# Direct Chemical Synthesis of 1α,25-Dihydroxy[26,27-$^3$H]vitamin $D_3$ with High Specific Activity: Its Use in Receptor Studies†

Joseph L. Napoli,‡ William S. Mellon,§ Mary A. Fivizzani, Heinrich K. Schnoes, and Hector F. DeLuca*

ABSTRACT: The first direct chemical synthesis of radiolabeled 1α,25-dihydroxyvitamin $D_3$ is reported. Unlike all previous syntheses, the new approach does not rely on enzymatic 1α-hydroxylation of radiolabeled precursors. Rather, isotope is introduced in the last synthetic step by reaction of [$^3$H]-methylmagnesium bromide with methyl 1α-hydroxy-26,27-dinorvitamin $D_3$-25-carboxylate to give 1α,25-dihydroxy-[26,27-$^3$H]vitamin $D_3$ with a specific activity of 160 Ci/mmol. Mass spectroscopy confirmed that the radiohormone consists of a single isomer with six tritium atoms bound to carbons 26 and 27. Synthetically produced 1α,25-dihydroxy[26,27-$^3$H]vitamin $D_3$ is indistinguishable from 1α,25-dihydroxy-[26,27-$^3$H]vitamin $D_3$ obtained from the enzymatic 1α-hydroxylation of 25-hydroxy[26,27-$^3$H]vitamin $D_3$ (160 Ci/mmol) by high-pressure liquid chromatography analysis and in the competitive binding assay using chick intestinal cytosol as the receptor source. Equilibrium dissociation constant measurements with the high specific activity radiohormone indicate a $K_d$ of $8.2 \times 10^{-11}$ M for the chick intestinal cytosol 1α,25-dihydroxyvitamin $D_3$ receptor—a value considerably lower than the constants in the range of $(1–5) \times 10^{-9}$ M previously reported.

Current evidence is consistent with the hormonal form of vitamin $D_3$, 1α,25-dihydroxyvitamin $D_3$ [1α,25-$(OH)_2D_3$],[1] mediates calcium metabolism by a classical steroid hormone mechanism (DeLuca & Schnoes, 1976; DeLuca, 1978; Haussler & McCain, 1977; Norman & Henry, 1974). After entering target cells, 1α,25-$(OH)_2D_3$ binds with a high-affinity, low-capacity binding protein specific for 1α,25-$(OH)_2$D-like molecules (Brumbaugh & Haussler, 1974a, 1975a,b; Kream et al., 1976, 1977a,b; Lawson & Wilson, 1974). The binding protein–hormone complex then apparently translocates to the nucleus in a temperature-dependent process (Brumbaugh & Haussler, 1974b). Autoradiography of intestinal sections prepared from rachitic chicks dosed with high specific radioactive 1α,25-$(OH)_2D_3$ has recently provided unambiguous experimental evidence for the localization of 1α,25-$(OH)_2D_3$ in crypt and mucosal cell nuclei (Zile et al., 1978). These results have recently been confirmed (Jones & Haussler, 1979). However, despite present awareness, much work remains to be done to characterize 1α,25-$(OH)_2$D tissue receptors and to provide an in-depth understanding of the hormone's mechanism of action.

Radiolabeled hormone has been essential to experiments that have thus far elucidated the biochemistry of 1α,25-$(OH)_2D_3$. Yet high specific activity material has become available only recently (Yamada et al., 1978; Muccino et al., 1978). Both of these syntheses, which have produced 25-hydroxy[23,24-$^3$H]vitamin $D_3$ (25-OH[23,24-$^3$H]$D_3$) with specific activities of 78 and 92 Ci/mmol, respectively, required multiple synthetic and chromatographic manipulations with highly radioactive intermediates. Moreover, these syntheses did not yield 1α,25-dihydroxy[23,24-$^3$H]vitamin $D_3$ [1α,25-$(OH)_2$[23,24-$^3$H]$D_3$] directly. The hormone could be obtained only by enzymatic conversion of 25-OH[23,24-$^3$H]$D_3$. A newly introduced route to radiolabeled vitamin D compounds has provided 25-OH[26,27-$^3$H]$D_3$ with a specific activity of 160 Ci/mmol (Napoli et al., 1979). One of the benefits of this most recent approach, besides doubling the ligand's specific radioactivity, is the insertion of tritium in the last synthetic step. But like the other synthesis, enzymatically catalyzed 1α-hydroxylation is needed to obtain 1α,25-dihydroxy-[26,27-$^3$H]vitamin $D_3$ [1α,25-$(OH)_2$[26,27-$^3$H]$D_3$]. Therefore, to date, no direct chemical synthesis of radiolabeled 1α,25-$(OH)_2D_3$ has been accomplished, although such a procedure obviously would be advantageous. This paper reports the first direct chemical synthesis of radiolabeled 1α,25-$(OH)_2$D of high specific activity.

## Materials and Methods

*General.* Ultraviolet (UV) absorbance spectra were taken in 95% ethanol with a Beckman Model 24 recording spectrophotometer. Nuclear magnetic resonance (NMR) spectra were taken in $CDCl_3$ with a Bruker WH-270 FT spectrometer. Mass spectra were obtained at 115 °C above ambient temperature with an AEI MS-9 spectrometer coupled with a DS-50 data system. High-pressure liquid chromatography (LC) was done on a microparticulate silica gel column (5-μm particles; 0.62 × 25 cm) with a Waters Associates Model ALC/GPC-204 liquid chromatograph. Thin-layer chromatography (TLC) was done on silica gel 60 F-254 aluminum-backed, precoated sheets with a bed thickness of 0.25 mm available from E. Merck, Darmstadt, West Germany. Sephadex LH-20 was purchased from Pharmacia Chemicals, Piscataway, NJ. Radioactivity was measured with a Packard Model 3255 liquid scintillation counter, except for the binding protein experiments. Crystalline 1α,25-$(OH)_2D_3$ was a gift from Dr. M. Uskokovic of the Hoffmann-La Roche Co., Nutley, NJ. The chemical synthesis of 25-OH[26,27-$^3$H]$D_3$ (160 Ci/mmol) and its enzymic conversion to 1α,25-$(OH)_2$-[26,27-$^3$H]$D_3$ were previously described (Napoli et al., 1979).

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received December 6, 1979.* The work was supported by National Institutes of Health Program-Project Grant AM-14881, Postdoctoral Training Grant DE-07031, and the Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation.

‡ Present address: Department of Biochemistry, University of Texas Health Sciences Center, Dallas, TX.

§ Present address: Department of Pharmacology, College of Pharmacy, University of Wisconsin—Madison, Madison, WI 53706.

[1] Abbreviations used: 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin $D_3$; 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$, 1α,25-dihydroxy[26,27-$^3$H]vitamin $D_3$; 1α,25-$(OH)_2$[23,24-$^3$H]$D_3$, 1α,25-dihydroxy[23,24-$^3$H]vitamin $D_3$; 25-OH[23,24-$^3$H]$D_3$, 25-hydroxy[23,24-$^3$H]vitamin $D_3$; 25-OH-$D_3$, 25-hydroxyvitamin $D_3$; UV, ultraviolet; NMR, nuclear magnetic resonance; LC, high-pressure liquid chromatography.

0006-2960/80/0419-2515$01.00/0 

*Rat Bioassays.* Weanling male rats from the Holtzman Co. (Madison, WI) were individually housed in overhanging wire cages and fed a vitamin D deficient diet containing normal calcium (0.47%) and normal phosphorus (0.3%) levels for 2 weeks after receipt. They were then placed on a low-calcium (0.02%), normal-phosphorus, vitamin D deficient diet for an additional 2 weeks (Suda et al., 1970). Twenty-four hours before sacrifice the animals were divided into groups of six and were dosed intrajugularly with test compounds in 70 μL of ethanol or with ethanol alone. The rats were decapitated, and the first 10 cm of their small intestines was removed for measurement of active intestinal calcium transport by the everted gut sac method of Martin & DeLuca (1969).

*Synthesis. (1) Tosylation of 26,27-Dinorvitamin $D_3$-25-carboxylic Acid Methyl Ester.* Vitamin D ester **1** (Napoli et al., 1979) (25 mg) in dry pyridine (0.3 mL) was treated with *p*-toluenesulfonyl chloride (50 mg) for 72 h in the dark at 5 °C. The reaction mixture was added to 10% sodium bicarbonate on ice. The aqueous phase was extracted with ether–chloroform (4:1) several times. The combined organic phases were washed with 1 N hydrochloric acid, dilute sodium bicarbonate, water, and saturated sodium chloride and dried over magnesium sulfate. Evaporation of the solvent gave the tosylate **2**, homogeneous on TLC (ethyl acetate–hexane, 2:3; $R_f$ 0.62).

*(2) Solvolysis of Tosylate to Cyclovitamin Ester* **3**. The total sample of tosylate **2** was warmed at 60 °C under nitrogen in a mixture of dry methanol (0.5 mL), sodium bicarbonate, and dichloromethane (0.1 mL). Additional solvent was added as needed to compensate for evaporation. After 11.5 h, ether and water were added. The phases were separated, and the organic phase was washed several times with water and saturated sodium chloride and dried with magnesium sulfate. TLC (ethyl acetate–hexane, 1:4) revealed a major spot (after heating) with an $R_f$ of 0.42 (compound **3**) and several minor spots.

*(3) Allylic Oxidation to 1α-Hydroxycyclovitamin Ester* **4**. To selenium dioxide (2.8 mg) in dry dichloromethane (1 mL) at 0 °C was added *tert*-butyl hydroperoxide (11 μL). The mixture was stirred for 30 min under nitrogen. Cyclovitamin **3** in dichloromethane (1.0 mL) was added in one portion. The mixture was stirred for 10 min at 0 °C and an additional 7 min at ambient temperature and quenched with saturated sodium bicarbonate. The reaction mixture was extracted with dichloromethane several times, and the combined organic phase was washed with water and saturated sodium chloride and dried with magnesium sulfate. The residue was purified by TLC (ethyl acetate–hexane, 1:1) to give **4** (8.5 mg; $R_f$ 0.35) and the keto product **5** (5.8 mg; $R_f$ 0.58). Compound **4** was homogeneous on TLC.

*(4) 1α-Acetoxycyclovitamin Ester* **7**. 1α-Hydroxycyclovitamin **4** (8.5 mg) and acetic anhydride (0.1 mL) in dry pyridine (0.2 mL) were heated at 55 °C under nitrogen for 1.5 h. The reaction mixture was poured onto ice and potassium carbonate was added until effervescence ceased. The mixture was extracted with ether, washed with water, and dried with magnesium sulfate. Evaporation of the solvent gave 8.7 mg of acetate **7**: TLC (ethyl acetate–hexane, 2:3), $R_f$ 0.55.

*(5) Reduction of 1-Ketocyclovitamin* **5**. The mixture containing **5** (5.8 mg) was dissolved in tetrahydrofuran (0.6 mL) and methanol (0.1 mL) to which $NaBH_4$ (1 mg) was added. When the reaction was complete (TLC), water and ether were added and the phases were separated. Evaporation of the solvent after workup afforded a mixture of **4** and its 1β-hydroxy epimer **6** which was resolved by TLC (ethyl acetate–hexane, 1:1; $R_f$ 0.30 and 0.39, respectively).

*(6) 1α-Acetoxy-26,27-dinorvitamin $D_3$-25-carboxylic Acid Methyl Ester* **9**. A solution of acetate **7** (8.7 mg) in dry 1,2-dimethoxyethane (0.3 mL) was treated with formic acid (0.1 mL) at 55 °C under nitrogen for 15 min. The reaction mixture was poured onto ice, and sodium bicarbonate was added until effervescence ceased. Extraction with ether, followed by washing with water and saturated sodium chloride solution and drying over magnesium sulfate, gave a mixture of triester **8** and its 5,6-trans isomer as the major product. This mixture, in tetrahydrofuran (0.1 mL) and methanol (0.3 mL), was treated with several drops of saturated sodium bicarbonate for 5 min at ambient temperature. Water and ether were added, and the phases were separated. The ether phase was washed with water and saturated sodium chloride and dried with magnesium sulfate. The residue obtained after evaporating the solvent was purified by TLC. The band with an $R_f$ of 0.2 contained a mixture of **9** and its 5,6-trans isomer: UV $\lambda_{max}$ 269, $\lambda_{min}$ 223 nm. The mixture could be resolved by LC (2-propanol–hexane, 2.5:97.5) with two passes through the column (recycle mode) to give pure **9** (2.0 mg): UV $\lambda_{max}$ 266 and 248, $\lambda_{min}$ 224 nm.

*(7) 1α-Hydroxy-26,27-dinorvitamin $D_3$-25-carboxylic Acid Methyl Ester* **10**. A solution of **9** in ether (0.2 mL) and 0.1 M methanolic potassium hydroxide (0.075 mL) at ambient temperature was monitored by TLC [ethyl acetate–hexane, 4:1; $R_f$ 0.55 (for **9**) and 0.32 (for **10**)]. After ~1 h, hydrolysis was complete. Water and ether were added and the phases were separated. The aqueous phase was extracted with ether. The combined ether extracts were washed with water and saturated sodium chloride. Evaporation of the ether gave **10** (1.87 mg) which exhibited one spot on TLC analysis and was homogeneous on LC analysis (2-propanol–hexane, 7.5:92.5): UV $\lambda_{max}$ 265 nm, $\lambda_{min}$ 228 nm, $\lambda_{max}/\lambda_{min}$ 1.72; NMR δ 0.54 (s, 18-$CH_3$), 0.94 (d, $J$ = 6.2 Hz, 21-$CH_3$), 3.63 (s, $-CO_2CH_3$), 4.21 (m, 3α-H), 4.40 (m, 1β-H), 4.96, 5.28 (19 *E* and *Z* H's), 5.97, 6.32 (AB quartet, $J$ = 10.8 Hz, 6 and 7 H's); mass spectrum *m/e* (composition, *m/e* calculated, relative intensity) 416.2930 ($C_{26}H_{40}O_4$, 416.2927, 10), 398.2812 ($C_{26}H_{38}O_3$, 398.2821), 380.2694 ($C_{26}H_{36}O_2$, 380.2716, 22), 152.0840 ($C_9H_{12}O_2$, 152.0837, 29), 134.0745 ($C_9H_{10}O$, 134.0732, 100).

*(8) 1α,25-Dihydroxy[26,27-$^3H$]vitamin $D_3$* **11**. 1α-Hydroxy-26,27-dinorvitamin $D_3$-25-carboxylic acid methyl ester, **10** (1.0 mg, 2.2 μmol), in 1:1 dry benzene–diethyl ether was treated with $C^3H_3MgBr$ (22 μmol) (80 Ci/mmol) in the laboratories of New England Nuclear, Boston, MA. After workup,[2] the radiolabeled materials (270 mCi) were purified by chromatography on a Sephadex LH-20 column (2 × 65 cm) developed with chloroform–hexane (13:7) to give 98 mCi of **10** (160 Ci/mmol) which eluted from 900 to 1100 mL. LC analysis (2-propanol–hexane, 12:88) showed that **11** was homogeneous: UV $\lambda_{max}$ 262 nm; mass spectrum *m/e* (relative intensity) 428 ($M^+$), 410 ($M^+ - H_2O$, 9), 392 ($M^+ - 2H_2O$, 10), 287 ($M^+$ – side chain, 3), 285 (287 – 2H, 4), 269 (287 – $H_2O$, 5), 267 (285 – $H_2O$, 3), 152 (ring A plus carbons 6 and 7, 29), 134 (152 – $H_2O$, 100), 71 [$[(C^3H_3)_2C{=}OH]^+$, 100].

*Equilibrium Binding Studies.* One-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, WI) were fed a vitamin D deficient soy protein diet (Omdahl et al., 1971)

[2] The actual Grignard reaction and its workup were performed in the laboratories of New England Nuclear, Boston, MA. Purification of 1α,25-$(OH)_2$[26,27-$^3H$]vitamin $D_3$ and all other reactions and experiments were conducted in our laboratories.

and received water ad libitum for 4–6 weeks. All animals were maintained in a vivarium at 25–26 °C on an alternating 12-h light and dark cycle.

Chickens were fasted for 16–20 h after which time they were sacrificed by cervical dislocations, and the duodenal loop was freed of pancreas and excised. Unless otherwise stated, all procedures were carried out between 0 and 4 °C. Mucosa was scraped free of serosa and washed 3 times in several volumes (w/v) of buffer consisting of 50 mM Tris-HCl, 300 mM KCl, 1.5 mM EDTA, and 5.0 mM DTT, pH 7.4. Washed mucosa was homogenized in 2 volumes of buffer by using a Polytron, type PT-20 (Brinkman Instruments, Westbury, NY). Cytosol was the supernatant fluid (without the fluffy lipid layer) obtained by centrifuging the homogenate at 78000*g* for 90 min in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) using a 30 rotor. The cytosol was lyophilized and stored at −70 °C until use. Cytosol protein concentration was determined by the method of Bradford (1976) using crystalline bovine serum albumin as a standard.

1,25-$(OH)_2$[26,27-$^3$H]$D_3$ (0.025–5.0 nM) was dissolved in 0.025 mL of absolute ethyl alcohol which was mixed with cytosolic protein (0.225 mg) and sufficient homogenization buffer to make the final incubation volume of 0.5 mL. The nonspecific 1,25-$(OH)_2D_3$ binding was measured by a parallel incubation of the cytosol with 1,25-$(OH)_2D_3$ containing a 100-fold excess of nonradioactive 1,25-$(OH)_2D_3$. The assay tubes were incubated with shaking in a water bath for 1 h at 25 °C and then placed on ice. Subsequently, the unbound 1,25-$(OH)_2D_3$ was removed from the cytosol by absorption to 0.5% activated charcoal and 0.05% dextran for 10 min at 0–4 °C. The charcoal was removed by centrifugation at 2300*g* for 5 min in a Sorvall LR-5 refrigerated centrifuge (Du Pont Instruments, Newtown, CT) using an HS4 rotor. A 0.5-mL aliquot of the supernatant was removed and added to 3.5 mL of a scintillation fluid mixture consisting of 1.32 L of Triton X-100, 8.0 g of PPO, and 0.2 g of POPOP per 4 L of toluene. The radioactivity was determined by liquid scintillation counting using a Beckman LS-100C instrument with an efficiency for tritium of 36%. Quench correction was monitored through the use of automatic external standardization. Analysis of the data was performed by the method of Scatchard (1949) to determine the equilibrium dissociation constant ($K_d$) from the slope of the plot with an $x$-intercept value equaling the molarity of binding in solution. Regression analyses were performed to obtain the best fit.

## Results

The synthesis of 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$, **11** (Figure 1), proceeded from vitamin D ester **1** which was obtained as previously described for the synthesis of 25-OH[26,27-$^3$H]$D_3$ (Napoli et al., 1979). Introduction of the 1α-hydroxyl group into **1** was achieved essentially by the method of Paaren et al. (1978). The secosteroid **1** was tosylated, and the resulting diester **2** was solvolyzed in methanolic sodium bicarbonate to give cyclovitamin **3** in high yield. Selenium dioxide/*tert*-butyl hydroperoxide oxidation of **3** gave a mixture of the desired 1α-hydroxycyclovitamin **4** and the 1-ketocyclovitamin **5** in a 1.5:1 ratio. Reduction of **5** with methanolic sodium borohydride produced a mixture (containing chiefly 1α-hydroxycyclovitamin **4** and its 1β-hydroxy epimer **6**) from which additional amounts of pure **4** could be obtained by TLC separation. The cyclovitamin **4** was acetylated to give **7**, and this acetate was then solvolyzed to secosteroid **8** and its 5,6-trans isomer. After removal of the formyl group by hydrolysis (yielding a mixture of 1α-acetoxyvitamin **9** and the corresponding 5,6-*trans*-1α-acetoxyvitamin) pure *cis*-triene **9** was

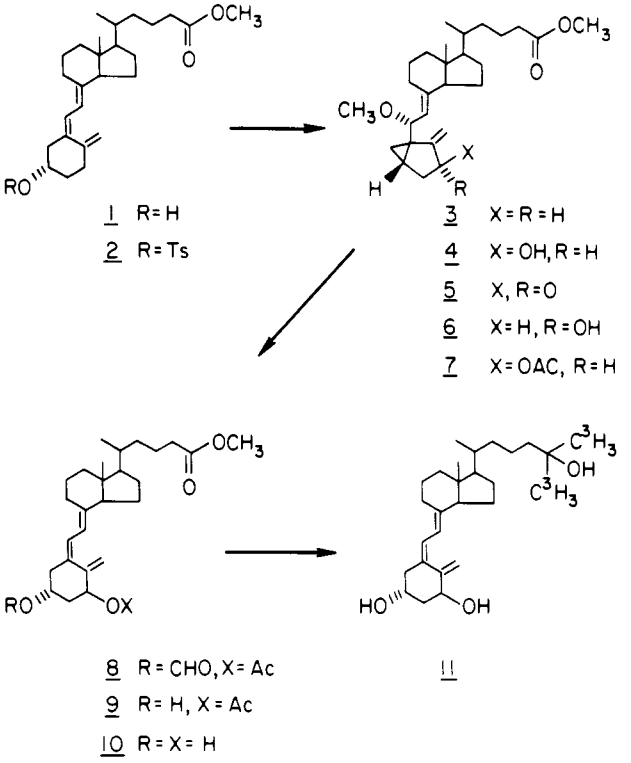


FIGURE 1: Direct chemical synthesis of 1,25-$(OH)_2$[26,27-$^3$H]$D_3$ with a specific activity of 160 Ci/mmol. 26,27-Dinorvitamin $D_3$ ester, **1**, was converted via the tosylate **2** to the cyclovitamin **3**. Oxidation, rearrangement, and hydrolysis ultimately gave the 1α-hydroxy-26,27-dinorvitamin D ester **10**. Treatment of **10** with [$^3$H]methylmagnesium bromide (80 Ci/mmol) produced 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$, **11**, which was purified to homogeneity by Sephadex LH-20 chromatography.

obtained from the mixture by LC and deacylated in the presence of methanolic potassium hydroxide to give 1α-hydroxy-26,27-dinorvitamin $D_3$-25-carboxylic acid methyl ester **10**, in ~7.5% overall yield.

The immediate unlabeled precursor **10** to the radiohormone **11** was characterized by UV, NMR, and high-resolution mass spectrometry. The UV absorbance maximum at 265 nm and a minimum at 228 nm are characteristic of the 5,6-*cis*-triene chromophore, and the NMR spectrum with doublets at δ 5.97 and 6.32 (protons at C-6 and C-7, respectively) and one-proton signals at δ 4.96 and 5.28 (C-19 hydrogens) confirmed this assignment. The NMR spectrum further exhibited the expected carbinyl protons [δ 4.40 (1β-H) and 4.21 (3α-H)] and the resonance for the methyl ester group (singlet, δ 3.63). The high-resolution mass spectrum indicated a molecular ion of 416.2930 which corresponds to the expected formula of $C_{26}H_{40}O_4$. Consecutive losses of $H_2O$ ($m/e$ 398.2812 and $m/e$ 380.2694) confirmed the presence of two hydroxyl groups. The peaks at $m/e$ 152.0840 ($C_{19}H_{12}O_2$) and 134.0745 (152 − $H_2O$, base peak) are characteristic for 1,3-dihydroxyvitamin D compounds. These spectra, plus the homogeneity of **9** on LC at an elution volume of 78 mL, indicated that **9** was pure.

The final synthetic step involved treating ester **10** with the Grignard reagent [$^3$H]$CH_3$MgBr (80 Ci/mmol) which gave vitamin **11** with a specific activity of 160 Ci/mmol because two [$^3$H]$CH_3$ radicals were incorporated into each molecule of **11**. The 270 mCi of material recovered from the Grignard reaction was purified by Sephadex LH-20 chromatography (Figure 2) to give 98 mCi (26% from **10**) of 1α,25-$(OH)_2$-[26,27-$^3$H]$D_3$. This material was checked for purity by LC (Figure 3). All of the radioactivity applied to the LC column was recovered in one peak, demonstrating that the sample not

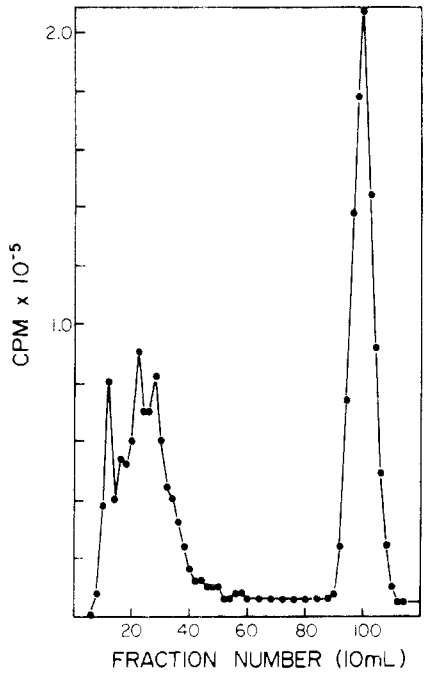


FIGURE 2: Purification of crude chemically synthesized 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$. The entire radioactive material recovered from the reaction of 26,27-dinorvitamin D ester **10** (Figure 1) with [$^3$H]methylmagnesium bromide (80 Ci/mmol) was placed on a Sephadex LH-20 column (2 × 65 cm) equilibrated with chloroform–hexane (13:7). Ten-milliliter fractions were collected and aliquots (10 $\mu$L) of every other fraction were diluted to 1 mL; 10 $\mu$L of the resulting solution was counted for radioactivity. The material in fractions 8–60 was discarded. Fractions 91–110 contained pure 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$.

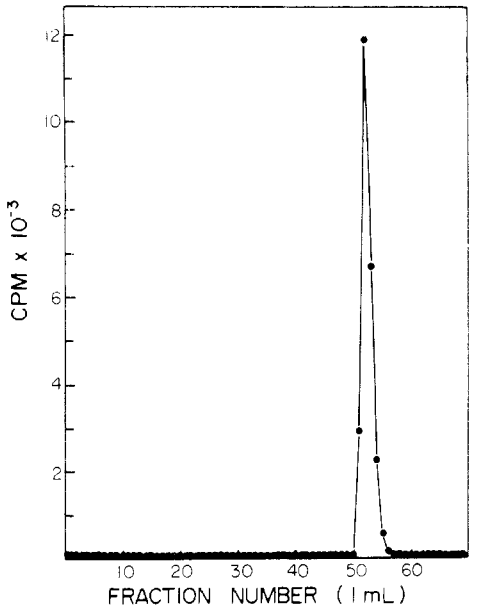


FIGURE 3: High-pressure liquid chromatogram of 1$\alpha$,25-$(OH)_2$-[26,27-$^3$H]$D_3$ recovered from the Sephadex LH-20 column shown in Figure 2. The microparticulate silica gel column was developed with 2-propanol–hexane (3:22) at a flow rate of 2 mL/min. The radioactive material eluted in about 8.5 column volumes and was homogeneous. Recovery of the radioactivity applied to the column was quantitative. Authentic unlabeled 1$\alpha$,25-$(OH)_2D_3$ which had been mixed with the radiolabeled material prior to chromatography eluted with a maximum peak height (UV, 254 nm) occurring in fraction 51.

only was homogeneous with respect to other vitamin D compounds but also did not contain extraneous radioactive substances which might not elute from the column.

The structure of 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ was confirmed by ultraviolet and mass spectroscopy. The compound showed a UV absorbance maximum at 262 nm, and the mass spectrum (Figure 4) displayed the typical pattern of 1$\alpha$-hydroxylated vitamin $D_3$ compounds. The molecular ion *m/e* 428 in the mass spectrum of 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ and corresponding 12 mass unit shifts of other peaks in the upper mass region established the presence of six tritium atoms in the labeled hormone. Further confirmation of tritium content and location at C-26 and C-27 is provided by peaks in the low-mass region. For example, the peak at *m/e* 59 $[(CH_3)_2C{=}OH]^+$ resulting from C-24–C-25 bond scission in all 25-hydroxylated vitamin D compounds is absent in the spectrum of 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ where instead an intense peak at *m/e* 71 $[(C^3H_3)_2C{=}OH]^+$ is observed. On the other hand, the expected peaks at *m/e* 152 and 134 representing the characteristic A-ring fragments in the spectrum of 1$\alpha$,25-$(OH)_2D_3$ are also present in the spectrum of 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$.

Additional confirmatory evidence for this structural assignment was obtained by comparing **11** to 1$\alpha$,25-$(OH)_2$-[26,27-$^3$H]$D_3$ synthesized by enzymatic 1$\alpha$-hydroxylation of 25-OH[26,27-$^3$H]$D_3$ (160 Ci/mmol). Radiohormone from each source was mixed with authentic 1$\alpha$,25-$(OH)_2D_3$, and the mixtures were analyzed individually by LC (Figure 5). A semipreparative microparticulate silica gel column capable of resolving the known vitamin D metabolites in one pass (Jones & DeLuca, 1975) was used in the recycle mode. After a total of four passes through the column, chemically synthesized 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ eluted in the same position as enzymatically synthesized 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$. However, both radioligands were somewhat more polar than, and clearly distinguishable from, nontritiated 1$\alpha$,25-$(OH)_2D_3$. This small but noticeable difference—base-line resolution was not achieved—is not unexpected in view of the isotopic differences between the compounds and the high resolving power of the chromatographic techniques used.

Chemically synthesized and enzymically produced 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ samples were also virtually identical in their interactions with the chick intestinal cytosolic binding protein specific for 1$\alpha$,25-$(OH)_2D$ (Figure 6). The radiohormones gave similar competitive equilibrium displacement curves when increasing concentrations of unlabeled 1,25-$(OH)_2D_3$ were added to the radioligand–binding protein complexes. Linear regression analyses of the data in the mass range of 4.6–74.5 pg resulted in a correlation coefficient of 0.99 for each curve. The curves were also parallel with a slope of −58 for the chemically synthesized radioligand and a slope of −60 for enzymatically produced material. The mass that would give 50% displacement was calculated to be 17.8 pg for chemically synthesized 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ and 14.9 pg for enzymatically synthesized radiotracer. Thus, as well as being chromatographically identical, the two ligands are identical with respect to the intestinal receptor.

The equilibrium dissociation constant, $K_d$, for 1$\alpha$,25-$(OH)_2D_3$ binding to the chick intestinal cytosolic binding protein was measured by using 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$. The data were plotted by the method of Scatchard (1949) and analyzed by linear regression (Figure 7). The correlation coefficient, *r*, was 0.95 and the slope was $-12.2$ nM$^{-1}$ which corresponds to a $K_d$ of $8.2 \times 10^{-11}$ M. 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ from direct chemical synthesis was used to obtain the data in Figure 7; however, in a separate experiment enzymically produced 1$\alpha$,25-$(OH)_2$[26,27-$^3$H]$D_3$ gave essentially the same results with a $K_d$ of $7.1 \times 10^{-11}$ M (Mellon & DeLuca, 1979).

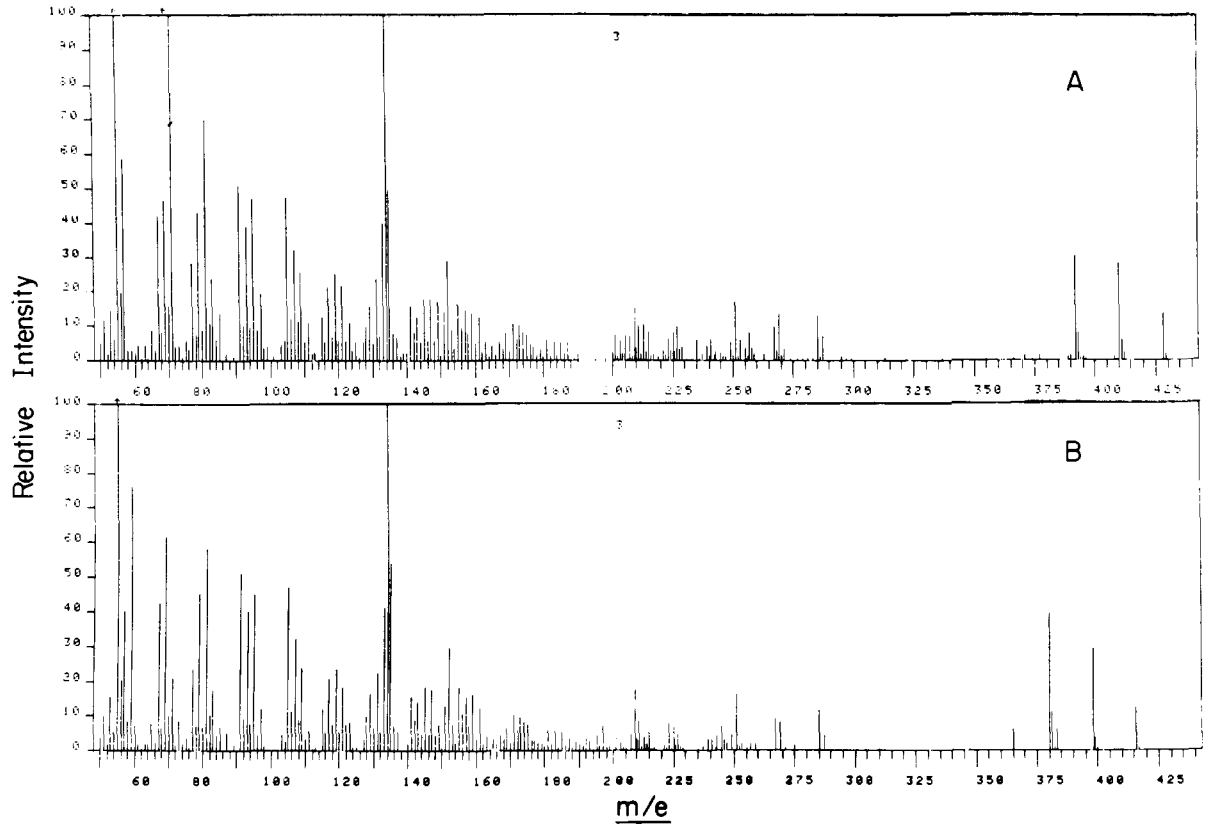


FIGURE 4: Mass spectra of 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ (A) and authentic 1α,25-$(OH)_2D_3$ (B). The peaks of $m/e$ >200 have been amplified threefold for clarity. Note that compared to 1α,25-$(OH)_2D_3$ which has a molecular ion of $m/e$ 416, 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ shows a molecular ion of $m/e$ 428, indicating incorporation of six tritium atoms. Also note the isotopic homogeneity of the tritiated sample.

Table I: Intestinal Calcium Transport in Vitamin D Deficient Rats Administered either 1α-OH-$D_3$ or 1α-Hydroxy-26,27-dinorvitamin $D_3$-25-carboxylic Acid Methyl Ester (Compound **10** in Figure 1)[a]

| group | compd | dose (μg) | [$^{45}$Ca]serosal/ [$^{45}$Ca]mucosal |
|---|---|---|---|
| 1 | ethanol | | 2.6 ± 0.3 |
| 2 | 1α-OH-$D_3$ | 0.0125 | 4.9 ± 0.4[b] |
| 3 | compound **10** | 0.125 | 2.5 ± 0.2 |
| 4 | compound **10** | 1.25 | 2.6 ± 0.2 |
| 5 | compound **10** | 12.50 | 2.2 ± 0.1 |

[a] Data are expressed as the mean ± SEM of values from six animals. [b] Significantly different from control; $p < 0.005$.

The synthetic intermediate, 1α-hydroxy-26,27-dinorvitamin $D_3$-25-carboxylic acid methyl ester (**10**), is a new vitamin D analogue. Hence, it was assayed for its ability to stimulate intestinal calcium transport in vitamin D deficient rats fed a low-calcium diet. As the data in Table I show, compound **10** did not promote intestinal calcium transport in a dose range 10–1000-fold greater than the dose of 1α-hydroxyvitamin $D_3$ that produces a maximal intestinal calcium transport response (Holick et al., 1975).

## Discussion

Readily obtainable radiolabeled 1α,25-$(OH)_2D_3$ of high specific activity is essential to continued progress in understanding the biochemistry of vitamin D and to the clinical application of that knowledge. However, the sole method of producing radiolabeled 1α,25-$(OH)_2D_3$ has been by enzymatic conversion of radiolabeled 25-hydroxyvitamin $D_3$ (25-OH-$D_3$). This necessity of a biochemical conversion, using homogenates from vitamin D deficient chicks, has limited the quantity and distribution of radiohormone. The totally chemical route to 1α,25-$(OH)_2$[$^3$H]$D_3$ has several notable features: it does not rely on enzymatic conversion of 25-OH[$^3$H]$D_3$ to 1α,25-$(OH)_2$[$^3$H]$D_3$ but yields 1α,25-$(OH)_2$[$^3$H]$D_3$ directly; the radioactivity is introduced in the final synthetic step so that multistep handling of highly radioactive intermediates is avoided; radioligand with a specific activity of 160 Ci/mmol is produced; the yields are relatively good as shown by the recovery of 98 mCi of pure product from 1 mg of immediate precursor; purification is simple—one Sephadex LH-20 column procedure is sufficient to provide homogeneous material.

Using vitamin D ester **10** as precursor to radiolabeled 1α,25-$(OH)_2D_3$ has the further advantage that **10** may be stored indefinitely, to be then reacted as needed, with a suitable Grignard reagent, to give radiolabeled 1,25-$(OH)_2D_3$. Since the Grignard reaction and subsequent purification are brief and straightforward, this is indeed an attractive alternative. In this manner losses of radiohormone from autoradiolysis in storage could be circumvented, and the precursor **10** would be continually available to product any 26,27-isotopically labeled 1α,25-$(OH)_2D_3$ for which there were an appropriate Grignard reagent or organometallic reagent. For example, this route could also provide 1α,25-$(OH)_2$[26,27-$^{14}$C]$D_3$ with a specific activity of 120 mCi/mmol or 26,27-hexadeuterio-1α,25-$(OH)_2D_3$.

Another important aspect of this synthesis is that it produces a homogeneous product for which the exact isotopic location and degree of incorporation are known. In contrast, 1α,25-$(OH)_2$[23,24-$^3$H]$D_3$, obtained enzymatically from 25-OH-[23,24-$^3$H]$D_3$, is actually a mixture of compounds differing in tritium content and containing isotope distributed between carbons 23 and 24 (and likely others) with unknown degrees of incorporation. This is the consequence of synthesizing 25-OH[23,24-$^3$H]$D_3$ by catalytic tritiation of 23,24-acetylenic

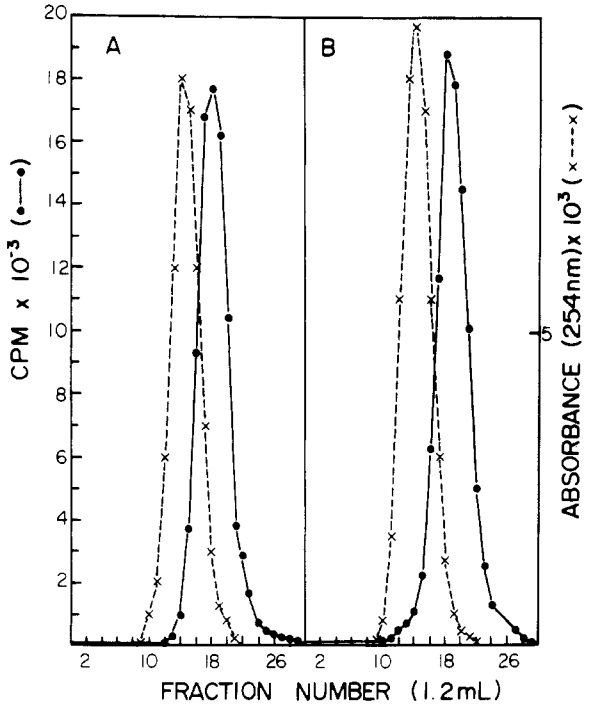


FIGURE 5: High-pressure liquid chromatographic comparisons of 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$, prepared by chemical or enzymic synthesis, to authentic unlabeled 1α,25-$(OH)_2D_3$. In the first run (A), a mixture of 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$, obtained by direct chemical synthesis as described in this paper, and authentic unlabeled 1α,25-$(OH)_2D_3$ were coinjected onto a microparticulate silica gel column eluted with 2-propanol–hexane, 3:22. After a total of four passes through the column (recycle mode), at a flow rate of 3 mL/min, fractions were collected. Fraction 1 began 84 min after injection. The UV peak of unlabeled 1α,25-$(OH)_2D_3$ (×) eluted at 44 column volumes and was clearly distinguishable from 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ (●). In the second run (B), which was done immediately following the first run, a mixture of 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$, obtained by enzymic hydroxylation of 25-OH[26,27-$^3$H]$D_3$ (160 Ci/mmol), was chromatographed with unlabeled 1α,25-$(OH)_2D_3$ exactly as described for the first run. The same separation between 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ and 1α,25-$(OH)_2D_3$ occurred. Moreover, 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ in (A) and (B) eluted in corresponding fractions as did 1α,25-$(OH)_2D_3$.

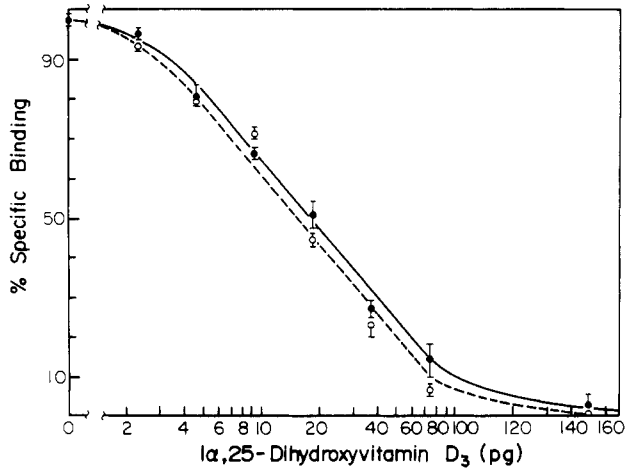


FIGURE 6: Competition of 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ and unlabeled 1α,25-$(OH)_2D_3$ for the chick intestinal cytosolic binding protein specific for 1α,25-$(OH)_2$D. Percent specific binding of 1α,25-$(OH)_2$-[26,27-$^3$H]$D_3$ obtained by direct chemical synthesis (●) or from enzymic hydroxylation of 25-OH[26,27-$^3$H]$D_3$ (○) is plotted against log [picograms of unlabeled 1α,25-$(OH)_2D_3$]. Values are expressed as mean ± SEM of triplicate determinations.

precursors. It has been shown, for example, that catalytic deuteration of the 23,24-acetylenic bond results in incorporation of one to five deuterium atoms with a relative abundance

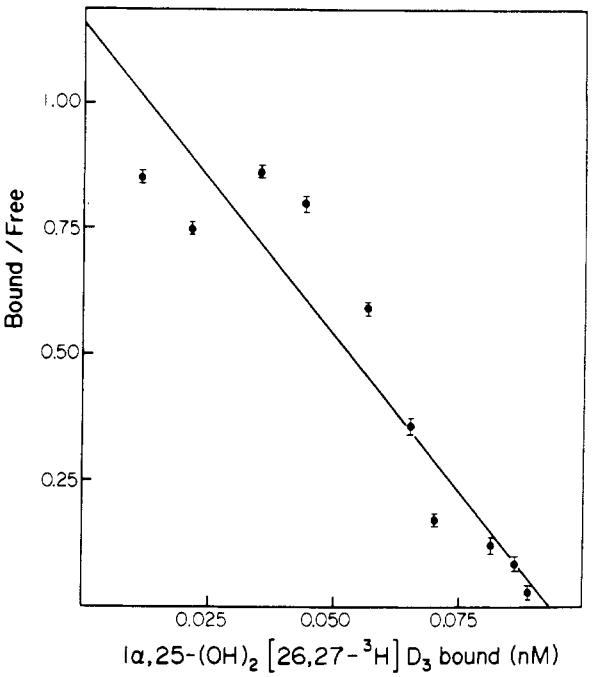


FIGURE 7: Scatchard analysis of binding between 1α,25-$(OH)_2$-[26,27-$^3$H]$D_3$ and the chick intestinal cytosolic binding protein specific for 1α,25-$(OH)_2$D. The ratio of bound to free material is plotted against concentration (nM) of bound 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$.

of 4, 22, 37, 26, and 12%, respectively (Muccino et al., 1978). The same is likely to be true for the synthesis of 25-OH-[23,24-$^3$H]$D_3$ and, consequently, 1α,25-$(OH)_2$[23,24-$^3$H]$D_3$. In some experiments, this ambiguity of label location and degree of incorporation at specific sites could present difficulties in the interpretation of results, a problem effectively circumvented by the present preparation for which isotope location and content are known precisely.

The biological competency of 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ is demonstrated by its ability to compete with unlabeled 1α,25-$(OH)_2D_3$ for the chick intestinal cytosolic binding protein. Nearly superimposable curves were obtained with 1α,25-$(OH)_2$[26,27-$^3$H]$D_3$ made directly or with that made enzymatically from 25-OH[26,27-$^3$H]$D_3$. These curves are virtually the same as those obtained previously with 1α,25-$(OH)_2$[23,24-$^3$H]$D_3$ (Eisman et al., 1976a,b). Therefore, the new label will probably prove most useful in this assay and should increase the assay's sensitivity.

One immediate benefit of the new very high specific activity label has been the demonstration that the equilibrium dissociation constant, $K_d$, of the 1α,25-$(OH)_2$D–intestinal receptor complex at $8.2 \times 10^{-11}$ M is lower than the previously reported values of $(1-5) \times 10^{-9}$ M (Brumbaugh & Haussler, 1974a; Lawson & Wilson, 1974; Tsai & Norman, 1973). These earlier determinations of dissociation constants were based on kinetic measurements of 1,25-$(OH)_2D_3$ binding carried out under nonsaturating conditions and/or with 1,25-$(OH)_2D_3$ of low specific activity. Use of the new hexatritio-1,25-$(OH)_2D_3$ allows the unambiguous demonstration of a truly high-affinity receptor site and the accurate determination of receptor–ligand binding constants.

Caution should be exercised when using 1α,25-$(OH)_2$-[26,27-$^3$H]$D_3$ as a chromatographic marker. Because of its high isotope content, it can be distinguished chromatographically from 1α,25-$(OH)_2D_3$ on efficient LC columns. The chromatographic differences between the two compounds should cause no serious problems, however, during routine analysis on Sephadex LH-20 or similar systems.

Compound **10** is a new vitamin D analogue similar in structure to the 1α,25-$(OH)_2D_3$ side-chain cleavage product (Esvelt et al., 1979). Therefore, its effect on intestinal calcium transport in vitamin D deficient rats on a low-calcium diet was measured; the analogue was inactive at the doses tested.

Acknowledgments

We thank Alan Hamstra for the competitive binding protein assay, Sean Hehir and Joe Alper for technical assistance with the NMR spectra, and Mel Micke for technical assistance with the mass spectra. We are grateful to Dr. Richard Young (New England Nuclear, Boston, MA) and his colleagues for performing the Grignard reaction.

References

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Brumbaugh, P. F., & Haussler, M. R. (1974a) *J. Biol. Chem. 249*, 1258.
Brumbaugh, P. F., & Haussler, M. R. (1974b) *J. Biol. Chem. 249*, 1251.
Brumbaugh, P. F., & Haussler, M. R. (1975a) *J. Biol. Chem. 250*, 1588.
Brumbaugh, P. F., & Haussler, M. R. (1975b) *Life Sci. 16*, 353.
DeLuca, H. F. (1978) *Handb. Lipid Res. 2*, 69.
DeLuca, H. F., & Schnoes, H. K. (1976) *Annu. Rev. Biochem. 45*, 631.
Eisman, J. A., Hamstra, A. J., Kream, B. E., & DeLuca, H. F. (1976a) *Science 193*, 1021.
Eisman, J. A., Hamstra, A. J., Kream, B. E., & DeLuca, H. F. (1976b) *Arch. Biochem. Biophys. 176*, 235.
Esvelt, R. E., Schnoes, H. K., & DeLuca, H. F. (1979) *Biochemistry 18*, 3977.
Haussler, M. R., & McCain, T. A. (1977) *N. Engl. J. Med. 297*, 974.
Holick, M. F., Kasten-Schraufrogel, P., Tavela, T., & DeLuca, H. F. (1975) *Arch. Biochem. Biophys. 166*, 63.
Jones, G., & DeLuca, H. F. (1975) *J. Lipid Res. 12*, 448.
Jones, P. G., & Haussler, M. R. (1979) *Endocrinology 104*, 313.
Kream, B. E., Reynolds, R. D., Knutson, J. C., Eisman, J. A., & DeLuca, H. F. (1976) *Arch. Biochem. Biophys. 176*, 779.
Kream, B. E., Yamada, S., Schnoes, H. K., & DeLuca, H. F. (1977a) *J. Biol. Chem. 252*, 4501.
Kream, B. E., Jose, M., Yamada, S., & DeLuca, H. F. (1977b) *Science 197*, 1086.
Lawson, D. E. M., & Wilson, P. W. (1974) *Biochem. J. 144*, 573.
Martin, D. L., & DeLuca, H. F. (1969) *Am. J. Physiol. 216*, 1351.
Mellon, W. S., & DeLuca, H. F. (1979) *Arch. Biochem. Biophys. 197*, 90.
Muccino, R. R., Vernice, G. G., Cupano, J., Olinetto, E. P., & Liebman, A. A. (1978) *Steroids 31*, 645.
Napoli, J. L., Fivizzani, M. A., Hamstra, A. J., Schnoes, H. K., & DeLuca, H. F. (1979) *Anal. Biochem. 96*, 481.
Norman, A. W., & Henry, H. (1974) *Recent Prog. Horm. Res. 30*, 413.
Omdahl, J., Holick, M., Suda, T., Tanaka, Y., & DeLuca, H. F. (1971) *Biochemistry 10*, 2935.
Paaren, H. E., Hamer, D. E., Schnoes, H. K., & DeLuca, H. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2080.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Suda, T., DeLuca, H. F., & Tanaka, Y. (1970) *J. Nutr. 100*, 1049.
Tsai, H. C., & Norman, A. W. (1973) *J. Biol. Chem. 248*, 5967.
Yamada, S., Schnoes, H. K., & DeLuca, H. F. (1978) *Anal. Biochem. 85*, 34.
Zile, M., Bunge, E. C., Barsness, L., Yamada, S., Schnoes, H. K., & DeLuca, H. F. (1978) *Arch. Biochem. Biophys. 186*, 15.