Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1031-1037

CHEMICAL SYNTHESIS OF [1β-^{3}H]1α,25-DIHYDROXYVITAMIN D_3 AND [1α-^{3}H]1β,25-DIHYDROXYVITAMIN D_3: BIOLOGICAL ACTIVITY OF 1β,25-DIHYDROXYVITAMIN D_3

Sally A. Holick, Michael F. Holick, and Julia A. MacLaughlin

Endocrine Unit, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

Received October 23,1980

SUMMARY

The simple three-step preparation of [1β-^{3}H]1α,25-dihydroxyvitamin D_3 and [1α-^{3}H]1β,25-dihydroxyvitamin D_3 from 1α,25-dihydroxyvitamin D_3 is described. In the rat, 1β,25-dihydroxyvitamin D_3, when compared with its α-epimer, did not stimulate intestinal calcium transport or bone calcium mobilization at doses 1000-fold higher than the doses of the natural hormone, 1α,25-dihydroxyvitamin D_3.

INTRODUCTION

The synthesis of tritium-labeled vitamin D_3 and 25-hydroxyvitamin D_3 (25-OH-D_3) of high specific activity has been instrumental in elucidating the metabolism of vitamin D_3 and its 25-hydroxy derivative to biologically active metabolites (1—3). For the study of the metabolism of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3), tritium-labeled 1α,25-$(OH)_2$-D_3 was biochemically synthesized by incubation in vitro of [^{3}H]25-OH-D_3 with chicken kidney homogenates (1—3).

Recently, Napoli et al. (4) chemically synthesized [26,27-^{3}H]1α,25-$(OH)_2$-D_3 from homocholenate in twelve steps. In a simpler fashion we prepared 1α,25-$(OH)_2$-D_3 with the tritium label in the A-ring. Our procedure is based upon the novel method of Mazur and colleagues (5,6) for oxidation of the 1α-hydroxyl group of 1α-hydroxyvitamin D_3 (1α-OH-D_3) to 1-oxoprevitamin D_3. Reduction of this ketone with lithium aluminum hydride ($LiAlH_4$) and subsequent thermal isomerization yielded a mixture of 1β-hydroxyvitamin D_3 (1β-OH-D_3) and 1α-OH-D_3 in a ratio of 2.8:1, whereas reduction with sodium borohydride ($NaBH_4$) resulted in a single product, 1β-OH-D_3. Using similar methods, Paaren et al. (7) oxidized 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3 to their corresponding 1-oxo

0006-291X/80/231031-07$01.00/0

derivatives and, after reduction with $LiAlH_4$, obtained a mixture of 1:4 of 1α- and 1β-epimers. This simple three-step synthesis suggested that this procedure would be ideal for the synthesis of radiolabeled 1α- and 1β-hydroxy derivatives by use of either ^3H-$LiAlH_4$ or ^3H-$NaBH_4$ as the reducing agent. We now report the synthesis of [1β-^{3}H]1α,25-$(OH)_2$-D_3 and [1α-^{3}H]1β,25-$(OH)_2$-D_3, with a specific activity of 15 Ci/mM, and the first detailed analysis of the biological activity of 1β,25-$(OH)_2$-D_3.

METHODS

Chemical Synthesis of 1β,25-Dihydroxyvitamin D_3: Activated manganese dioxide was prepared by simultaneous addition and stirring, for 1 h, of aqueous solutions of manganese sulfate monohydrate and sodium hydroxide to a hot aqueous solution of potassium permanganate. The brown precipitate was washed until free of potassium permanganate and then dried at 110°C for 24 hours.

Activated manganese dioxide, 200 mg, was added in increments to 40 mg of 1α,25-$(OH)_2$-D_3 (kindly provided by Dr. Milan Uskoković, Hoffmann-La Roche, Nutley, NJ) in 30 ml of dry methylene chloride, and the reaction mixture was stirred for 3 hours at room temperature when thin-layer chromatography (chloroform:ethyl acetate, 1:9, vol/vol) indicated approximately 50% oxidation. The reaction mixture was dried under nitrogen and then applied to a glass column (2 cm X 17 cm) packed with Sephadex LH-20, (Pharmacia, New Brunswick, NJ), slurried, and developed in 7:3 vol/vol chloroform:n-hexane. Fractions (4.0 ml) were collected, and fractions 15 through 25, having the ultraviolet (uv) absorption spectrum λ_{max}(ether)288,238 nm of 1-oxo-25-hydroxyprevitamin D_3, were combined to give 15 mg of product (m/e 414 (M^+, 21%), 396 (82), and 378 (100)). $NaBH_4$, 20 mg, was added to a solution, at 0°C, of 1-oxo-25-hydroxyprevitamin D_3 in 10 ml of methanol and 100 μl of distilled water. The reduction was continued for 1 h at 0°C, at which time the uv absorption spectrum showed the disappearance of the 288- and 238-nm peaks and the appearance of a 260-nm peak. The solution was distributed between ether and water; the aqueous layer was withdrawn and extracted with ether; the ether layers were combined, and the procedure was repeated twice. The 1β,25-dihydroxyprevitamin D_3 (1β,25-$(OH)_2$-preD_3) and 1α,25-dihydroxyprevitamin D_3 (1α,25-$(OH)_2$-preD_3) were isolated by high-pressure liquid chromatography (LC) (Waters Associates, Milton, MA) on a μPorasil column (0.4 X 30 cm) using 5% vol/vol isopropanol/n-hexane at 3 ml/min. Under these conditions 1β,25-$(OH)_2$-preD_3 (Fig. 1) is eluted much earlier (t_r, 14 min) than 1α,25-$(OH)_2$-preD_3 (t_r, 28 min) is.

Thermal isomerization of 1β,25-$(OH)_2$-preD_3 (MeOH, 60°C, 3 h) followed by purification on LC yielded 1β,25-$(OH)_2$-D_3 with a t_r of 24 min. Its uv absorption spectrum indicated λ_{max}265 nm, λ_{min}228 nm, and its ^1H nuclear magnetic resonance spectrum (80 MHz, $CDCl_3$) was as follows: δ 0.54 and 1.20 (s, 13-Me, 25-Me_2), 4.10 (m, 3-H), 4.32 (m, 1-H), 5.00 (d, J 2.0 Hz (*Z*)-H, 5.28 (d, J 1.4 Hz, 19 (*E*)-H), and 6.44 and 6.04 (AB_q, J 11.8 Hz, 6- and 7-H). Thermal isomerization of 1α,25-$(OH)_2$-preD_3 (MeOH, 60°C, 3 h) followed by LC yielded 1α,25-$(OH)_2$-D_3 with a t_r of 26 min and λ_{max}265 nm, λ_{min}228 nm.

Further characterization of the products was achieved by reducing 1-oxo-25-hydroxyprevitamin D_3 with sodium borodeuteride (^2H-$NaBH_4$) using a procedure

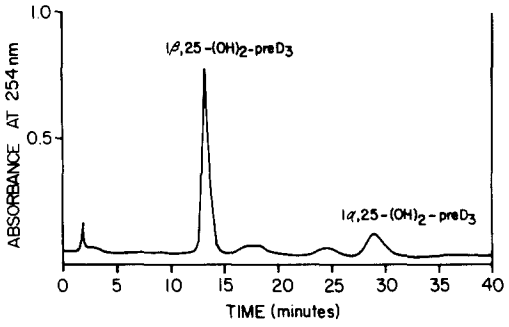

Figure 1. High-pressure liquid chromatographic separation of 1β,25-dihydroxyprevitamin D_3 and 1α,25-dihydroxyprevitamin D_3 on a μPorasil column (5% vol/vol, isopropanol/n-hexane, 3 ml/min).

similar to the reduction with $NaBH_4$. The deuterated previtamins were isolated by LC and were then thermally isomerized to the deuterated vitamins, which were purified by LC. The mass spectrum of [1β-^{2}H]1α,25-$(OH)_2$-D_3 (taken on a Kratos Model MS-50 mass spectrometer (Manchester, England) at 70 eV using a direct probe for sample introduction and a source temperature of 100°C above ambient temperature) showed m/e 417 (M^+,22%), 153 (25), and 135 (100). The mass spectrum of [1α-^{2}H]1β,25-$(OH)_2$-D_3 showed m/e 417 (M^+, 50%), 153 (94), and 135 (100).

In an analogous sequence (Fig. 2), 1-oxo-25-hydroxyprevitamin D_3 (2.0 mg) was dissolved in 5 ml of MeOH and reduced with 1.0 mg of ^{3}H-$NaBH_4$ (specific activity 80 Ci/mM, obtained from New England Nuclear Corp., Boston, MA) at 0°C for 60 min. The excess ^{3}H-$NaBH_4$ was reacted with acetone and dried in vacuo. The reaction mixture was dissolved in 1.0 ml of 65:35 (vol/vol) of $CHCl_3$:n-hexane and applied to a glass column (1.5 X 30 cm) containing 15 g Sephadex LH-20 that was slurried in the same solvent. The products, [1α-^{3}H]1β,25-$(OH)_2$-preD$_3$ and [1β-^{3}H]1α,25-$(OH)_2$-preD$_3$, eluted between 70 and 100 ml and 150 and 200 ml, respectively. The isolated previtamin-D_3 epimers were warmed at 60°C for 6 h in MeOH, which thermally isomerizes the previtamin D's to the corresponding [1α-^{3}H]1β,25-$(OH)_2$-D_3 and [1β-^{3}H]1α,25-$(OH)_2$-D_3 in an equilibrium ratio of preD$_3$ to D_3 of approximately 1:4.

The equilibrium reactions were chromatographed separately on LC chromatography as described above. The products [1α-^{3}H]1β,25-$(OH)_2$-D_3 and [1β-^{3}H]1α,25-$(OH)_2$-D_3 had identical uv absorption spectra (λ_{max}265 nm, λ_{min}228 nm), characteristic of the 5,6-cis-triene chromophore. Identity and radioactive purity of [1β-^{3}H]1α,25-$(OH)_2$-D_3 were established by cochromatography with authentic crystalline 1α,25-$(OH)_2$-D_3. Compound IVa eluted identically with crystalline 1α,25-$(OH)_2$-D_3 on LC (Fig. 3).

Bioassays: Weanling male rats (Holtzman Co., Madison, WI) were fed a vitamin-D-deficient diet adequate in calcium and phosphorus for two weeks, and were then switched to a vitamin-D-deficient low-calcium (0.02%) diet for an additional 2 wk. Groups of 6 rats received either 20 ng of standard 1α,25-$(OH)_2$-D_3 (obtained from Hoffmann-La Roche, Nutley, NJ), 20 ng of synthetic 1α,25-$(OH)_2$-D_3 (obtained from the reduction of 1-oxo-25-hydroxy-previtamin D_3), or 20 μg of synthetic 1β,25-$(OH)_2$-D_3 (obtained from the reduction of 1-oxo-25-hydroxy-previtamin D_3) intrajugularly in 50 μl of 95% EtOH, whereas a control group received only the vehicle. The animals were decapitated 24 h after adminis-

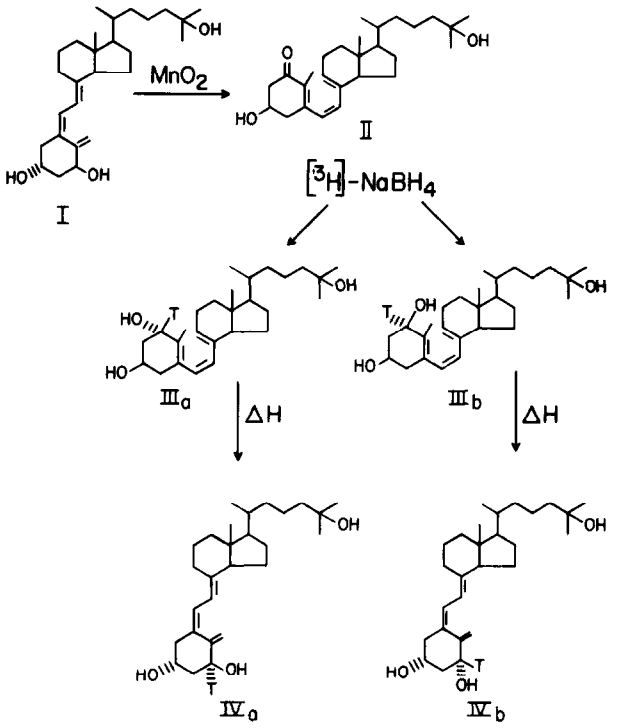

Figure 2. Procedure for the preparation of [1β-^{3}H]1α,25-dihydroxyvitamin D_3 (IV_a) and [1α-^{3}H]1β,25-dihydroxyvitamin D_3 (IV_b).

tration, and their duodena and blood were collected. Intestinal calcium transport activity was measured by the everted-gut-sac technique (8), and bone calcium mobilization was determined based upon serum calcium measurements (9).

RESULTS AND DISCUSSION

We synthesized 1-oxo-25-hydroxyprevitamin D_3, as previously described (5—7), and have reduced this product with $NaBH_4$ as outlined in Figure 2. In contrast to the experiment of Mazur and colleagues (5,6), which yielded only 1β-OH-preD_3 (100%) after the reduction of 1-oxo-preD_3 with $NaBH_4$, our experiment yielded a mixture of 1β- and 1α-epimers in a ratio of 93:7, respectively, after the reduction of the 25-hydroxy derivative of 1-oxo-preD_3 (compound II). The products had the characteristic uv absorption spectra for the 6,7-cis-triene-chromophore, and they thermally equilibrated to 5,6-cis-triene isomers. Further characterization was obtained by synthesizing [1β-^{2}H]1α,25-$(OH)_2$-D_3 and [1α-^{2}H]1β,25-$(OH)_2$-D_3, which gave the appropriate mass spectra (7). The

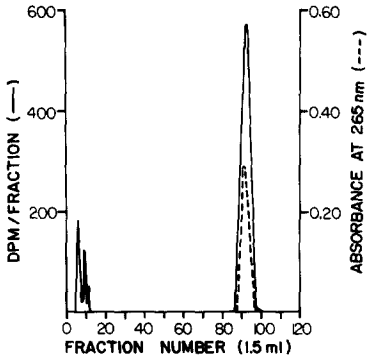

Figure 3. High-pressure liquid co-chromatography of [1β-^{3}H]1α,25-dihydroxyvitamin D_3 (——) with crystalline 1α,25-dihydroxyvitamin D_3 (---) on a μPorasil column (5% vol/vol, isopropanol/n-hexane, 3 ml/min).

biologic activities of the synthesized 1α,25-$(OH)_2$-D_3 and 1β,25-$(OH)_2$-D_3 were determined. Synthetic 1α,25-$(OH)_2$-D_3 (20 ng) elicited intestinal calcium-transport and bone calcium-mobilization responses identical with equal amounts of standard 1α,25-$(OH)_2$-D_3. Synthetic 1β,25-$(OH)_2$-D_3 at a dose of 20 μg was unable to stimulate either intestinal calcium transport or bone calcium mobilization 24 h after administration (Table 1). Thus, based upon spectroscopic, chromatographic, and bioassay data, the structures of the synthesized 1α,25-$(OH)_2$-D_3 and 1β,25-$(OH)_2$-D_3 were confirmed.

With an identical sequence of reactions, 1-oxo-25-hydroxyprevitamin D_3 was reduced with ^{3}H-$NaBH_4$ to yield [1β-^{3}H]1α,25-$(OH)_2$-preD_3 and [1α-^{3}H]1β,25$(OH)_2$-preD_3. After thermal isomerization and purification on LC the products demonstrated uv absorption spectra characteristic of the 5,6-cis-triene system for the D vitamins. The specific activity of each product was determined to be 15 Ci/mM, and the radiochemical purity of each was based upon coelution with authentic products.

We report a simple chemical synthesis of [1β-^{3}H]1α,25-$(OH)_2$-D_3. Although the yield of the reduction with $NaBH_4$ is only 7%, compared with 25 to 30% when $LiAlH_4$ is used, this method of reduction and tritium incorporation was chosen because of the availability of ^{3}H-$NaBH_4$ of high specific activity. (The specific activity for the ^{3}H-$LiAlH_4$ that is commercially available at the

TABLE 1

Effect of synthetic 1β,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-D_3 on intestinal calcium transport and bone calcium mobilization 24 h after administration to vitamin-D-deficient rats.[a]

Dose	^{45}Ca serosal/ ^{45}Ca mucosal (mean ± SEM)	Serum calcium levels (mg/dL)
50 µl 95% EtOH	2.4 ± 0.1	4.4 ± 0.2
20 ng 1α,25-$(OH)_2$-D_3[b]	4.4 ± 0.1	7.5 ± 0.1
20 ng 1α,25-$(OH)_2$-D_3[c]	4.3 ± 0.1	7.3 ± 0.1
20 µg 1β,25-$(OH)_2$-D_3	2.2 ± 0.1	4.4 ± 0.2

[a] There were six animals in each group.
[b] Obtained from the reduction of 1-oxo-25-hydroxyprevitamin D_3.
[c] Crystalline synthetic standard obtained from Hoffmann-La Roche, Nutley, NJ.

present time is approximately 1/80 that of $NaBH_4$.) Furthermore, our procedure labels the 1α,25-$(OH)_2$-D_3 in the A-ring, making it valuable for investigation of the side-chain cleavage metabolism of this metabolite.

Lawson et al. (6) reported that as much as 10 µg of 1β-OH-D_3 was unable to produce calcium binding protein in chick intestine, and 0.25 µg of 1β-OH-D_3 failed to produce any effect on endochondral calcification in the rat. Based upon these data, these authors stated that there was an absolute requirement of the 1α-hydroxyl group to be in the α position. However, they presumed that 1β-OH-D_3 was hydroxylated on C-25 in vivo to 1β,25-$(OH)_2$-D_3 without providing evidence for this. Paaren et al. (7) further noted that in vitro testing in the intestinal cytosol binding assay showed that 1β-OH-D_3 and 1β,25-$(OH)_2$-D_3 were 1.65×10^5 and 3.0×10^3 times less potent than 1α,25-$(OH)_2$-D_3 in displacing bound [^{3}H]1α,25-$(OH)_2$-D_3. Our analysis of the in vivo biological activity of 1α,25-$(OH)_2$-D_3 compared with its 1β isomer clearly demonstrates that a change in the stereochemical position of the 1-hydroxyl from α to β completely abolishes the biological activity of 1,25-$(OH)_2$-D_3.

ACKNOWLEDGEMENTS

We are indebted to Dr. J.T. Potts, Jr. for enthusiastic support, to L.B. Fred for editorial assistance, and to the Harvard University Chemistry Department for the use of their Varian FT-80 NMR spectrometer and of their MS-50 mass spectrometer.

This work was supported by Fellowship #1 F 32 AM05856-01 and by Grant AM 25506-01 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

REFERENCES

1. Holick, M.F., and Clark, M.B. (1978) Fed. Proc. 37, 2567-2574.
2. DeLuca, H.F., and Schnoes, H.K. (1976) Annu. Rev. Biochem. 45, 631-666.
3. Norman, A.W., and Henry, H. (1974) Recent Prog. Horm. Res. 30, 431-479.
4. Napoli, J.L., Mellon, W.S., Fivizzani, M.A., Schnoes, H.K., and DeLuca, H.F. (1980) Biochemistry 19, 2515-2521.
5. Sheves, M., Friedman, N., and Mazur, Y. (1977) Org. Chem. 42, 3597-3599.
6. Lawson, D.E.M., Friedman, N., Sheves, M., and Mazur, Y. (1977) FEBS Lett. 80, 137-140.
7. Paaren, H.E., Schnoes, H.K., and DeLuca, H.F. (1977) J. Chem. Soc. Chem. Commun. 23, 890-892.
8. Schachter, D., Kimberg, D.V., and Schenker, H. (1961) Am. J. Physiol. 200, 1263-1271
9. Holick, M.F., Garabedian, M., and DeLuca, H.F. (1972) Biochemistry 11, 2715-2719.