-\(\text{trimethyllysine}\)\(^\dagger\)

Robert K. Leśniak,\(^a\) Suzana Markolovic,\(^a\) Kaspars Tars\(^b\) and Christopher J. Schofield\(^{a,3}\)

\(\text{N}^e\)-\(\text{Trimethyllysine hydroxylase (TMLH)}\) catalyses the first step in mammalian biosynthesis of carnitine, which plays a crucial role in fatty acid metabolism. The stereochemistry of the 3-hydroxy-\(\text{N}^e\)-\(\text{trimethyllysine}\) product of TMLH has not been defined. We report enzymatic and asymmetric synthetic studies, which define the product of TMLH catalysis as (2S,3S)-3-hydroxy-\(\text{N}^e\)-\(\text{trimethyllysine}\).

Carnitine plays key roles in mammalian metabolism by enabling the transport of fatty acids into mitochondria as O-acyl carnitine esters and in maintaining acetyl group homeostasis.\(^1^-^3\) There is considerable biomedical interest in carnitine and its biosynthesis. Carnitine is biosynthesised from (2S)-\(\text{N}^e\)-\(\text{trimethyllysine}\) (TML, (1)),\(^4\) which is derived from naturally-occurring TML residues in proteins following proteolysis.\(^5^-^7\) Two 2-oxoglutarate (2OG)-dependent oxygenases, \(\text{N}^e\)-\(\text{trimethyllysine hydroxylase (TMLH)}\) and \(\gamma\)-\(\text{butyrobetaine hydroxylase (BBOX)}\), catalyse the first and final steps of carnitine biosynthesis, respectively (Fig. 1).\(^8^-^9\) BBOX is one of the proposed targets of Meldonium (Mildronate, THP, Met-88),\(^10^-^{11}\) a drug that is used for treatment of cardiovascular disease\(^12\) and by athletes due to perceived performance-enhancing properties.\(^13^-^{14}\) Carnitine is proposed to promote atherosclerosis by acting as a precursor for trimethylamine oxide.\(^15\) There are also reported links between TMLHE gene mutations and autism in males.\(^16^-^{18}\) Whilst BBOX has been extensively characterised, including by detailed kinetic and biophysical studies,\(^19^-^{20}\) relatively little is reported on TMLH,\(^21\) notably including on the stereochemistry of the product of its catalysis.

To define the stereochemistry of the TMLH-catalysed 3-hydroxy-\(\text{N}^e\)-\(\text{trimethyllysine}\) (3HO-TML) product (2), we investigated the asymmetric synthesis of (2S,3R)-3HO-TML (13) (Scheme 1).

\(^a\) Department of Chemistry, University of Oxford, Chemistry Research Laboratory, 12 Mansfield Road, Oxford OX1 3TA, UK. E-mail: christopher.schofield@chem.ox.ac.uk

\(^b\) Biomedical Research and Study Centre, Ratsupites 1, LV1067 Riga, Latvia

\(^\dagger\) Electronic supplementary information [ESI] available: Synthesis procedures, assay conditions, NMR assignments and spectra, and MS analyses. See DOI: 10.1039/c6cc08381a

We employed the Dixon methodology,\(^22\) which involves Ag(i)-catalysed aldol-type reactions in the presence of a cinchona alkaloid-derived pre-catalyst (7).\(^22^-^{23}\) We envisaged this approach could enable the requisite introduction of differently protected \(\text{N}^e\)- and \(\text{N}^e\)-amino groups in a precursor of (13). Thus, dibenzyl aldehyde (6), prepared in two steps from (5), was reacted with tert-butyl isocyanocacetate in the presence of Ag2O and the pre-catalyst (7) to yield trans-oxazoline (8) (\(J_{2-3} = 7.0\) Hz)\(^22^-^{24}\) in good yield (78%; Scheme 1).

Importantly, high diastereoselectivity favouring the trans-diastereomers (2S,3R/2R,3S : 2S,3S/2R,3R; d.r. > 95:1) of (8) was observed (2S,3R : 2R,3S : 3 : 1 inferred from analyses on (9)). Oxazoline (8) was unstable at room temperature (and over prolonged periods at \(-20^\circ\)C), decomposing to give formamide (9). We found that conversion of (8) to (9) is promoted by aqueous citric acid, as reported for other oxazolines,\(^22\) or by aqueous acetic acid in THF in near quantitative yield (Scheme 1). The stereochemistry of the major stereoisomer of formamide (9)
was assigned as (2S,3R) by $^1$H NMR analysis of the corresponding Mosher’s esters (Fig. S1, ESI$^\dagger$). (2S,3R)-3-Hydroxylysine (3HO-Lys) (11) was efficiently obtained from (8) using H$_2$/Pd/C in aqueous citric acid followed by the removal of formamide and tert-butyl ester protecting groups via acid hydrolysis to give (11) (Scheme 1). The reduction and hydrolysis steps to give (10) from (8) via (9) were initially carried out separately; however, use of MeOH/5% citric acid as a solvent during hydrogenation enabled one-pot conversion of (8) to (10) in high yield (96%). Comparison of the optical rotation of 3HO-Lys (11) with that of enantiopure (25,3S)-3-hydroxy-L-lysine (13) and (2S,3R)-3-hydroxylysine (11) via oxazoline (8). The oxazoline ring hydrolysis and reduction steps can be carried out separately or via a one-pot reaction, as displayed.

Scheme 1  Stereoselective synthesis of (2S,3R)-3-hydroxy-N'-trimethyllysine (13) and (2S,3R)-3-hydroxylysine (11) via oxazoline (8). The oxazoline ring hydrolysis and reduction steps can be carried out separately or via a one-pot reaction, as displayed.

Fig. 2  TMLH catalysis produces (2S,3S)-3-hydroxy-N'-trimethyllysine (14). (A) $^1$H NMR assignment of the product resulting from TMLH-catalysed C-3 hydroxylation of (2S)-N'-trimethyllysine (1). Superimposition of $^1$H NMR spectra of the reaction mixture before (blue) and after (red) addition of TMLH shows 3HO-TML formation. Signals arising between $\delta = 3.5–3.75$ ppm (including glycerol) are omitted for clarity. (B) Overlaid extracted ion chromatograms ($m/z = 375.2$, corresponding to the mass of derivatised 3HO-TML) for (i) TML incubated with (red) or without (black; at baseline) TMLH and (ii) TMLH-treated TML (red) and TMLH-treated TML spiked with synthetic (2S,3R)-3HO-TML (13), black). (C) The stereochemistry of TMLH- and BBOX-catalysed hydroxylation is the same relative to the quaternary ammonium and carboxylate groups.

The overall results define the stereochemical outcome of the TMLH-catalysed hydroxylation of TML as (2S,3S)-3-hydroxy-N'-trimethyllysine (14). Interestingly, BBOX catalyses hydroxylation of $\gamma$-butyrobetaine (3) to give carnitine (4) with the (3R)-stereochemistry (Fig. 1). Thus, the stereochemical outcomes of TMLH and BBOX catalysis are the same relative to the trimethylammonium and carboxylic acid groups (Fig. 2C), reflecting the likely common evolutionary origins of TMLH and BBOX, as revealed by structural analyses$^{19,29,30}$.
The results also highlight the continued important role of synthesis, including via efficient asymmetric catalysis, for biomolecular structural assignments. Modern proteomic and other mass spectrometry (MS) methodologies are identifying many new potential post-translational modifications (e.g. JMD4-catalysed formation of C-4 hydroxylsine), the regio- and stereochemistries of which need to be confirmed, e.g. by NMR, high resolution MS analyses and, at least in our view, wherever possible by comparison with synthetic standards.

We thank the Wellcome Trust, Biotechnology and Biological Sciences Research Council, the British Heart Foundation (R. K. L.), and the Clarendon Fund (S. M.) for funding. We thank Dr Jürgen Brem, Dr Michael A. McDonough, and Dr Sarah E. Wilkins for helpful advice.

Notes and references

1. R. R. Ramsay, R. D. Gandour and F. R. van der Leij, Biochim. Biophys. Acta, 2001, 1546, 21–43.
2. J. D. McGarry, Biochem. Soc. Trans., 1995, 23, 321–324.
3. C. Hoppel, Am. J. Kidney Dis., 2003, 41, S4–S12.
4. D. S. Sachan and C. L. Hoppel, Biochem. J., 1980, 188, 529–534.
5. J. Llabadie, W. A. Dunn and N. N. Aronson, Jr., Biochem. J., 1976, 160, 85–95.
6. W. A. Dunn, G. Rettura, E. Seifert and S. England, J. Biol. Chem., 1984, 259, 10764–10770.
7. L. Servillo, A. Giovane, D. Castaldo and M. L. Balestrieri, Plos One, 2014, 9, e84589.
8. R. P. Hausinger, Crit. Rev. Biochem. Mol. Biol., 2004, 39, 21–68.
9. C. Loenarz and C. J. Schofield, Nat. Chem. Biol., 2008, 4, 152–156.
10. E. Liepinsh, R. Vilskerts, D. Loca, O. Kirjanova, O. Pugovics, I. Kalvinsh and M. Dambrova, J. Cardiovasc. Pharmacol., 2006, 48, 314–319.
11. E. Liepinsh, I. Konrads, E. Skapare, O. Pugovics, S. Grinberga, J. Kuka, I. Kalvinsh and M. Dambrova, J. Pharm. Pharmacol., 2013, 65, 1195–1201.
12. V. Dzerve and M. I. S. Group, Medicina, 2011, 47, 544–551.
13. C. Gorgens, S. Guédart, J. Dib, H. Geyer, W. Schanzer and M. Thevis, Drug Test. Anal., 2015, 7, 973–979.
14. H. K. Greenblatt and D. J. Greenblatt, Clin. Pharmacol. Drug Dev., 2016, 5, 167–169.
15. R. A. Koeth, Z. Wang, B. S. Levison, J. A. Buffa, E. Org, B. T. Sheehy, E. B. Britt, X. Fu, Y. Wu, L. Li, J. D. Smith, J. A. DiDonato, J. Chen, H. Li, G. D. Wu, J. D. Lewis, M. Warrier, J. M. Brown, R. M. Krauss, W. H. Tang, F. D. Bushman, A. J. Lusis and S. L. Hazen, Nat. Med., 2013, 19, 576–585.
16. R. O. Rostl, A. A. Sadek, K. K. Vaux and J. G. Gleeson, Dev. Med. Child Neurol., 2014, 56, 12–18.
17. C. Nava, F. Lamari, D. Heron, C. Mignot, A. Rastetter, B. Keren, D. Cohen, A. Faudet, D. Bouteiller, M. Gilleron, A. Jacquette, S. Whalen, A. Afenjar, D. Perisse, C. Laurent, C. Dupuits, C. Gautier, M. Gerard, G. Huguet, S. Caillat, B. Leheup, M. Leboyer, C. Gillberg, R. Delorme, T. Bourgeron, A. Brice and C. Depienne, Transl. Psychiatry, 2012, 2, e179.
18. P. B. Celestino-Soper, C. A. Shaw, S. J. Sanders, J. Li, M. T. Murtha, A. G. Ercan-Sencicek, L. Davis, S. Thomson, T. Gambin, A. C. Chinault, Z. Ou, J. R. German, A. Milosavljevic, J. S. Sutcliffe, E. H. Cook, Jr., P. Stankiewicz, M. W. State and A. L. Beaudet, Hum. Mol. Genet., 2011, 20, 4360–4370.
19. M. A. McDonough, C. Loenarz, R. Chowdhury, I. J. Clifton and C. J. Schofield,Curr. Opin. Struct. Biol., 2010, 20, 659–672.
20. A. M. Rydzik, I. K. Leung, G. T. Kochan, N. D. Loik, L. Henry, M. A. McDonough, T. D. Claridge and C. J. Schofield, Org. Biomol. Chem., 2014, 12, 6354–6358.
21. A. H. K. Al Temimi, B. J. G. E. Pieters, Y. Reddy Vijayendar, P. B. White and J. Mecinovic, Chem. Commun., 2016, 52, 12849–12852.
22. F. Sladojevich, A. Trabocchi, A. Guarna and D. J. Dixon, J. Am. Chem. Soc., 2011, 133, 1710–1713.
23. A. Franchino, P. Jakubec and D. J. Dixon, Org. Biomol. Chem., 2016, 14, 93–96.
24. P. F. Hughes, S. H. Smith and J. T. Olson, J. Org. Chem., 1994, 59, 5799–5802.
25. H. S. M. A. Dale, J. Am. Chem. Soc., 1973, 95, 512–519.
26. T. R. Hoyer, C. S. Jeffrey and F. Shao, Nat. Protoc., 2007, 2, 2451–2458.
27. C. E. Masse, A. J. Morgan and J. S. Panek, Org. Lett., 2000, 2, 2571–2573.
28. A. Kazaks, M. Makrecka-Kuka, J. Kuka, T. Voronkova, I. Akopjana, S. Grinberga, O. Pugovics and K. Tars, Protein Expression Purif., 2014, 104, 1–6.
29. I. K. Leung, T. J. Krojer, G. T. Kochan, L. Henry, F. von Delft, T. D. Claridge, U. Oppermann, M. A. McDonough and C. J. Schofield, Chem. Biol., 2010, 17, 1316–1324.
30. K. Tars, J. Rummieks, A. Zeltins, A. Kazaks, S. Ketelovica, A. Leonciks, J. Sharipo, A. Viksna, J. Kuka, E. Liepinsh and M. Dambrova, Biochem. Biophys. Res. Commun., 2010, 398, 634–639.
31. T. Feng, A. Yamamoto, S. E. Wilkins, E. Sokolova, L. A. Yates, M. Munzel, P. Singh, R. J. Hopkins, R. Fischer, M. E. Cockman, J. Shelley, D. C. Trudgian, J. Schodel, J. S. O. McCullagh, W. Ge, B. M. Kessler, R. J. Gilbert, L. Y. Frolova, E. Alkalaeva, P. J. Ratcliffe, C. J. Schofield and M. L. Coleman, Mol. Cell, 2014, 53, 645–654.
32. S. Markolovic, S. E. Wilkins and C. J. Schofield, J. Biol. Chem., 2015, 290, 20712–20722.