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

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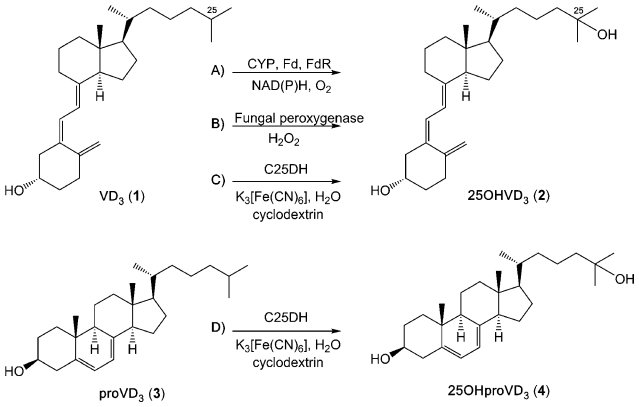
Dedicated to Georg Fuchs on the occasion of his 70th birthday

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

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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

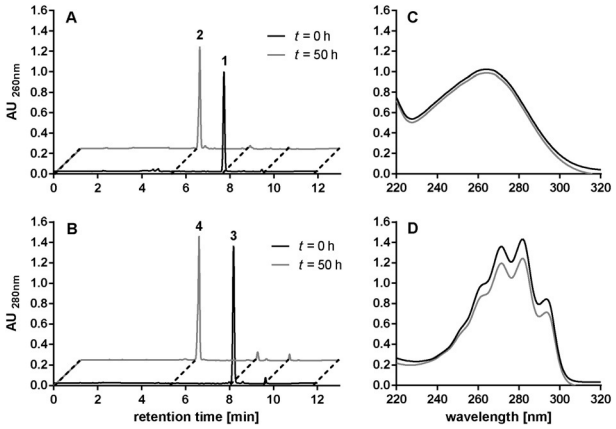


Figure 1. Enzyme assays for the hydroxylation of **1** and **3**. A, B) Ultra-performance liquid chromatography (UPLC) analysis of the enzymatic conversion of **1** and **3** into their hydroxylated products **2** and **4**, respectively. C, D) UV spectra of **1**–**4**.

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

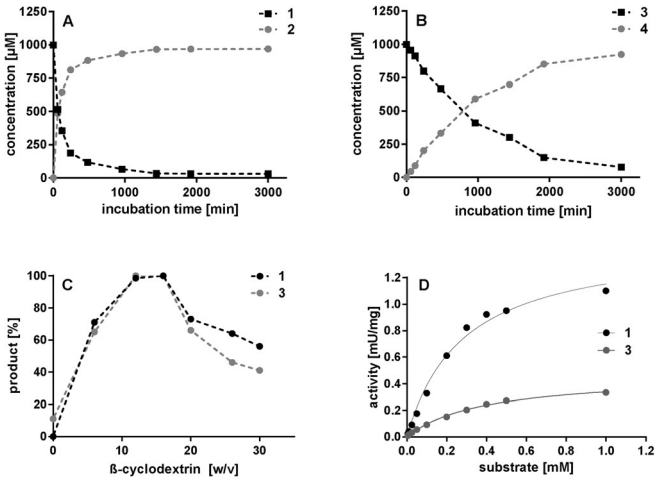


Figure 2. Properties of the enzymatic hydroxylation of **1** and **3**. A) Time-dependent conversion of **1** into **2** in the presence of 0.5 mM $AgNO_3$. B) Time-dependent conversion of **3** into **4**. Both reactions contained 15% HPCD C) HPCD dependence of the conversion of **1** and **3**. D)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

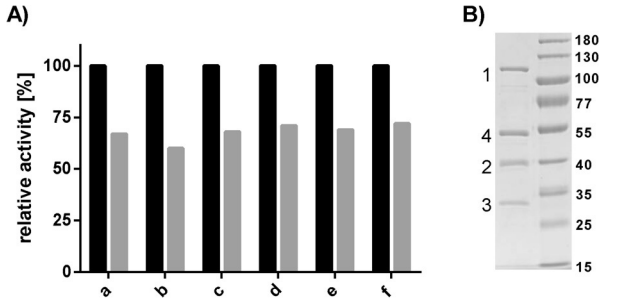
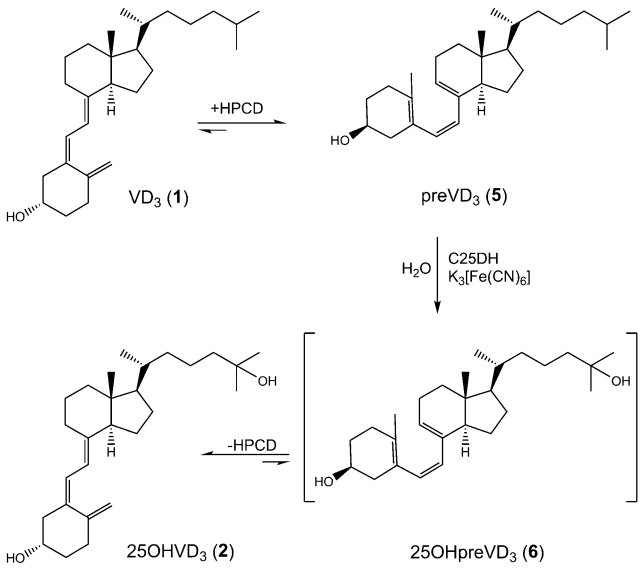


Figure 3. Enrichment of the VD_3 (**1**) hydroxylating enzyme. A) Relative specific conversion rates of VD_3 (**1**, grey columns) and cholest-4-en-3-one (black columns) obtained during the solubilization and purification steps. a) cell extract; b) solubilized membrane fraction; c–f) fractions obtained 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.

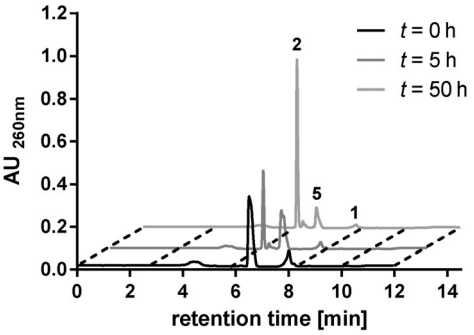


Figure 4. UPLC analysis of enzymatic VD_3 (**1**) hydroxylation in the presence of HPCD after steroid extraction with *n*-hexane. At $t = 0$ h, before enzyme addition, around 80 % of the added VD_3 was HPCD-dependently isomerized into $preVD_3$ (**5**).

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.

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