Biocatalysis

International Edition: DOI: 10.1002/anie.201510331
German Edition: DOI: 10.1002/ange.201510331

25-Hydroxyvitamin D_3 Synthesis by Enzymatic Steroid Side-Chain Hydroxylation with Water

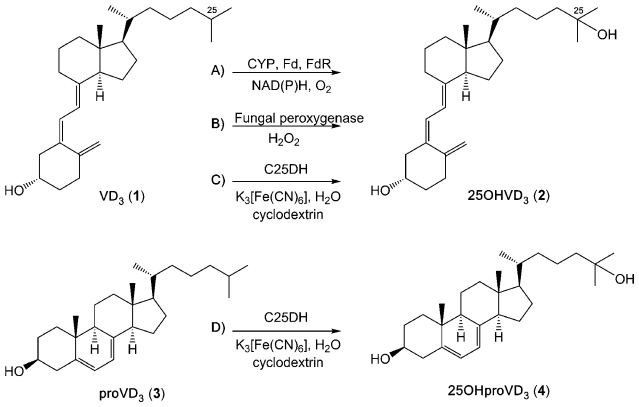
*Markus Warnke, Tobias Jung, Juri Dermer, Karin Hipp, Nico Jehmlich, Martin von Bergen, Sascha Ferlaino, Alexander Fries, Michael Müller, and Matthias Boll**

Dedicated to Georg Fuchs on the occasion of his 70th birthday

Abstract: *The hydroxylation of vitamin D_3 (VD_3, cholecalciferol) side chains to give 25-hydroxyvitamin D_3 ($25OHVD_3$) is a crucial reaction in the formation of the circulating and biologically active forms of VD_3. It is usually catalyzed by cytochrome P450 monooxygenases that depend on complex electron donor systems. Cell-free extracts and a purified Mo enzyme from a bacterium anaerobically grown with cholesterol were employed for the regioselective, ferricyanide-dependent hydroxylation of VD_3 and $proVD_3$ (7-dehydrocholesterol) into the corresponding tertiary alcohols with greater than 99 % yield. Hydroxylation of VD_3 strictly depends on a cyclodextrin-assisted isomerization of VD_3 into $preVD_3$, the actual enzymatic substrate. This facile and robust method developed for $25OHVD_3$ synthesis is a novel example for the concept of substrate-engineered catalysis and offers an attractive alternative to chemical or O_2/electron-donor-dependent enzymatic procedures.*

Vitamin D_3 (VD_3, cholecalciferol, **1**) is generally known for its regulatory function in calcium and phosphorous homeostasis, but it is also recognized as an antiproliferative factor in dividing cells and tissues.[1] VD_3 is formed from $proVD_3$ (7-dehydrocholesterol, **3**) by UV-B irradiation to give the *seco* form, followed by a thermal [1,7] sigmatropic hydrogen shift. It is converted into its biologically active form by consecutively acting cytochrome P450 monooxygenases (CYPs): hydroxylation in the liver yields 25-hydroxy-VD_3 ($25OHVD_3$, calcidiol, **2**), which is converted into calcitriol ($1\alpha,25(OH)_2VD_3$) in the kidney. Insufficient levels of biologically active VD_3 are linked to numerous forms of cancer but also to cardiovascular diseases, immunodeficiency, and diabetes.[2] $25OHVD_3$ (**2**) represents the circulating form of VD_3, and its plasma concentration has been associated with VD_3-linked disorders.[3]

Classical chemical $25OHVD_3$ (**2**) synthesis procedures involve multiple steps with low yields and specificities,[4] although recently more convenient procedures, for example, methods involving anchoring on a solid phase, have been developed.[5] For enzymatic synthesis, CYPs either from liver, fungi, or actinomycetes have been employed.[6] However, the dependence on complex electron-donor systems consisting of NAD(P)H, ferredoxin (Fd), and ferredoxin reductase (FdR) has limited biotechnological applications; promising approaches employ whole cells expressing the genes encoding CYP, Fd, and FdR (Scheme 1 A). As an alternative, an H_2O_2-dependent peroxygenase has been used for hydroxylation of low concentrations of VD_3 and ergocalciferol (VD_2; Scheme 1 B).[7]



Scheme 1. Enzymatic conversions of VD_3 (**1**) and $proVD_3$ (**3**) into $25OHVD_3$ (**2**) and $25OHproVD_3$ (**4**), respectively. A) Hydroxylation of **1** by O_2-dependent CYPs in the presence of an electron-donor system including NAD(P)H, Fd, and FdR. B) Hydroxylation of **1** by H_2O_2-dependent peroxygenase. C, D) Water- and $K_3[Fe(CN)_6]$-dependent hydroxylation of **1** and **3** by C25DH. (A) and (B) were previously established,[6,7] (C) and (D) were studied in this work.

A fundamentally different mode of enzymatic steroid side-chain hydroxylation was identified during the anaerobic degradation of cholesterol to CO_2 in the denitrifying bacterium *Sterolibacterium denitrificans*: the regioselective hydroxylation of cholesterol into 25-hydroxycholesterol with

[*] M. Sc. M. Warnke, T. Jung, Dr. J. Dermer, K. Hipp, Prof. Dr. M. Boll
Faculty of Biology - Microbiology
Albert-Ludwigs-Universität Freiburg
Schänzlestrasse 1, 79104 Freiburg (Germany)
E-mail: matthias.boll@biologie.uni-freiburg.de
S. Ferlaino, Dr. A. Fries, Prof. Dr. M. Müller
Institute of Pharmaceutical Sciences
Albert-Ludwigs-Universität Freiburg
Albertstrasse 25, 79104 Freiburg (Germany)
Dr. N. Jehmlich, Prof. Dr. M. von Bergen
Department of Proteomics
Helmholtz Centre of Environmental Sciences
Permoserstr. 15, 04318 Leipzig (Germany)

Supporting information for this article (including experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201510331.

water catalyzed by steroid C25 dehydrogenase (C25DH).[8] This enzyme belongs to the DMSO reductase (DMSOR) family of Mo enzymes that catalyze O_2-independent hydroxy- and oxo-transfer reactions.[9] A C–H bond functionalization with water to a tertiary alcohol via a carbocation transition state has been proposed.[8] C25DH transfers electrons to electrochemically regenerative electron acceptors such as $K_3[Fe(CN)_6]$ (Scheme 1C). The genome of *S. denitrificans* contains at least eight homologous genes encoding putative Mo-dependent steroid hydroxylases,[8] thus making this organism a promising biological platform for water-dependent hydroxylations of various steroids. In this work, we explored the use of *S. denitrificans* enzyme(s) for regioselective synthesis of the biologically relevant hydroxylated forms of VD_3 (**1**) and proVD_3 (**3**). The hydroxylated 25OHproVD_3 (**4**, Scheme 1D) can easily be converted into 25OHVD_3 (**2**) by established UV irradiation methods.[10]

In the presence of 2-hydroxypropyl-β-cyclodextrin (HPCD), crude extracts from *S. denitrificans* anaerobically grown with cholesterol catalyzed the $K_3[Fe(CN)_6]$-dependent conversion of VD_3 (**1**) and proVD_3 (**3**) into the single products **2** and **4** (Figure 2A,B), respectively, with substrate conversions of >99% (**2**) and 97% (**4**; Figure 1A,B). After the

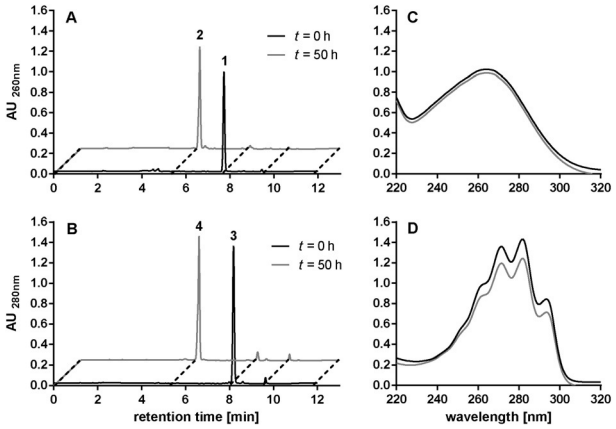


Figure 1. Enzyme assays for the hydroxylation of **1** and **3**. A, B) Ultra-performance liquid chromatography (UPLC) analysis of the enzymatic conversion of **1** and **3** into their hydroxylated products **2** and **4**, respectively. C, D) UV spectra of **1**–**4**.

conversion of VD_3 (**1**) reached 92% (16 h), the product **2** was slowly degraded by unspecific enzymatic reactions, which could be fully prevented by the addition of 0.5 mM $AgNO_3$, a known inhibitor of the downstream reactions of steroid catabolism.[8] The UV/Vis spectra of the products were identical to those of the substrates, which is in agreement with side-chain hydroxylations distant from the conjugated triene (**1**) and diene (**3**) chromophors (Figure 1C,D).

Conversion of **1** and **3** was HPCD dependent, with an optimum at 15% (*w*/*v*; Figure 2C). The initial rate of the reaction followed Michaelis–Menten kinetics, with $v_{max} = 1.48 \pm 0.05$ nmol min^{-1} per mg crude extract protein for VD_3 (**1**), which is threefold higher than the rate of proVD_3 (**3**) conversion (0.47 ± 0.02 nmol min^{-1} per mg crude extract protein; Figure 2D). The conversions of **1** and **3** were strictly

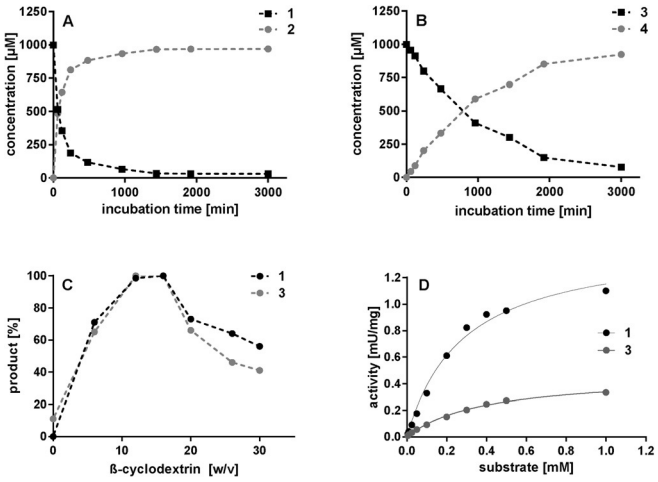


Figure 2. Properties of the enzymatic hydroxylation of **1** and **3**. A) Time-dependent conversion of **1** into **2** in the presence of 0.5 mM $AgNO_3$. B) Time-dependent conversion of **3** into **4**. Both reactions contained 15% HPCD C) HPCD dependence of the conversion of **1** and **3**. D) Substrate concentration dependence of the conversion of **1** and **3**.

dependent on electron acceptors with standard redox potentials higher than 200 mV, such as $[Fe(CN)_6]^{3-}$ (ferricyanide), with the highest initial rates between 5–10 mM $K_3[Fe(CN)_6]$. This rather high value can be explained by competing unspecific $K_3[Fe(CN)_6]$ oxidizing reactions in cell extracts.

The products formed from **1** and **3** were analyzed by chemical ionization mass spectrometry. For the product of **1** ($M_r = 384.65$), compound **2**, an ion with *m*/*z* $[M+H]^+$ of 401.3, was determined, thus indicating the addition of a hydroxy group (Figure S1 in the Supporting Information). Likewise, the conversion of **3** ($M_r = 384.65$) yielded a product with an identical *m*/*z* $[M+H]^+$ (Figure S2). Both results strongly suggest the hydroxylation of VD_3 (**1**) and proVD_3 (**3**).

To unambiguously determine the regioselectivity of the reaction, the products were characterized by NMR spectroscopy. The ^1H and ^{13}C NMR spectra of authentic **1** and **3** standards were highly similar to their products **2** and **4**, respectively, with the exception of the chemical shifts for C26, C27 methyl groups (^1H spectra), and C25 (^{13}C spectra). For **1** and **3**, the C26 and C27 methyl groups are observed as doublets at $\delta = 0.86$ ppm in the ^1H spectra. For **2** and **4**, the ^1H NMR signal for CH_3-26,27 is observed as a singlet at $\delta = 1.23$ ppm. A downfield shift is observed in the ^{13}C NMR spectrum for the C25 signal ($\delta = 71.1$ ppm versus 22.8 ppm for **1** and **3**), thus indicating the hydroxylation of C25, as described for 25-hydroxycholest-4-en-3-one.[11]

The ^1H and ^{13}C NMR spectroscopic data are in perfect agreement with those published for 25OHVD_3.[14] The complete NMR spectroscopic data are presented in the Supporting Information (^1H and ^{13}C NMR spectra/shifts, 2D NMR spectra for **2**, and ^1H NMR spectrum of **4**; Figures S3–S5 and Table S1).

To identify the enzyme(s) involved in VD_3 (**1**) hydroxylation, we enriched the activity from extracts of cells grown with cholesterol. After four chromatographic steps, four

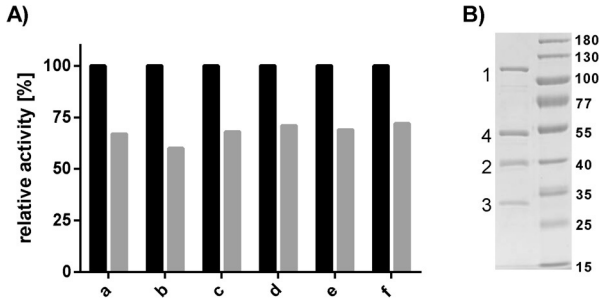
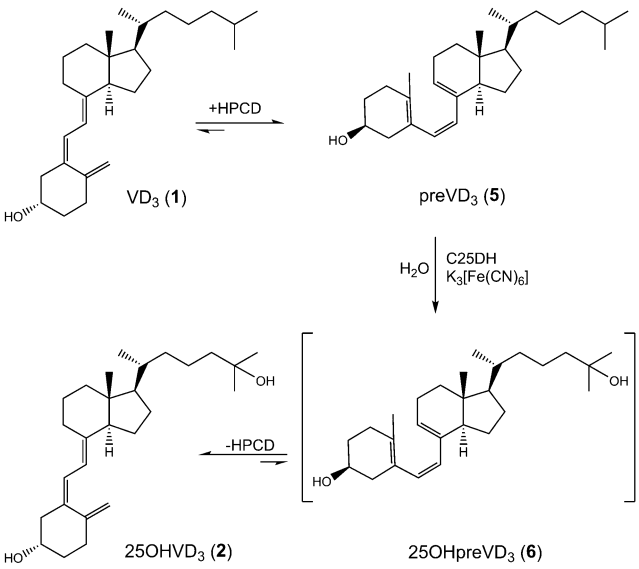


Figure 3. Enrichment of the VD_3 (**1**) hydroxylating enzyme. A) Relative specific conversion rates of VD_3 (**1**, grey columns) and cholest-4-en-3-one (black columns) obtained during the solubilization and purification steps. a) cell extract; b) solubilized membrane fraction; c–f) fractions obtained after chromatography on DEAE-Sepharose, ceramic hydroxyapatite, Reactive Red agarose, and Mono Q anion exchange chromatography, respectively. B) SDS gel of enriched enzyme after four chromatographic steps. Left lane: bands 1–3 were identified as αβγ-subunits of C25DH; band 4 is a degradation product of band 1 (Table S2). Right lane: Molecular mass standard with masses in kDa as indicated.



Scheme 2. Proposal for HPCD-dependent hydroxylation of VD_3 by C25DH. In the presence of HPCD, the equilibrium between VD_3 (**1**) and $preVD_3$ (**5**), which are interconverted through a thermal [1,7] hydrogen shift, is shifted to the right. The *cZc* conformer of **5** is likely the substrate for C25DH. The hydroxylated **6** was not detected owing to rapid isomerization to **2**.

protein bands were highly enriched with a specific VD_3 (**1**) hydroxylation activity of 80 nmol min^{-1} mg^{-1} (Figure 3 A). Mass spectrometric analysis of tryptic digests of the enriched protein bands identified the αβγ-subunits of C25DH (JQ292991-3) plus a fourth band corresponding to a degradation product of the α-subunit (JQ292991; Table S2). After each purification step, the relative specific hydroxylation rates of VD_3 (**1**) and cholest-4-en-3-one[8] remained almost constant in all activity-containing fractions (around 70 % with **1**), thus confirming that both reactions are catalyzed by the same enzyme (Figure 3 B). VD_3 (**1**) hydroxylation activity was highest in extracts of cells grown with cholesterol (100 %), and significantly lower in cells grown with sitosterol (18 %), ergosterol (15 %), or stigmasterol (5 %), which is in line with the induction of C25DH during growth with cholesterol.

The conversion of cholest-4-en-3-one and $proVD_3$ (**3**) by C25DH was observed in the presence of various detergents and HPCD. In contrast, conversion of VD_3 (**1**) was negligible when HPCD was replaced by a number of commonly used detergents (Figure S6). This finding suggests that HPCD plays a specific role in VD_3 conversion rather than serving as a general solubilizing agent. The equilibrium $preVD_3$ (**5**) ⇌ VD_3 (**1**) through thermal [1,7] sigmatropic rearrangement lies far to the right in aqueous detergent solutions (Scheme 2, upper reaction).[12] However, in the presence of HPCD, an inclusion complex is formed at a 2:1 ratio (HPCD/VD_3), in which the thermodynamically unfavorable *cZc* conformer of $preVD_3$ (**5**) is stabilized.[12] This finding led us to the assumption that $preVD_3$ (**5**) rather than VD_3 (**1**) is the actual substrate of C25DH.

By replacing the commonly used ethyl acetate for steroid extraction by n-hexane, more than 80 % of VD_3 (**1**, 1 mM) added to the HPCD-containing assay mixture was identified as $preVD_3$ (**5**) by UPLC analysis. In the course of the enzymatic reaction, $preVD_3$ (**5**) was time-dependently converted into $25OHVD_3$ (**2**), with virtually no formation of the expected $25OHpreVD_3$ (**6**) intermediate (Figure 4). In contrast, during extraction with ethyl acetate, $preVD_3$ (**5**) was never identified during VD_3 (**1**) hydroxylation (Figure 1 A). To substantiate the HPCD-dependent conversion of VD_3 (**1**) into $preVD_3$ (**5**) as an essential process for enzymatic side-chain hydroxylation, we replaced VD_3 (**1**) by authentic $preVD_3$ (**5**) and observed an identical product pattern as during VD_3 (**1**) conversion (Figure S7). The rates of VD_3 (**1**) and $preVD_3$ (**5**) hydroxylation were nearly identical, thus suggesting that the HPCD-dependent conversion of VD_3 (**1**) into $preVD_3$ (**5**) is not rate limiting.

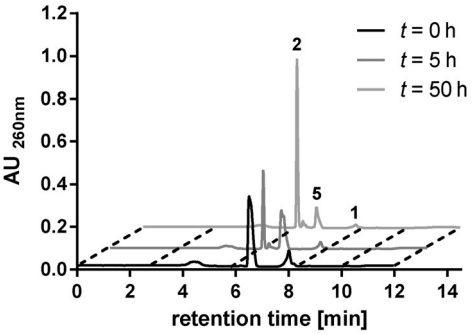


Figure 4. UPLC analysis of enzymatic VD_3 (**1**) hydroxylation in the presence of HPCD after steroid extraction with *n*-hexane. At $t = 0$ h, before enzyme addition, around 80 % of the added VD_3 was HPCD-dependently isomerized into $preVD_3$ (**5**).

In summary, the results indicate that $preVD_3$ (**5**) but not VD_3 (**1**) is the substrate for C25DH. The failure to detect any trace of a 25-OH-$preVD_3$ intermediate indicates a rapid isomerization into $25OHVD_3$ (Scheme 2).

The procedure presented in this work represents a new enzymatic route for the regioselective hydroxylation of VD_3

(**1**), proVD$_3$ (**3**), and preVD$_3$ (**5**) at C25, and expands our knowledge of tertiary alcohol synthesis.[13] Since cell extracts of *S. denitrificans* can be used for these reactions, it circumvents tedious enzyme purification or genetic manipulation procedures and offers a facile method for a mechanistically demanding chemical reaction. The method strictly depends on HPCD for VD$_3$ (**1**) solubilization and isomerization and represents a novel example of substrate-range expansion. In comparison to CYP-dependent hydroxylations it does not depend on electron-donor systems. The only cofactor used, $K_3[Fe(CN)_6]$, can be regenerated by electrodes as has been demonstrated for the related ethyl benzene dehydrogenase.[14] Moreover, the method does not depend on oxygen and nor is it oxygen sensitive. The synthetic method for 25OHVD$_3$ synthesis may be useful for the treatment of 25OHVD$_3$ deficiencies and to satisfy the increasing demand for monitoring of its levels in blood plasma.

Acknowledgements

This work was funded by DFG (BO 1565/11-1) in frame of the Scientific Priority Programme SPP1319. We thank Dr. P. Bisel, Freiburg, for help with NMR measurements.

Keywords: biotechnology · C–H activation · enzyme catalysis · steroids · vitamins

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1881 – 1884
Angew. Chem. **2016**, *128*, 1913 – 1916

[1] a) M. F. Holick, *N. Engl. J. Med.* **2007**, *357*, 266 – 281; b) S. V. Ramagopalan, A. Heger, A. J. Berlanga, N. J. Maugeri, M. R. Lincoln, A. Burrell, L. Handunnetthi, A. E. Handel, G. Disanto, S. M. Orton, C. T. Watson, J. M. Morahan, G. Giovannoni, C. P. Ponting, G. C. Ebers, J. C. Knight, *Genome Res.* **2010**, *20*, 1352 – 1360.

[2] a) M. Wacker, M. F. Holick, *Nutrients* **2013**, *5*, 111 – 148; b) A. Hossein-nezhad, M. F. Holick, *Mayo Clin. Proc.* **2013**, *88*, 720 – 755.

[3] C. J. Farrell, S. Martin, B. McWhinney, I. Straub, P. Williams, M. Herrmann, *Clin. Chem.* **2012**, *58*, 531 – 542.

[4] G. D. Zhu, W. H. Okamura, *Chem. Rev.* **1995**, *95*, 1877 – 1952.

[5] D. Nicoletti, A. Mourino, M. Torneiro, *J. Org. Chem.* **2009**, *74*, 4782 – 4786.

[6] a) K. Hayashi, H. Sugimoto, R. Shinkyo, M. Yamada, S. Ikeda, S. Ikushiro, M. Kamakura, Y. Shiro, T. Sakaki, *Biochemistry* **2008**, *47*, 11964 – 11972; b) K. Hayashi, K. Yasuda, H. Sugimoto, S. Ikushiro, M. Kamakura, A. Kittaka, R. L. Horst, T. C. Chen, M. Ohta, Y. Shiro, T. Sakaki, *FEBS J.* **2010**, *277*, 3999 – 4009; c) D. J. Kang, J. H. Im, J. H. Kang, K. H. Kim, *Bioprocess Biosyst. Eng.* **2015**, *38*, 1281 – 1290; d) D. J. Kang, J. H. Im, J. H. Kang, K. H. Kim, *Biotechnol. Lett.* **2015**, *37*, 1895 – 1904; e) T. Sakaki, H. Sugimoto, K. Hayashi, K. Yasuda, E. Munetsuna, M. Kamakura, S. Ikushiro, Y. Shiro, *Biochim. Biophys. Acta Proteins Proteomics* **2011**, *1814*, 249 – 256; f) K. Yasuda, M. Endo, S. Ikushiro, M. Kamakura, M. Ohta, T. Sakaki, *Biochem. Biophys. Res. Commun.* **2013**, *434*, 311 – 315; g) Y. Yasutake, T. Nishioka, N. Imoto, T. Tamura, *ChemBioChem* **2013**, *14*, 2284 – 2291.

[7] E. D. Babot, J. C. Del Rio, M. Canellas, F. Sancho, F. Lucas, V. Guallar, L. Kalum, H. Lund, G. Grobe, K. Scheibner, R. Ullrich, M. Hofrichter, A. T. Martinez, A. Gutierrez, *Appl. Environ. Microbiol.* **2015**, *81*, 4130 – 4142.

[8] J. Dermer, G. Fuchs, *J. Biol. Chem.* **2012**, *287*, 36905 – 36916.

[9] R. Hille, J. Hall, P. Basu, *Chem. Rev.* **2014**, *114*, 3963 – 4038.

[10] S. Fuse, Y. Mifune, N. Tanabe, T. Takahashi, *Org. Biomol. Chem.* **2012**, *10*, 5205 – 5211.

[11] Y. R. Chiang, W. Ismail, M. Müller, G. Fuchs, *J. Biol. Chem.* **2007**, *282*, 13240 – 13249.

[12] X. Q. Tian, M. F. Holick, *J. Biol. Chem.* **1995**, *270*, 8706 – 8711.

[13] M. Müller, *ChemBioEng. Rev.* **2014**, *1*, 14 – 26.

[14] P. Kalimuthu, J. Heider, D. Knack, P. V. Bernhardt, *J. Phys. Chem. B* **2015**, *119*, 3456 – 3463.

Received: November 6, 2015
Published online: December 22, 2015

