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Evidence That Trimethyllysine Hydroxylase Catalyzes the Formation of (2*S*,3*S*)-3-Hydroxy-*N*$^{\epsilon}$-trimethyllysine

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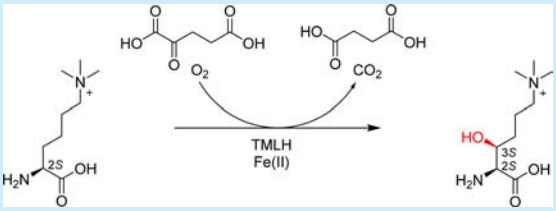
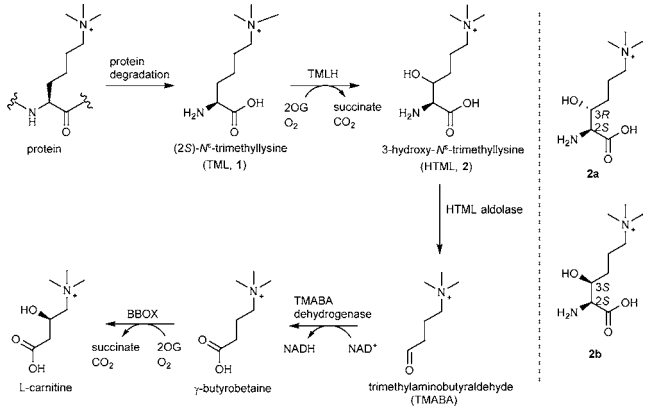
Supporting Information

ABSTRACT: Trimethyllysine hydroxylase (TMLH) is an Fe(II) and 2-oxoglutarate (2OG) dependent oxygenase involved in the biomedically important carnitine biosynthesis pathway. A combination of synthetic and NMR studies provides direct evidence that human TMLH catalyzes the stereoselective conversion of (2*S*)-*N*$^{\epsilon}$-trimethyllysine to (2*S*,3*S*)-3-hydroxy-*N*$^{\epsilon}$-trimethyllysine.



Enzymes have time and again served as an immense source of inspiration for organic chemists, most notably in the rational design of small molecular systems that catalyze challenging chemical transformations.[1] In the past decade, a special effort has been made in developing bioinspired, selective transformations of highly abundant and inert C−H bonds.[2] Members of Fe(II) and 2-oxoglutarate-dependent oxygenases catalyze stereoselective hydroxylations of unactivated C−H bonds in various (bio)molecules, including proteins, DNA, and small molecule metabolites.[3] Pioneering biochemical studies on trimethyllysine hydroxylase (TMLH), an Fe(II)/2OG oxygenase involved in the first step of physiologically important carnitine biosynthesis in humans, revealed that the enzyme catalyzes C-3 hydroxylation of (2*S*)-*N*$^{\epsilon}$-trimethyllysine (L-trimethyllysine, **1**) to produce 3-hydroxy-*N*$^{\epsilon}$-L-trimethyllysine (**2**), which then undergoes three additional enzymatic steps to the final L-carnitine (Scheme 1).[4] Recent advanced NMR spectroscopic studies on recombinantly expressed human TMLH ultimately confirmed that the enzymatic hydroxylation occurs at the C-3 site of (2*S*)-*N*$^{\epsilon}$-trimethyllysine substrate; however, such analyses did not unravel the stereochemistry of the hydroxy group at the C-3 position of the product, presumably due to the freely rotatable C_2-C_3 single bond.[5] Due to the intrinsic nature of enzymes to efficiently catalyze transformations of molecules in a highly stereoselective fashion, it is likely that TMLH-catalyzed C-3 hydroxylation of (2*S*)-*N*$^{\epsilon}$-trimethyllysine leads to only one product (i.e., 3*R* or 3*S*). In this regard, it is noteworthy that the final step in carnitine biosynthesis is catalyzed by γ-butyrobetaine hydroxylase (BBOX), another Fe(II)/2OG oxygenase, that specifically produces enantiopure L-carnitine (Scheme 1).[6]

Scheme 1. Role of Trimethyllysine Hydroxylase (TMLH) in the Carnitine Biosynthesis Pathway[a]



[a]Structures of two potential TMLH-catalyzed products **2a** and **2b** are shown on the right.

In order to unquestionably assign the stereochemistry of the TMLH-catalyzed formation of 3-hydroxy-*N*$^{\epsilon}$-L-trimethyllysine, it is essential to carry out comparative NMR spectroscopic studies on the enzymatically produced (2*S*)-3-hydroxy-*N*$^{\epsilon}$-trimethyllysine and the two enantiopure synthetic standards that possess 3*R* and 3*S* stereochemistry, respectively. Herein, we report concise syntheses of enantiopure (2*S*,3*R*)-3-hydroxy-*N*$^{\epsilon}$-trimethyllysine (**2a**) and (2*S*,3*S*)-3-hydroxy-*N*$^{\epsilon}$-trimethyllysine (**2b**) that enabled full elucidation of the stereochemistry at the C-3 site of the TMLH-catalyzed formation of the 3-hydroxy-*N*$^{\epsilon}$-trimethyllysine product (Scheme 1). Our studies provide direct evidence that TMLH exclusively catalyzes the formation of (2*S*,3*S*)-3-hydroxy-*N*$^{\epsilon}$-trimethyllysine, not forming any (2*S*,3*R*)-3-hydroxy-*N*$^{\epsilon}$-trimethyllysine diastereoisomer.

We conceived that both diastereoisomers of (2*S*)-3-hydroxy-*N*$^{\epsilon}$-trimethyllysine could be synthesized from readily available Garner's aldehyde **3** via a sequential set of transformations (Scheme 2 and Supporting Information).[7] The enantiopure alkyne **4** was prepared using a literature protocol by metal-mediated diastereoselective propargylation of Garner's aldehyde followed by benzyl protection of the newly formed alcohol in good yield.[8] To incorporate the dimethyl group on

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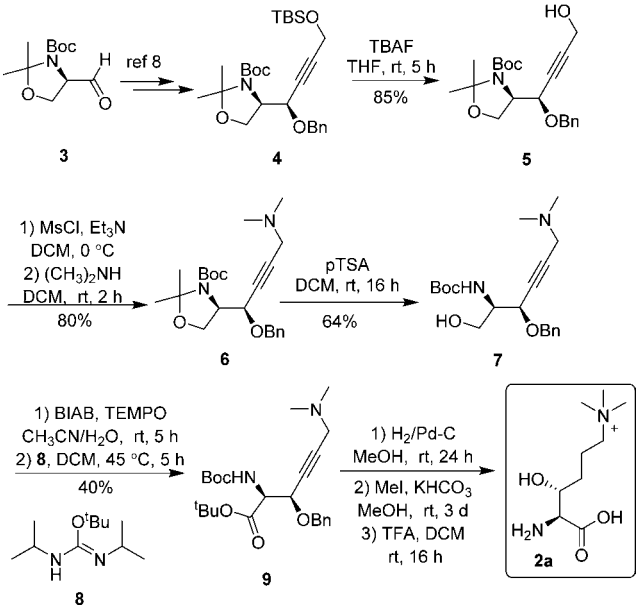
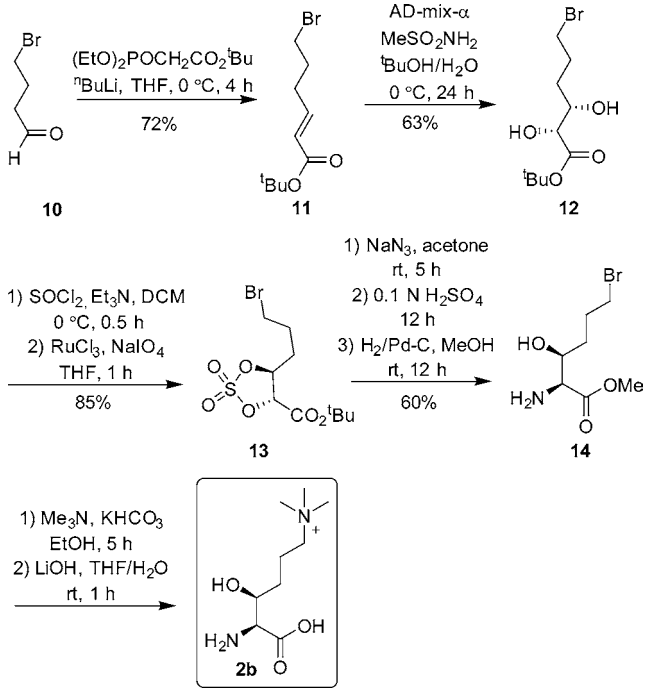
Scheme 2. Synthesis of (2*S*,3*R*)-3-Hydroxy-N^{ϵ}-trimethyllysine (2a)



Scheme 3. Synthesis of (2*S*,3*S*)-3-Hydroxy-N^{ϵ}-trimethyllysine (2b)



the side chain, the TBS protecting group on the primary alcohol was cleaved by TBAF in THF. The resulting alcohol **5** was then treated with MsCl and Et_3N in CH_2Cl_2 to deliver the sulfonate, thus setting the stage for incorporation of dimethylamino functionality. The S_N2 attack on the mesylate by $(Me)_2NH$ afforded compound **6** in very good yield. In order to generate the required carboxylic acid functionality at the C-1 position of the final (2*S*,3*R*)-3-hydroxy-N^{ϵ}-trimethyllysine, the 1,2-acetonide protecting group was cleaved by pTSA in CH_2Cl_2 to yield the enantio- and diastereopure alcohol **7** (>96%, based on the ^{19}F NMR analysis of the corresponding Mosher's ester, Figure S1). Alcohol **7** was transformed to a carboxylic acid in the presence of BIAB and TEMPO in a mixture of CH_3CN and H_2O. Subsequently, the formed crude acid was converted to *tert*-butyl ester **9** (no racemization at the C_α was observed) in the presence of 2-*tert*-butyl-1,3-diisopropylisourea **8** in CH_2Cl_2 at 45 °C for 5 h. Finally, a simultaneous one-step reduction of the alkyne and deprotection of the benzyl group of the alcohol was achieved by hydrogenation with Pd−C in MeOH for 24 h. The treatment of the resulting N^{α}-Boc protected (2*S*,3*R*)-3-hydroxy-N^{ϵ}-dimethyllysine with MeI under basic conditions followed by acid-promoted hydrolysis afforded the targeted diastereopure product (2*S*,3*R*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2a**).

After successfully accomplishing the stereoselective synthesis of (2*S*,3*R*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2a**), we envisaged the synthesis of (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**) by the same protocol. Although we were able to proceed with the preparation of the 2*S*,3*S* diastereoisomer of **9** starting from Garner's aldehyde **3**, the last step in the synthesis of **2b** failed, presumably due to decomposition of the final product **2b** under highly acidic conditions. Thus, we established an alternative approach for the preparation of the (2*S*,3*S*)-isomer that relies on the Sharpless asymmetric dihydroxylation methodology (Scheme 3).

The preparation of *tert*-butyl-protected α,β-unsaturated ester **11** and its subsequent multistep conversion to the (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**) are outlined in Scheme 3.

Aldehyde **10** was prepared from ethyl bromobutyrate using an established protocol; treatment of ethyl bromobutyrate by DIBAL-H at −78 °C led to the formation of **10**,[9] which was subjected to Horner−Emmons olefination with *tert*-butyl diethylphosphonoacetate to give the requisite olefin **11** in 72% yield. The asymmetric dihydroxylation of **11** proceeded as expected, affording the *syn*-dihydroxy ester **12** with an excellent level of enantioselectivity.[10]

In order to incorporate the amine functionality at the C-2 position, the diol was treated with $SOCl_2$ and Et_3N to afford the cyclic sulfite, which was, upon treatment with $NaIO_4$ and catalytic amount of $RuCl_3$, further oxidized to sulfate **13**. In accordance with Corey's method,[10b] the regiospecific (C_α) nucleophilic opening of the electrophilic cyclic sulfate **13** with sodium azide in acetone afforded the azidosulfate, which underwent further acidic hydrolysis and Pd−C mediated hydrogenation to afford an exclusive formation of the aminoalcohol **14** that possesses the desired 2*S*,3*S* stereochemistry. ^{19}F NMR analyses of the Mosher's amide of **14** and the Mosher's ester of the preceding azidoalcohol confirmed high degrees (>98.5%) of enantiopurity and diastereopurity of both compounds (Figure S2). Finally, treatment of **14** with trimethylamine and $KHCO_3$ in EtOH, followed by saponification with LiOH in THF/H_2O, afforded the final diastereopure product (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**).

Having synthesized enantiopure (2*S*,3*R*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2a**) and (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**), we aimed to provide a conclusive answer about the stereochemistry at the C-3 position of the product of TMLH-catalyzed C-3 hydroxylation of (2*S*)-N^{ϵ}-trimethyllysine. An incubation of (2*S*)-N^{ϵ}-trimethyllysine, $FeSO_4$, 2-oxoglutarate, ascorbate, and TMLH in buffered solution of TRIS-d_{11} (20 mM, pD = 7.5) for 1 h at 37 °C resulted in almost quantitative formation (>95%) of (2*S*)-3-hydroxy-N^{ϵ}-trimethyllysine product, as demonstrated by ^1H NMR analysis (Figure 1). In agreement with our recent study, two indicative signals at 4.07

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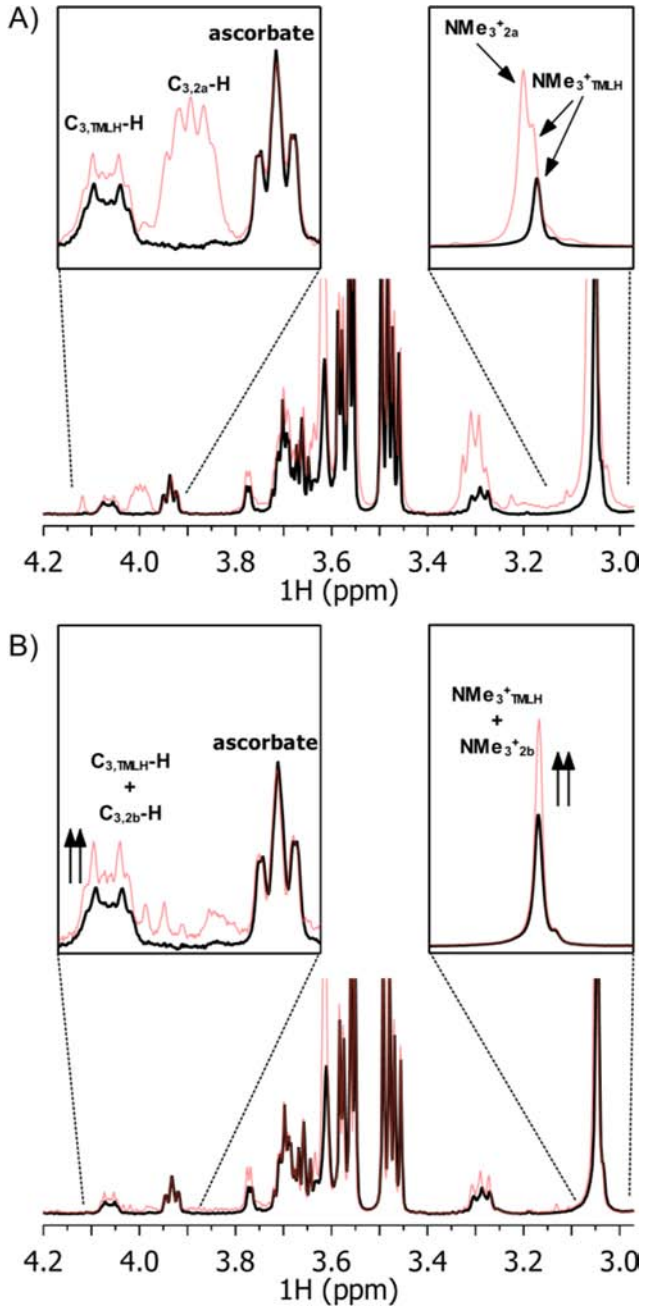

Figure 1. NMR spectroscopic analyses of TMLH-catalyzed hydroxylation of (2*S*)-3-hydroxy-N^{ϵ}-trimethyllysine. (A) Addition of the **2a** isomer to the enzymatic mixture. (B) Addition of the **2b** isomer to the enzymatic mixture. (Black spectrum = enzymatic reaction, red spectrum = added authentic sample of **2a**/**2b** to the enzymatic reaction).

and 3.78 ppm were assigned as C_3-H and C_2-H, respectively.[5] We then carried out ^{1}H NMR-based doping experiments. To two separate samples of the enzymatic mixture that contained the TMLH-catalyzed (2*S*)-3-hydroxy-N^{ϵ}-trimethyllysine product we added an authentic sample of synthetic enantiopure (2*S*,3*R*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2a**) and (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**), respectively.

Addition of synthetic **2a** resulted in the observation of new peaks in the enzymatic mixture spectrum (Figure 1A). The most prevalent new peaks appeared at 4.00, 3.31, and 3.06 ppm, which were identified as $C_{3,2a}$-H, $C_{6,2a}$-H, and $NMe_3^+{}_{2a}$, respectively. A minor increase in the C_3-H peak intensity (at 4.07 ppm) was also observed; however, this increase derives from a small impurity in the synthetic compound **2a** (Figure S3). Additionally, it appears that NMe_3^+ increases ~2-fold upon addition of **2a**, but this is rationalized by the effect of coadding two peaks with similar chemical shifts. The spectral deconvolution of the methyl region revealed that NMe_3^+ intensity basically remains unchanged (see Table S1 and Figures S4−S6 in the Supporting Information). ^{1}H−^{1}H COSY analysis, moreover, confirmed the appearance of new indicative peaks upon the addition of the authentic sample **2a** to the enzymatic mixture (Figure S7). Collectively, these results clearly indicate that (2*S*,3*R*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2a**) is not produced in the TMLH-catalyzed reaction, suggesting that it is very likely that the (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**) isomer is formed in the enzymatic reaction.

Indisputable proof about the correct assignment of the stereochemistry at the C-3 site of the enzymatic reaction was provided by the enhancement experiment in the presence of synthetic (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**). Notably, addition of (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**) to the enzymatic mixture resulted in no new observed peaks in the ^{1}H NMR spectrum (small peaks in the region of 3.95−4.05 ppm derive from little impurities in the authentic sample **2b**). Instead, a ~2-fold increase in peak intensity relative to the ascorbate present was observed for C_2-H, C_3-H, C_6-H, and NMe_3^+ (Figure 1B and Table S1). In all cases, the integrals were normalized to ascorbate, and similar results were also obtained when the well-resolved 2OG peaks were used instead as a reference (Table S1). Importantly, the comparative analyses of the multiplicity-edited ^{1}H−^{13}C HSQC spectra revealed that the TMLH-catalyzed 3-hydroxy-N^{ϵ}-L-trimethyllysine product[5] and the synthetic (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**) possess exactly the same values of ^{13}C NMR chemical shifts for the C_{α} (at 59.3 ppm) and the C_{β} (at 68.8 ppm) (**2a** exhibits distinguishable resonances at 59.0 ppm for C_{α} and at 69.4 ppm for C_{β}). Taken together, these observations clearly indicate that 3-hydroxy-N^{ϵ}-trimethyllysine produced by TMLH is not consistent with **2a** but rather **2b**, the (2*S*,3*S*) diastereoisomer.

In conclusion, based on newly developed stereoselective syntheses of (2*S*,3*R*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2a**) and (2*S*,3*S*)-3-hydroxy-N^{ϵ}-trimethyllysine (**2b**) and NMR doping experiments, we have provided conclusive evidence that the 3-hydroxy-N^{ϵ}-trimethyllysine product of the human TMLH-catalyzed reaction possesses the 2*S*,3*S* stereochemistry. It is envisioned that this study will not only provide a valuable insight into the rational design of small molecule inhibitors of biomedically important carnitine biosynthesis pathway but will also importantly contribute to a flourishing field of bioinspired chemistry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03608.

Synthetic procedures and NMR analyses (PDF)

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) (a) Breslow, R. *J. Biol. Chem.* **2009**, *284*, 1337. (b) Wiester, M. J.; Ulmann, P. A.; Mirkin, C. A. *Angew. Chem., Int. Ed.* **2011**, *50*, 114.

(2) (a) Bigi, M. A.; Reed, S. A.; White, M. C. *Nat. Chem.* **2011**, *3*, 218. (b) Chen, K.; Baran, P. S. *Nature* **2009**, *459*, 824. (c) Davies, H. M. L.; Morton, D. *J. Org. Chem.* **2016**, *81*, 343. (d) Kamata, K.; Yonehara, K.; Nakagawa, Y.; Uehara, K.; Mizuno, N. *Nat. Chem.* **2010**, *2*, 478. (e) Labinger, J. A.; Bercaw, J. E. *Nature* **2002**, *417*, 507. (f) Mas-Ballesté, R.; Que, L. *Science* **2006**, *312*, 1885. (g) Narayan, A. R. H.; Jiménez-Osés, G.; Liu, P.; Negretti, S.; Zhao, W.; Gilbert, M. M.; Ramabhadran, R. O.; Yang, Y.-F.; Furan, L. R.; Li, Z.; Podust, L. M.; Montgomery, J.; Houk, K. N.; Sherman, D. H. *Nat. Chem.* **2015**, *7*, 653. (h) Newhouse, T.; Baran, P. S. *Angew. Chem., Int. Ed.* **2011**, *50*, 3362. (i) Que, L.; Tolman, W. B. *Nature* **2008**, *455*, 333.

(3) Loenarz, C.; Schofield, C. J. *Nat. Chem. Biol.* **2008**, *4*, 152.

(4) (a) Hulse, J. D.; Ellis, S. R.; Henderson, L. M. *J. Biol. Chem.* **1978**, *253*, 1654. (b) Huszar, G. *J. Mol. Biol.* **1975**, *94*, 311. (c) Kazaks, A.; Makrecka-Kuka, M.; Kuka, J.; Voronkova, T.; Akopjana, I.; Grinberga, S.; Pugovics, O.; Tars, K. *Protein Expression Purif.* **2014**, *104*, 1. (d) Kerner, J.; Hoppel, C. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **2000**, *1486*, 1. (e) Novak, R. F.; Swift, T. J.; Hoppel, C. L. *Biochem. J.* **1980**, *188*, 521. (f) Steiber, A.; Kerner, J.; Hoppel, C. L. *Mol. Aspects Med.* **2004**, *25*, 455. (g) Strijbis, K.; Vaz, F. M.; Distel, B. *IUBMB Life* **2010**, *62*, 357. (h) Vaz, F. M.; Wanders, R. J. A. *Biochem. J.* **2002**, *361*, 417.

(5) Al Temimi, A. H. K.; Pieters, B. J. G. E.; Reddy, Y. V.; White, P. B.; Mecinović, J. *Chem. Commun.* **2016**, *52*, 12849.

(6) (a) Kamps, J. J. A. G.; Khan, A.; Choi, H.; Lesniak, R. K.; Brem, J.; Rydzik, A. M.; McDonough, M. A.; Schofield, C. J.; Claridge, T. D. W.; Mecinović, J. *Chem. - Eur. J.* **2016**, *22*, 1270. (b) Leung, I. K. H.; Krojer, T. J.; Kochan, G. T.; Henry, L.; von Delft, F.; Claridge, T. D. W.; Oppermann, U.; McDonough, M. A.; Schofield, C. J. *Chem. Biol.* **2010**, *17*, 1316. (c) Rydzik, A. M.; Leung, I. K. H.; Kochan, G. T.; Loik, N. D.; Henry, L.; McDonough, M. A.; Claridge, T. D. W.; Schofield, C. J. *Org. Biomol. Chem.* **2014**, *12*, 6354.

(7) Passiniemi, M.; Koskinen, A. M. P. *Beilstein J. Org. Chem.* **2013**, *9*, 2641.

(8) Gruza, H.; Kiciak, K.; Krasiński, A.; Jurczak, J. *Tetrahedron: Asymmetry* **1997**, *8*, 2627.

(9) Somekawa, K.; Okuhira, H.; Sendayama, M.; Suishu, T.; Shimo, T. *J. Org. Chem.* **1992**, *57*, 5708.

(10) (a) Masse, C. E.; Morgan, A. J.; Panek, J. S. *Org. Lett.* **2000**, *2*, 2571. (b) Saravanan, P.; Corey, E. J. *J. Org. Chem.* **2003**, *68*, 2760.

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