# Synthesis of 25-hydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$ and 1α,25-dihydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$

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*Syntheses of polydeuterated analogs of 25-hydroxyvitamin $D_3$ and 1α,25-dihydroxy vitamin $D_3$ are described. These analogs, containing stable isotope atoms at metabolically stable positions, are potentially useful in studies involving catabolism of hydroxylated metabolites of vitamin $D_3$. (Steroids* **57**:142–146, 1992)

**Keywords:** synthesis; 25-hydroxyvitamin $D_3$; 1α,25-dihydroxyvitamin $D_3$; deuterium labeling; thermospray mass spectrometry; metabolic studies; steroids

## Introduction

Advances made in the field of mass spectrometry during the last two decades have greatly expanded the use of stable isotope-labeled molecules of biological importance.[1,2] These analogs have been used as internal standards in biochemical assays using isotope-dilution mass spectrometry.[3] A combination of stable isotope-labeled compounds and mass spectrometric analysis has also been an important tool in the in vivo and in vitro metabolic studies of these molecules.[4] In the vitamin D area, isotope-dilution mass spectrometric technique has provided an accurate, and probably more reliable, alternative to the classical biological measurements of vitamin D and its various metabolites in a variety of body fluids. Typically, these methods have used synthetic analogs of vitamin D and its metabolites, labeled with several deuterium atoms ($^2H$) in the steroidal side chain.[5] Synthetic methods for obtaining such $^2H$-labeled analogs and mass spectrometric techniques used for such assays have been described in a recent review.[6] Despite the success of isotope dilution mass spectrometry in assaying vitamin D and its metabolites, the $^2H$-labeled analogs have enjoyed a limited application in conducting metabolic studies. The reason for that lies, at least in part, in the fact that the majority of the $^2H$-labeled analogs of vitamin D metabolites reported so far contain $^2H$ atoms at C-26(27) positions in the steroidal side chain, which is prone to metabolic transformations and cleavage, leading to the loss of the tracer atoms.[7] In this communication, we report the syntheses of 25-hydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$ and 1α,25-dihydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$, and analogs of 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) and 1α,25-dihydroxyvitamin $D_3$ (1,25-$(OH)_2D_3$), respectively, in which the $^2H$ atoms are located in positions from where they cannot be lost by further metabolism of the molecules.

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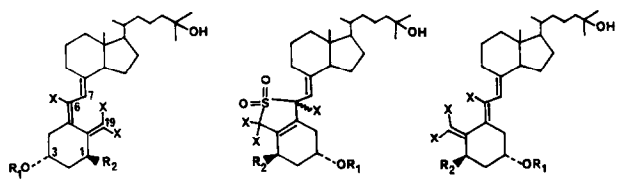
## Experimental

Dimethylformamide (DMF, Aldrich Chemical Co., Milwaukee, WI, USA) was dried by distilling from calcium hydride and storing over 4-Å molecular sieves in an argon atmosphere. Eosin Y (0.5% solution in 90% ethanol) and deuterium oxide ($^2H_2O$, >99% isotopic purity) were purchased from Sigma Chemical Co., St. Louis, MO, USA, and Aldrich Chemical Co., respectively.

Thin-layer chromatography (TLC) plates were obtained from Analtech Co. (Vineland, NJ, USA). Ultraviolet (UV) spectra of the synthetic compounds were obtained in methanol in a Perkin Elmer 552A UV/VIS spectrophotometer (Norwalk, CT, USA). High-performance liquid chromatographic (HPLC) analyses of synthetic compounds were performed on a Waters HPLC system consisting of an M-40 solvent delivery pump and a Lambda Max-480 UV detector (Waters Inc., Milford, MA, USA). The wavelength of the detector was set at 254 nm. Silica (ECONOSIL, 5 μm) and $C_{18}$ (MICROSORB, 5 μm) HPLC columns were obtained from Rainin Instruments Co., Woburn, MA, USA. $^1H$ NMR spectra were obtained in a Bruker 250-MHz instrument with samples in $CDCl_3$ as solvent. TMS was used as the internal standard, wherever appropriate. Otherwise, δ7.26 peak of $CHCl_3$ was used as the internal standard. Isotope-enrichment of the target compounds were determined by HPLC-mass spectrometry (HPLC-MS) due to its availability to us. In this method, synthetic compounds were eluted from a 3-μm Axxiom ODS



1: $R_1$ = TBDMS, $R_2$ = X = H
2: (2a + 2b): $R_1$ = TBDMS, $R_2$ = X = H
3: $R_1$ = TBDMS, $R_2$ = H, X = $^2H$
4: $R_1$ = TBDMS, $R_2$ = H, X = $^2H$
5. $R_1$ = TBDMS, $R_2$ = H, X = $^2H$
6: $R_1$ = $R_2$ = H, X = $^2H$
7: $R_1$ = X = H, $R_2$ = OH
8: $R_1$ = X = H, $R_2$ = OH
9: $R_1$ = H, $R_2$ = OH, X = $^2H$
10: $R_1$ = TBDMS, $R_2$ = OTBDMS X = H
11: $R_1$ = TBDMS, $R_2$ = OTBDMS, X = H
12: $R_1$ = TBDMS, $R_2$ = OTBDMS, X = $^2H$

**Figure 1** Structures of various synthetic intermediates involved in the synthesis of 25-hydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$ and 1α,25-dihydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$.

column (4.6 mm × 7.5 cm) with 90% aqueous methanol. The effluent was mixed with 0.1 M ammonium acetate ($NH_4OAc$) and the mixture was introduced directly into a Vestec (Houston, TX, USA) thermospray mass spectrometer, Model No. 20 (block 230 C, vapor 200 C) under the control of a Teknivent (St. Louis, MO, USA) data system. $MH^+$ (for 25-OH-$D_3$) and $MH^+$-$H_2O$ (for 1,25-$(OH)_2D_3$) ions for both the deuterated and nondeuterated samples were monitored by selected ion monitoring (SIM) mode.

## *Sulfur dioxide-adduct of 25-OH-$D_3$-3β-*tert*-butyldimethylsilyl ether (2)*

Approximately 2 ml of liquid sulfur dioxide ($SO_2$) (obtained by dropwise addition of concentrated $H_2SO_4$ to sodium metabisulfite, and condensing the vapor at −78 C) was added to 50 mg of 25-OH-$D_3$-3β-*tert*-butyldimethylsilyl ether (**1**) at −78 C with stirring (Figure 1). Cooling bath was removed, and the yellow solution was refluxed for an hour. Removal of $SO_2$ by aspiration produced an almost quantitative yield of the $SO_2$ adduct (**2**). In the TLC [3 : 1 hexane/ethyl acetate (EtOAc)] two close non-UV-absorbing spots (detected by iodine absorption), corresponding to 6R- and 6S-adducts, were obtained. The two isomers were separable by flash chromatography over silica (Merck Grade 60, 60Å (Aldrich Chemical Co.) using 3 : 1 hexane/EtOAc as eluent with regulated argon pressure from a tank and an effluent flow rate of approximately 3 ml/minute. We concluded that the slower moving isomer was the 6R-isomer by comparison with the published data.[8] $^1H$ NMR of **2**(**2a** and **2b**): δ0.04 (6H, d, $Si(CH_3)_2$); δ0.55 and 0.64 (two singlets, together integrating 3H, $C_{18}$-$CH_3$ for 6R and 6S isomers); δ0.86 (9H, s, $C(CH_3)_3$); δ0.93 (3H, d, $C_{20}$-$CH_3$); δ1.19 (6H, s, $C_{26,27}$-$CH_3$); δ3.6 (2H, broad s, $C_{19}$-$CH_2$); δ3.9 (1H, m, $C_3$-H); δ4.52-4.85 (m, consisting of four narrow doublets, 2H, $C_{6,7}$-H). Partial $^1H$ NMR of purified 6R-isomer (**2a**): δ0.64 (3H, s, $C_{18}$-$CH_3$); δ4.5 (1H, d, J = 9.17 Hz, $C_6$-H), and δ4.69 (1H, d, J = 10 Hz, $C_7$-H).

## *Base-catalyzed $^1H$-$^2H$ exchange reaction of 2*

To an ice-cold solution of **2** (65 mg) in 0.6 ml of anhydrous DMF was added a solution of *t*-BuOK (88.2 mg in 0.2 ml of anhydrous DMF and 0.2 ml of $^2H_2O$) with stirring in an argon atmosphere. The ice-bath was removed and the tan-colored solution was stirred at 25 C under argon for 30 minutes. After this period the reaction mixture was chilled on ice and diluted with an excess of $^2H_2O$. The aqueous solution was extracted with ether. Flash chromatography of the reaction mixture on silica, using the method described above, produced 47.4 mg (73%) of the deuterated compound (**3**). In the $^1H$ NMR spectrum of **3**, the δ3.6 singlet peak (for $C_{19}$-$CH_2$ in **2**) was completely absent. There were, on the other hand, two singlets at δ4.68 and δ4.76 representing $C_7$-Hs of 6R and 6S-isomers of **3**. Judging from the integration values, it was concluded that deuteration at $C_{19}$ and $C_6$ sites were complete.

## *25-Hydroxy-5**E**-[6,19,19′-$^2H_3$]vitamin $D_3$-3β-*tert*-butyldimethylsilyl ether (4)*

A suspension of $NaHCO_3$ (16.8 mg) and (**3**) (24 mg) in 0.2 ml of anhydrous DMF was heated under argon at 90 C for 2.5 hours followed by cooling to 0 C and addition of water. The aqueous solution was extracted with ether. The yield of the desired product (**4**) was almost quantitative (after preparative TLC purification). UV spectrum of **4** had a $\lambda_{max}$ at 271 nm, typical for 5**E**-vitamin D chromophore. NMR of **4**: δ0.04 (6H, d, $Si(CH_3)_2$); δ0.57 (3H, s, $C_{18}$-$CH_3$); δ0.85 (9H, s, $C(CH_3)_3$); δ0.95 (3H, broad s, $C_{20}$-$CH_3$); δ1.32 (6H, narrow d, $C_{26,27}$-$CH_3$); δ3.96 (1H, m, $C_3$-H); δ5.92 (1H, s, $C_7$-H). Partial NMR of the nondeuterated counterpart of **4** (X = H): δ5.2 and 5.61 (2H, broad s, $C_{19}$-$CH_2$); δ6.2 (1H, d, J = 11.2 Hz, $C_7$-H); δ6.62 (1H, d, J = 12 Hz, $C_6$-H).

## *25-Hydroxy-5**Z**-[6,19,19′-$^2H_3$]vitamin $D_3$-3β-*tert*-butyldimethylsilyl ether (5)*

A solution of the 5**E**-compound (**4**) (5 mg) and eosin Y (0.5% solution in 90% ethanol, 0.3 ml) in a total of 1 ml of EtOH was irradiated in a quartz test tube under bubbling argon with a pre-equilibrated Hanovia medium-pressure Hg-arc lamp for 10 minutes. The reaction mixture was dried under argon and purified by preparative TLC (8 : 1 hexane/EtOAc) to provide the 5**Z**-isomer (**5**, 1.9 mg, 38%). UV spectrum of **5** had a typical 5**Z**-vitamin D-like absorption with a $\lambda_{max}$ at 265 nm and a $\lambda_{min}$ at 228 nm. In the NMR spectrum **5**, the olefinic region contained only a singlet peak at δ6.02 representing $C_7$-H.

## *25-Hydroxy-5**Z**-[6,19,19′-$^2H_3$]vitamin $D_3$ (6)*

To a solution of **5** (1.9 mg) in 0.6 ml of acetonitrile was added 0.1 ml of 5% aqueous hydrofluoric acid (HF). The solution was stirred at 25 C for 90 minutes and then neutralized with a saturated $NaHCO_3$ solution. The aqueous reaction mixture was extracted with EtOAc and dried under argon. The desilylated product (**6**) was purified by HPLC (silica column, 5% isopropanol (IPA) in hexane, 1.5 ml/minute). The yield of the product (**6**), which had an HPLC retention time identical with that of 25-OH-$D_3$, was 1.13 mg (75.8%). UV spectrum of **6** had $\lambda_{max}$ at 265 nm and $\lambda_{min}$ at 228 nm characteristic of 5**Z**-vitamin D chromophore. $^1H$ NMR of **6**: δ0.58 (3H, s, $C_{18}$-$CH_3$); δ0.95 (3H, narrow d, $C_{22}$-$CH_3$); δ1.2 (6H, s, $C_{26,27}$-$CH_3$); δ3.95 (1H, m, $C_3$-H); δ6.03 (1H, s, $C_7$-H). A thermospray mass spectrum of **6** was obtained by scanning from 100 to 450 amu (Figure 2). The percent deuterium enrichment was determined by comparing the intensity of the $MH^+$ ions for the labeled compound (m/z 404) and the unlabeled counterpart (m/z 401). Other significant peaks were 386 (383) [$MH^+$-$H_2O$], and 368 (365) [$MH^+$-$2H_2O$].

## *Base-catalyzed $^1H$-$^2H$ exchange reaction and thermolysis of the $SO_2$-adducts of 1α,25-(OH)$D_3$ (8) and 1,3-di-*tert*-butyldimethylsilyl ether of 1α,25-$(OH)_2D_3$(11)*

The $SO_2$ adducts (**8**) and (**11**) [prepared in the usual fashion from 1,25-$(OH)_2D_3$ (**7**) or the corresponding disilyl ether (**10**)] were treated with t-BuOK-DMF/$^2H_2O$ as described earlier. The reaction mixture in each case was extracted with EtOAc. The organic

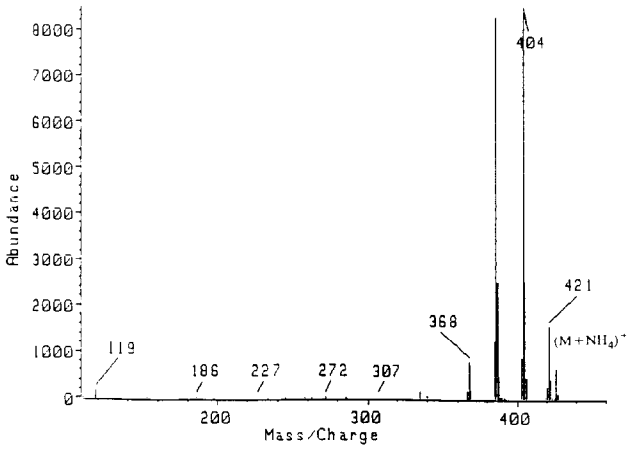



**Figure 2** Thermospray mass spectrum of 25-hydroxy-[6,19,19'-$^2H_3$]vitamin $D_3$. The base peak (m/z 404) represents the $MH^+$ ion.

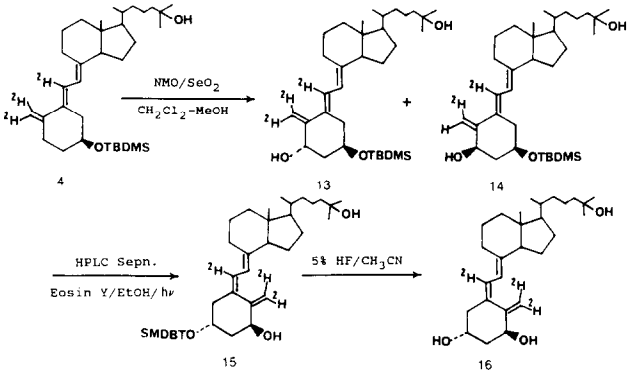



**Figure 3** Synthesis of 1α,25-dihydroxy-[6,19,19'-$^2H_3$]vitamin $D_3$.

extract, without further purification, was heated (3 : 1 EtOH/toluene, 60 C) for an hour under argon. In each case, the crude reaction mixture was applied to a TLC plate that was eluted with an appropriate solvent (1 : 1 hexane/EtOAc for 1,25-$(OH)_2D_3$, and 10 : 1 hexane/EtOAc for the corresponding disilyl ether). In each case the reaction product contained a complex mixture of UV-absorbing compounds. On a preparative scale, the bands corresponding to the 5**E**-isomers of 1,25-$(OH)_2D_3$ and its 1,3-disilyl ether were isolated, and the yields of the deuterated products, that is, 1α,25-dihydroxy-5**E**-vitamin $D_3$ (**9**), and its disilyl ether (**12**) were determined by UV. The yields of the desired products were only 1–4%.

## *1α,25-Dihydroxy-5**E**-[6,19,19'-$^2H_3$]vitamin $D_3$-3β-*tert*-butyldimethylsilyl ether* (**13**)

A solution of **4** (24 mg) and N-methylmorpholine N-oxide (NMO) (27.3 mg) in 1 ml dichloromethane was refluxed gently under argon for 15 minutes followed by cooling to room temperature and quick addition of a solution of selenium dioxide ($SeO_2$) (6.2 mg, dissolved in 1 ml methanol). The resulting mixture was refluxed for an additional hour, followed by cooling to room temperature, dilution with water, and extraction with ether. The organic extract was concentrated under argon, and was purified by preparative TLC on silica (3 : 1 hexane/EtOAc). The most intense UV-absorbing band was isolated. This product contained a mixture of 1α- (**13**) and 1β-hydroxy isomers (**14**) (Figure 3),

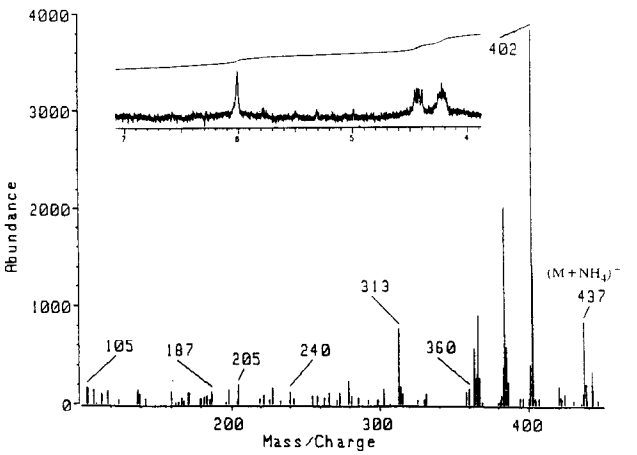



**Figure 4** Thermospray mass spectrum of 1α,25-dihydroxy-[6,19,19'-$^2H_3$]vitamin $D_3$. The base peak (m/z 402) represents $MH^+$-$H_2O$ ion. Inset: Partial NMR spectrum (δ4.0–7.0) of 1α,25-dihydroxy-[6,19,19'-$^2H_3$]vitamin $D_3$.

which were separated by HPLC (silica column, 2% IPA in hexane, 1.5 ml/min). Under these conditions, the desired 1α-isomer (**13**) had a longer retention time (13.7 minutes, 62%) than the 1β-isomer (**14**), 12 minutes, 10.2%). The UV spectrum of (**13**) had a $\lambda_{max}$ at 271 nm. $^1H$ NMR of (**13**): δ0.08 (6H, s, $Si(CH_3)_2$); δ0.46 (3H, s, $C_{18}$-$CH_3$); δ0.87 (9H, s, $(CH_3)_3C$); δ0.95 (3H, d, $C_{20}$-$CH_3$); δ1.23 (6H, s, $C_{26,27}$-$CH_3$); δ4.2 (1H, m, $C_3$-H); δ4.5 (1H, m, $C_1$-H); and δ5.8 (1H, s, $C_7$-H). NMR of the nondeuterated counterpart of **13**, X = H: δ 4.95 and 5.08 (2H, broad s, $C_{19}$-$CH_2$); δ5.86 (1H, d, J = 12.23 Hz, $C_7$-H); δ6.52 (1H, d, J = 11.04 Hz, $C_6$-H).

## *1α,25-Dihydroxy-5**Z**-[6,19,19'-$^2H_3$]vitamin $D_3$-3β-*tert*-butyldimethylsilyl ether* (**15**)

A solution of **13** (5 mg) and Eosin Y (1.34 mg) in 1 ml ethanol was irradiated under argon with stirring with a pre-equilibrated medium pressure Hg-arc lamp for 10 minutes. The irradiated mixture was purified by preparative TLC (3 : 1 hexane/EtOAc). The yield of the 5**Z**-isomer (**15**) was 2.77 mg (54.8%). UV spectrum of **15** had a maximum at 264 nm and a minimum at 228 nm. $^1H$ NMR: δ0.08 (6H, s, $Si(CH_3)_2$); δ0.53 (3H, s, $C_{18}$-$CH_3$); δ0.90 (9H, s, $C(CH_3)_3$); δ0.94 (3H, d, J = 6.2 Hz, $C_{21}$-$CH_3$); δ1.22 (6H, s, $C_{26,27}$-$CH_3$); δ4.26 (1H, m, $C_3$-H); δ4.38 (1H, m, $C_1$-H); δ6.02 (broad s, 1H, $C_7$-H). NMR of the nondeuterated compound (**15**, X = H): δ4.92 and 5.26 (broad s, 2H, $C_{19}$-$CH_2$); δ6.02 and 6.33 (ABq pattern, 2H, J = 11.1 Hz, $C_{6,7}$-H).

## *1α,25-Dihydroxy-5**Z**-[6,19,19'-$^2H_3$]vitamin $D_3$* (**16**)

A solution of **15** (1.95 mg) and 5% aqueous HF (0.1 ml) in 0.75 ml of acetonitrile was stirred at 25 C under argon for 90 minutes followed by addition of saturated sodium bicarbonate solution and extraction with EtOAc. The reaction mixture was purified by HPLC (silica column, 12% IPA in hexane, 2 ml/minute). The yield of the desilylated product (**16**) was 1.34 mg (88%). UV spectrum of **16** had a $\lambda_{max}$ at 264 nm and a $\lambda_{min}$ at 228 nm. The deuterated material was indistinguishable from a standard sample of the nondeuterated material, that is, 1,25-$(OH)_2D_3$ by both normal and reverse-phase HPLC. $^1H$ NMR: δ0.55 (3H, s, $C_{18}$-$CH_3$); δ0.93 (3H, d, J = 5.4 Hz, $C_{21}$-$CH_3$); δ1.21(6H, s, $C_{26,27}$-$CH_3$); δ4.22 (1H, m, $C_3$-H); δ4.44 (1H, m, $C_1$-H); δ6.01 (1H, broad s, $C_7$-H) (Figure 4, inset). In the thermospray mass spectrum of

16, the extent of the deuterium enrichment was determined by comparing m/z 402 and 399 ($MH^+$-$H_2O$) peaks of the labeled and the unlabeled compounds, respectively (Figure 4). Other significant peaks were 384 (381) [$MH^+$-$2H_2O$] and 366 (363) [$MH^+$-$3H_2O$].

## Results and discussion

It is well documented that 1,25-$(OH)_2D_3$, the hormonally active form of vitamin $D_3$, interacts with a nuclear receptor in its target tissues to bring about its observed physiologic functions including calcium and phosphorus homeostasis.[7] After serving its function, 1,25-$(OH)_2D_3$ undergoes a series of P450 cytochrome-mediated biologic oxidations at the side chain leading to the loss of four carbon atoms ($C_{24}$-$C_{27}$). The ultimate product, calcitroic acid, a $C_{23}$-carboxylic acid, is excreted in the bile. Despite recent research activity in this area,[9,10] details of this catabolic pathway are far from being established to date.

During the past few years, we have been interested in studying the antiproliferative effect of 1,25-$(OH)_2D_3$ on human skin.[11] These studies required that we closely investigate the above-mentioned catabolic process of 1,25$(OH)_2D_3$ in vitro as well as in vivo. Commercially available $^3H$-labeled analogs of 1,25-$(OH)_2D_3$, that is, 1$\alpha$,25-dihydroxy[26,27(n)-$^3H$]vitamin $D_3$ and 1$\alpha$,25-dihydroxy-[23,24(n)-$^3H$]vitamin $D_3$ were unsuitable for our studies for reasons mentioned earlier. Instead, we needed an analog of 1,25-$(OH)_2D_3$ that would ideally contain several atoms of $^2H$ or $^3H$ at nonmetabolizable positions. Before this report, syntheses of 1,25-$(OH)_2D_3$ analogs containing $^2H$ or $^3H$ atom at a metabolically stable 6- or 1-position have been described.[12–14] However, most metabolic studies, particularly those carried out in vivo, ideally require more than one tracer atom in the labeled analog to facilitate detection and analysis. Recently, the synthesis of [6,19,19′-$^2H_3$]vitamin $D_3$ by an efficient base-catalyzed exchange of 6,19,19′-hydrogens of vitamin $D_3$-sulfur dioxide adduct was reported.[15] The same synthetic methodology was also used to produce 24**R**,25-dihydroxy-[6,19,19′-$2H_3$]-vitamin $D_3$ and 24**R**,25-dihydroxy-[6,19,19′-$^3H_3$]vitamin $D_3$.[16] The simplicity and efficiency of the synthetic procedures led us to embark on the synthesis of the title compound. In a trial experiment, treatment of the 5**E**-1,25-$(OH)_2D_3$-$SO_2$ adduct (**8**) with *t*BuOK in anhydrous DMF and $^2H_2O$ (−78–25°C) followed by thermal extrusion of $SO_2$ produced the expected 5**Z**-1,25-$(OH)_2D_3$ (**9**) in very poor yield. The same sequence of reactions with the $SO_2$ adduct of 1,3-ditert-butyldimethylsilyl ether of 1,25-$(OH)_2D_3$ (**11**) also failed to produce the desired product.

This failure led us to introduce the 1$\alpha$-hydroxyl group in the molecule (labeled) at a later stage. Thus, the 3$\beta$-*tert*-butyldimethylsilyl (TBDMS) ether of 25-OH-$D_3$ (**1**) (Figure 1) was condensed with liquid $SO_2$ to produce the adduct (**2**). Next, the 6,19,19′ hydrogens of the adduct (**2**) were exchanged with deuterium ($^2H$) in anhydrous DMF-$^2H_2O$ (>99% isotopic purity) in the presence of *t*BuOK as the base-catalyst. The $^2H$-labeled product (**3**) was then converted to 25-hydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$ (**6**) in three steps. This labeled analog (**6**) was found to be identical with an authentic sample of unlabeled 25-OH-$D_3$ by HPLC. In the thermospray mass spectrum of **6**, using selective ion monitoring, the $M^+$ ion was increased by three mass units with >95% efficiency, indicating the introduction of three deuterium atoms (Figure 2). In the high-field NMR spectrum of **6** the olefinic region of the spectrum contained a single peak, integrating one proton. By comparison with the NMR spectrum of [6,19,19′-$^2H_3$]vitamin $D_3$,[15] this singlet was assigned to the C-7 proton.

The synthesis of 1$\alpha$,25-dihydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$ required the introduction of 1-hydroxyl group into the $^2H$-labeled 25-hydroxy-5**E**-vitamin $D_3$ analog (**4**) in a stereospecific manner. Pechet et al. have recently developed an efficient method of introducing a 1$\alpha$-hydroxyl group in the 5**E**-vitamin D system.[17] Thus, compound **4** upon oxidation ($NMO/SeO_2$) produced a mixture of 1-hydroxylated isomers (**13** and **14**), from which the predominant isomer, having the correct stereochemistry at 1-position (**13**),[8] was separated from the undesired stereoisomer (**14**) by HPLC (Figure 3). The 1$\alpha$-hydroxy analog (**13**) was then converted to the desired compound, 1$\alpha$,25-dihydroxy-[6,19,19′-$^2H_3$]vitamin $D_3$ (**16**) in the usual fashion. This deuterium-labeled analog (**16**), having HPLC properties identical to an authentic sample of unlabeled 1,25-$(OH)_2D_3$, was structurally identified by high-field NMR in which the characteristic olefinic absorptions (between $\delta$4.8 and 6.5) of 6, 7, and 19 hydrogens were replaced by a one-proton singlet ($\delta$6.05) representing the unlabeled hydrogen atom at the 7-position (Figure 4, inset). On the other hand, thermospray mass spectrum of the labeled analog (**16**) contained 89% $^2H_3$, 9% $^2H_2$ and a negligible amount of $^2H_0$ (Figure 4). These analyses clearly demonstrated that in this molecule, the 6, 19, and 19′ hydrogens were almost completely replaced by $^2H$ (>95% isotopic purity).

The $^2H$-labeled analogs of 25-OH-$D_3$ and 1,25-$(OH)_2D_3$, described above, are expected to be useful in carrying out a variety of in vivo and in vitro metabolic studies involving 25-OH-$D_3$ and 1,25-$(OH)_2D_3$ due to the stability of the tracer atoms in the catabolic processing of the molecules. Furthermore, these $^2H$-labeled analogs are expected to be largely devoid of primary kinetic isotope effect, which has been a problem in metabolic studies using isotopically labeled compounds. Halloran et al. have recently described an approximate 35% decrease in the metabolic clearance rate of 1,25-dihydroxy-[26,27(n)-$^3H$]vitamin $D_3$ and 1,25-dihydroxy-[23,24(n)-$^3H$]vitamin $D_3$, compared with 1,25-$(OH)_2D_3$, in rats.[18] Renal metabolic rates of these isotopically labeled analogs, and even the nature of metabolites produced thereof, were seriously affected. Clearly, these analogs, with tracer atoms positioned in close proximity with the metabolic oxidation centers, suffered serious primary kinetic isotope effects, leading to the observed alterations in the metabolic reaction rates. Uskokovic et al. have invoked this theory to synthesize 26,27-hexadeutero and 26(27)-trideutero an-

alogs of 1,25-$(OH)_2D_3$ with projected half-lives considerably longer than that of 1,25-$(OH)_2D_3$.[19] In the 6,19,19′-$^2$H-labeled analogs of 25-OH-$D_3$ and 1,25-$(OH)_2D_3$ described by us, the tracer atoms are positioned away from the steroidal side chain, which is the site of $P_{450}$ cytochrome-mediated oxidative activities. As a result, these analogs are expected to be free from any primary kinetic isotope effect.

In conclusion, we have described the syntheses of $^2$H-labeled analogs of 25-OH-$D_3$ and 1,25-$(OH)_2D_3$. These compounds are expected to be useful in carrying out in vivo and in vitro metabolic studies involving 25-OH-$D_3$ and 1,25-$(OH)_2D_3$.

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## References

1. Shackleton CHL (1985). Mass spectrometry: application to steroid and peptide research. *Endocr Rev* **6:**441–485.
2. Baillie TA (1981). The use of stable isotopes in pharmacological research. *Pharmacol Rev* **33:**81–142.
3. De Leenheer AP, Lefevre MF, Lambert WE, Colinet ES (1985). Isotope-dilution mass spectrometry in clinical chemistry. *Adv Clin Chem* **24:**111–161.
4. Vierhapper H, Nowotny P, Waldhausl W (1988). Estimation by gas chromatography–mass spectrometry with selected ion monitoring of urinary excretion rates of 3-androstanediol during/after i.v. administration of $^{13}$C-labeled testosterone in man. *J Steroid Biochem* **29:**105–109.
5. Whitney JG, Shackleton CHL, Edmonds CG, Burlingame AL, Piel CF (1979). Synthesis of deuterium-labeled vitamin $D_3$ metabolites: purification from human plasma for selected ion monitoring-mass spectrometry. In: Klein ER, Klein PD (eds), *Stable Isotopes: Proceedings of the Third International Conference*. Academic Press, New York, pp. 47–61.
6. Coldwell RD, Trafford DJH, Varley MJ, Kirk DN, Makin HLJ (1990). Stable isotope-labeled vitamin D, metabolites and chemical analogs: synthesis and use in mass spectrometric studies. *Steroids* **55:**418–432.
7. DeLuca HF (1979). The vitamin D system in the regulation of calcium and phosphorus metabolism. *Nutr Rev* **37:**161–193.
8. Calverley MJ (1987). Synthesis of MC903. A biologically active vitamin D metabolite analogue. *Tetrahedron* **43:**4609–4619.
9. Makin G, Lohnes D, Byford V, Ray R, Jones G (1989). Target cell metabolism of 1,25-dihydroxy vitamin $D_3$ to calcitroic acid. *Biochem J* **262:**173–180.
10. Reddy GS, Tserng K, Thomas BR, Dayal R, Norman AW (1987). Isolation and identification of 1,23-dihydroxy-24,25,26,27-tetranorvitamin $D_3$, a new metabolite of 1,25-dihydroxyvitamin $D_3$ produced in rat kidney. *Biochemistry* **26:**324–330.
11. Smith EL, Walworth NC, Holick MF (1986). Effect of 1$\alpha$,25-dihydroxy vitamin $D_3$ on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in culture. *J Invest Dermatol* **86:**709–713.
12. Holick MF, Tavela TE, Holick SA, Schnoes HK, DeLuca HF, Gallagher B (1976). Synthesis of 1$\alpha$-hydroxy[6-$^3$H]vitamin $D_3$ and its metabolism to 1$\alpha$,25-dihydroxy[6-$^3$H]vitamin $D_3$ in the rat. *J Biol Chem* **251:**1020–1025.
13. Paaren HE, Fivizzani MA, Schnoes HK, DeLuca HF (1981). Preparation of tritium or deuterium-labeled vitamin D analogs by a convenient general method. *Proc Natl Acad Sci USA* **78:**6173–6175.
14. Holick SA, Holick MF, McLaughlin JA (1980). Chemical synthesis of [1$\beta$-$^3$H]1$\alpha$,25-dihydroxyvitamin $D_3$ and [1$\alpha$-$^3$H]1$\beta$,25-dihydroxyvitamin $D_3$: biological activity of 1$\beta$-25-dihydroxyvitamin $D_3$. *Biochem Biophys Res Commun* **97:**1031–1037.
15. Yamada S, Suzuki T, Takayama H (1981). Novel regioselective C-6 and C-19 alkylation of vitamin $D_3$ via its sulfur dioxide adducts. *Tetrahedron Lett* **22:**3085–3088.
16. Yamada S, Shimizu M, Fukushima K, Niimura K, Maeda Y (1989). Syntheses of 24R,25-dihydroxy-[6,19,19′-$^3$H]vitamin $D_3$ and 24R,25-dihydroxy-[6,19,19′-$^2$H]vitamin $D_3$. *Steroids* **54:**145–157.
17. Andrews DR, Barton DHR, Hesse RH, Pechet MM (1986). Synthesis of 25-hydroxy- and 1$\alpha$,25-dihydroxyvitamin $D_3$ from vitamin $D_2$ (calciferol). *J Org Chem* **51:**4819–4828.
18. Halloran BP, Bikle DD, Castro ME, Gee E (1989). Isotopic labeling affects 1,25-dihydroxyvitamin D metabolism. *Biochemistry* **28:**1278–1281.
19. Uskokovic MR, Baggiolini E, Batcho A, Shiuey SJ, Wovkulic P, Horst RL (1988). Synthesis of 26,27-hexadeutero and epimeric 26-trideutero 1,25-dihydroxycholecalciferols. In: Norman AW, Schaefer K, Grioleit H-G, Herrath Dv (eds), *Vitamin D. Molecular, Cellular and Clinical Endocrinology*. de Gruyter, Berlin, pp. 22.