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# Synthesis of $^{13}C$ Labeled Vitamin D Metabolites for Their Use in LC-MS/MS: Valuable Tools for Cancer Research and Other Applications

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**Abstract.** *Background/Aim: Simultaneous assessment of various vitamin D metabolites in human biofluids by liquid chromatography tandem mass spectrometry (LC-MS/MS) represents a new promising tool for the differential diagnosis of vitamin D-related diseases. Particularly, besides 25(OH)$VD_{2/3}$, low-abundant medicinally relevant vitamin D metabolites, such as 24,25(OH)$_2VD_{2/3}$, 1,25(OH)$_2VD_{2/3}$, and 1,24,25(OH)$_3VD_{2/3}$, along with their 3-epi-derivatives have to be considered. Materials and Methods: The assessment of these metabolites by LC-MS/MS requires the development of calibration and reference standards, that is, their labeling with multiple deuterium-, or even better, $^{13}C$- atoms. Results: Overall, 10 $^{13}C$-labeled vitamin D metabolites have been chemically synthesized and obtained in good yield and high purity. Conclusion: Access to a wide variety of $^{13}C$-labeled highly pure vitamin D metabolites enables the advancement of LC-MS/MS applications towards a better understanding of differential diagnosis of vitamin D-related diseases.*

Increasing evidence demonstrates the relevance of vitamin D metabolites on the endocrine system, eventually associated to many types of cancer. Consequently, new convenient and reliable tools for the investigation of the increasingly recognized number of biologically active vitamin D metabolites in human biofluids and individual tissues are urgently needed. Simultaneous assessment of various vitamin D metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS) represents a new promising tool for the differential diagnosis of vitamin D-related diseases (1-3). Particularly, in addition to 25-hydroxy vitamin $D_3$ (25(OH)$VD_3$), low-abundant clinically relevant vitamin D metabolites, such as 24,25(OH)$_2VD_3$, 1α,25(OH)$_2VD_3$, and 1α,24,25(OH)$_3VD_3$ have to be considered. Furthermore, their $D_2$-analogs must not be neglected as well, due to their occurrence as a consequence of food fortification with vitamin $D_2$ (4). Consequently, simultaneous monitoring of various vitamin D metabolites allows for a deeper insight into the metabolic cascade, in order to understand their association with vitamin D-related diseases. In fact, the ratio of metabolites, for example the ratio of concentrations between 25(OH)$VD_3$ and 24,25(OH)$_2VD_3$ may reveal enzyme defects leading to vitamin D-dependent disorders, such as hypercalcemia in case of CYP24A1 deficiency (5) (high ratio) or various types of cancers in case of CYP24A1 overexpression (low ratio) (6). The measurement of these metabolites by LC-MS/MS requires availability of reference standards in high purity, which previously have to be assessed by quantitative nuclear resonance spectrometry (qNMR) (7). The isotopically labeled standards are favorably labeled with multiple deuterium-, or even better, $^{13}C$- atoms.

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*Metabolism of Vitamin D*. The main pathways of vitamin D metabolism considering the presumably clinically most relevant metabolites are shown in Figure 1 (8, 9). Following UV radiation and concomitant thermal exposure, 7-dehydrocholesterol **1** is converted in the skin to vitamin $D_3$ [**2**], that is hydroxylated in the liver to 25(OH)$VD_3$ [**3**], which, in turn, is hydroxylated in the kidneys to the presumably most active metabolite 1,25(OH)$_2VD_3$ (calcitriol) [**4**]. Further metabolism occurs mainly *via* two pathways, starting with hydroxylation at C24 by CYP24A1, and leading to either formation of 1,24,25(OH)$_3VD_3$ **5,** or 24,25(OH)$_2VD_3$ **6**. Vitamin $D_2$ **7** is metabolized similarly (10).

## Materials and Methods

*Materials*. Reagents were purchased from Fisher Scientific GmbH, Sigma Aldrich Corp., Acros Organics, Carbolution Chemicals GmbH or abcr GmbH and were used without further purification. All reactions involving oxygen or moisture sensitive compounds were carried out under dry nitrogen atmosphere. All solvents used for

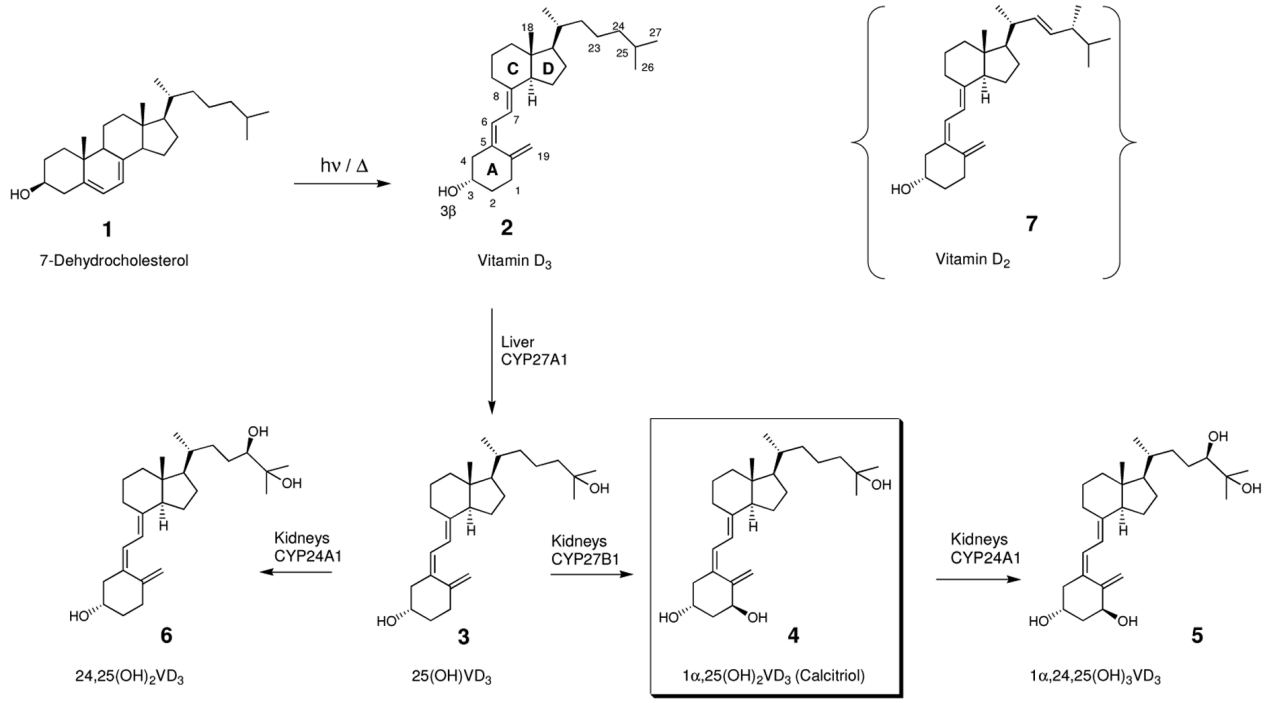


Figure 1. *Metabolic pathways of vitamin D.*

reactions and chromatographic purifications were purchased from Fisher Scientific GmbH in analytical grade. Organic extracts were dried over anhydrous $Na_2SO_4$, filtered and concentrated using rotary evaporator Heidolph Hei-VAP Advantage, pump Vacuubrand PC 3001 VARIO select at aspirator pressure (20-30 mmHg). Reactions were monitored by thin-layer chromatography (TLC) using aluminum-backed MERCK 60 silica gel plates (0.2 mm thickness). After visualization under ultraviolet light at 254 nm, the plates were developed by immersion in a solution containing either a mixture of *p*-anisaldehyde (2.5%), acetic acid (1%), and sulfuric acid (3.4%) in 95% ethanol or a solution of ceric ammonium nitrate (0.5 g) and ammonium molybdate (4.8 g) in $H_2O$ (100 ml) and $H_2SO_4$ (5.6 ml), followed by heating with a hot gun. Flash column chromatography was performed with Merck silica gel (230-400 mesh). NMR spectra were recorded in $CDCl_3$ (delta H 7.26, delta C 77.0) on a Bruker Avance I 500 MHz spectrometer (Bruker, BioSpin GmbH, Rheinstetten, Germany) equipped with a 5 mm TCI Probe ($^1$H 500 MHz, $^{13}$C 125 MHz) at 295 K using the standard pulse programs from TOPSPIN 2.4 software. HPLC analyses were performed on an Agilent 1260 HPLC System 600 bar, G1312B binary pump, G1379B degasser, G1367C HIP SL autosampler, and G1330B cooling module. Preparative HPLC separations and purifications were performed on a liquid chromatograph Interchim 5.250 puriFlash LC system, equipped with an integrated Nano-ELSD, UV-Vis DAD detector and software Intersoft X (ISX) with GENIUS, or on a Varian Star Chromatography Workstation Version 6.

*Methods*. The most practical and versatile synthesis of vitamin D metabolites starts with readily available vitamin $D_2$ [**7**] (11, 12) (Figure 2). In a connective (*i.e.*, convergent) synthesis (13), the *trans*-1,3-diene moiety of **7** is oxidatively cleaved to obtain an A-ring and a CD-ring building block. Both of them are modified separately and finally reconnected. By application of this strategy, vitamin $D_2$ [**7**] is converted in the corresponding *tert*-butyldimethylsilyl (TBDMS) ether and cleaved in an A-ring allylic alcohol **8** and a CD-ring diol ("Inhoffen-diol") **9**. **8** is converted *via* an allyl chloride in a phosphine oxide **10**. After a side chain of the CD-ring is prepared separately, **11** is obtained. Finally, both parts are connected by a Wittig-Horner-Washworth-Emmons reaction to obtain vitamin D metabolites of general formula **12**. If labeled compounds are needed, as for drug monitoring studies or as reference standards for measurement of any vitamin D metabolite in LC-MS/MS assays, accordingly labeled starting material can be used, leading to metabolites labeled favorably multifold by $^{13}$C in the side chain. By careful selection of commercially available $^{13}$C-labeled starting material, a wide variety of medicinally relevant $^{13}$C-labeled vitamin D metabolites can be synthesized by this strategy.

## Results

Overall, 10 $^{13}$C-labeled vitamin D metabolites have been chemically synthesized and obtained in good yield and high purity, as outlined by the following representative examples.

*Synthesis of 25(OH)VD$_3$$^{13}$C$_3$ and 1α,25(OH)$_2$VD$_3$$^{13}$C$_3$.* As outlined in Figure 3, Inhoffen-diol **9** is converted to the corresponding iodide **13**, that is connected with ethyl acrylate in a copper/zinc mediated reaction to give **14** (14). In this step,

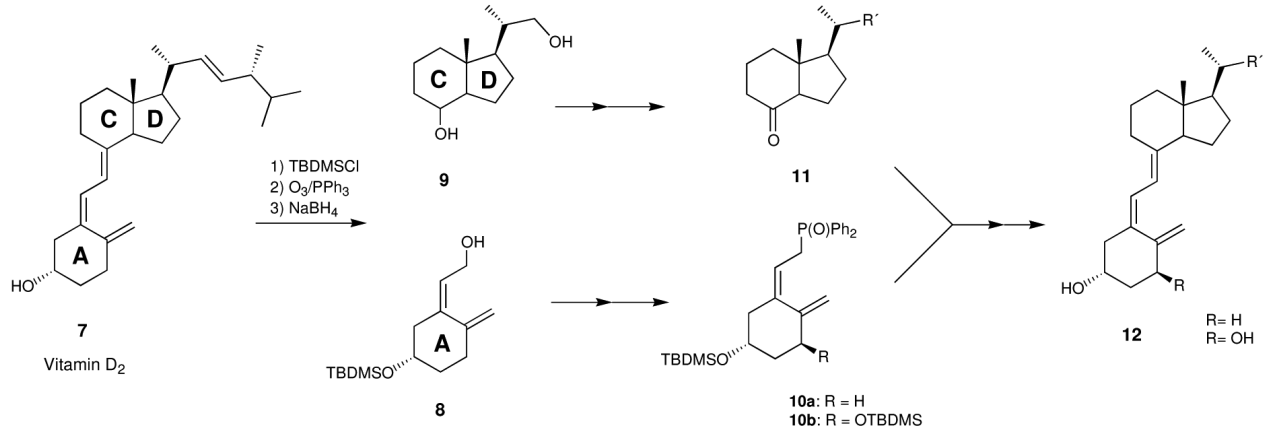


Figure 2. *Connective synthesis of vitamin D metabolites starting with vitamin $D_2$. $NaBH_4$: Sodium boron hydride; TBDMSCl: tert-butyldimethylsilyl chloride; $PPh_3$: triphenylphoshine.*

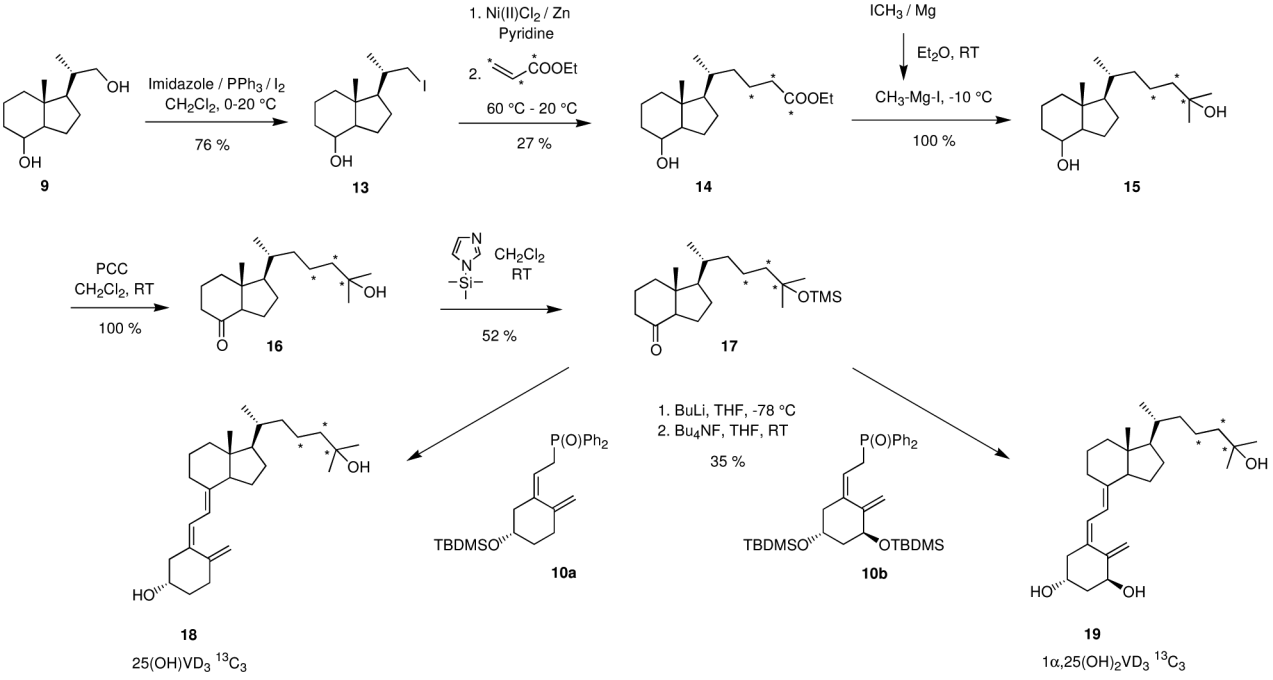


Figure 3. *Synthesis of 25(OH)$VD_3{}^{13}C_3$ and 1α,25(OH)$_2VD_3{}^{13}C_3$. $PPh_3$: Triphenylphosphine; RT: room temperature; THF: tetrahydrofurane; PCC: pyridinium chloro chromate; TMS: trimethylsilyl; TBDMS: tert-butyldimethylsilyl; BuLi: n-butyl lithium; $Bu_4NF$: tetra-n-butylammonium fluoride.*

commercially available 3-fold $^{13}C$-labeled acrylate is used to obtain the corresponding 3-fold labeled metabolite. By Grignard reaction of **14** with methylmagnesium iodide, diol **15** is obtained. Subsequent oxidation with pyridinium chlorochromate (PCC) leads to ketone **16**, and protection of the C25-OH group as a trimethylsilyl ether gives ketone **17**, that is coupled with diphenylphoshine oxide **10a**, leading after removal of the silyl protective group to 25(OH)$VD_3{}^{13}C_3$ [**18**]. Analogously, coupling of **17** with **10b** leads to 1α,25(OH)$_2VD_3{}^{13}C_3$ [**19**]. The synthesis of vitamin $D_2$ metabolites is somewhat more challenging than those of their $D_3$ counterparts, particularly because epimerization at C20 may occur during connection of a side chain to the CD-ring building block. Additionally, a chiral methyl group at C24 has

to be installed. Therefore, in some cases the side chain favorably may be attached after A-ring and CD-ring building blocks have already been connected (15).

## Discussion

As an established methodology, deuterium (D) labeled biomarkers are commonly used as calibration and reference standards in LC-MS/MS based diagnostic tools, such as for therapeutic drug monitoring or other metabolism studies. In order to achieve a high accuracy determining the concentration of an analyte of interest, the presence of unlabeled reference standard in the probe has to be excluded (D0=0). Multifold labeling is an alternative, however, the occurrence of unlabeled reference standard can not rigorously excluded, due to H-D-exchange in the course of the synthesis. Therefore, labeling of reference standards by $^{13}C$ appears advantageous, although their synthesis is more challenging. By appropriate choice of labeled starting material, the positions of labeling and number of labeled atoms can be selected on demand. Apparently, 1-3 fold $^{13}C$-labeled ethyl acrylate (Figure 3) is commercially available. In the following Grignard addition $^{13}C$ labeled methyl iodide can be used to introduce two $^{13}C$ atoms at position C26 and C27. By application of other methodologies to build up the CD-ring *de novo*, such as a Pauson-Khand reaction (16, 17), labeling is also possible at various positions at the CD-ring, particularly at C18. Finally, various positions at the A-ring can be $^{13}C$ labeled as well, for example if the A-ring is employed as an acyclic enyne precursor in a Pd(0)-mediated reaction (18), either by reaction of an accordingly labeled enyne with a CD-ring vinyl bromide (19) or an enyne triflate with a CD-ring boronate (20).

## Conclusion

Access to a wide variety of $^{13}C$-labeled highly pure vitamin D metabolites by application of a convergent synthesis, starting with vitamin $D_2$, enables the application of LC-MS/MS-based assays towards a better understanding of vitamin D-related diseases by differential diagnosis.

## Conflicts of Interest

The Authors declare no conflicts of interests.

## Authors' Contributions

Lars Kattner conducted the research, provided the materials (reagents, instrumentation, computing resources and other synthesis and analysis tools), surveyed, headed and coordinated the research activities, prepared the presentation of the published work, specifically its visualization (drawing of Figures) and text, has written the initial and revised manuscript, and acquired the financial support for the project leading to this publication. Erik Rauch designed the methodology, performed the experiments, created and analyzed the data.

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