Synthesis and Biological Activity of 2α-Hydroxyvitamin D_3 †

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From 2α-hydroxycholesterol, obtained by hydroboration–oxidation of cholesta-1,5-dien-3β-ol, the 2α-hydroxylated analogue of cholesta-5,7-dien-3β-ol (provitamin D) has been prepared *via* the usual four-step procedure: (i) acetylation, (ii) bromination, (iii) dehydrobromination, and (iv) hydrolysis. 2α-Hydroxycholecalciferol (2α-OH-D_3), produced by irradiation of cholesta-5,7-dien-2α,3β-diol, did not show any significant biological activity on bone and intestine in rats, suggesting that the 2α-hydroxy-function does not mimic (at least significantly) the 1α-hydroxy-function. Vitamin D_3 itself was much more potent than 2α-OH-D_3 in inducing calcium transport activity. The presence of the 2α-hydroxy-function seems to be inhibitory to the metabolism of vitamin D_3.

MUCH evidence indicates that cholecalciferol (vitamin D_3) is converted first in the liver into 25-hydroxycholecalciferol (25-OH-D_3) and then in the kidney into 1α,25-dihydroxycholecalciferol [1α,25-$(OH)_2$-D_3] before it exerts its biological action on bone and intestine.[1-3] Thus, 1α,25-$(OH)_2$-D_3 is effective in anephric animals, whereas vitamin D_3 and 25-OH-D_3 are totally ineffective. Further studies demonstrated that vitamin D_3 analogues, such as 1α-hydroxycholecalciferol (1α-OH-D_3),[4-6] 5,6-*trans*-vitamin D_3 (5,6-*trans*-D_3),[7] and dihydrotachysterol$_3$ (DHT_3),[8] were also capable of exerting their physiological action in anephric animals. All the above vitamin D analogues possess a hydroxy-function at position 1α or in the same geometric position as normally occupied by the 1α-hydroxy-group of 1α,25-$(OH)_2$-D_3. In this connection, it seemed worthwhile to synthesize 2α-hydroxycholecalciferol [2α-OH-D_3 (5)] and test its biological activity, because a 2α-hydroxy-function would be closer in position than any other possible hydroxy-functions to the 1α-hydroxy-group of vitamin D, and might be expected to mimic the latter group in biological systems.

Synthesis.—The synthesis was achieved in six steps from 2α-hydroxycholesterol (1a).[6,9] Acetylation with acetic anhydride-pyridine afforded the diacetate (1b), which was brominated with 1,3-dibromo-5,5-dimethylhydantoin in benzene–petroleum and then dehydrobrominated with trimethyl phosphite in boiling xylene. The product was reduced with lithium aluminium hydride in ether and the resulting dienes were separated by chromatography on Sephadez LH-20. The overall yield of the 5,7-diene (3a) from (1a) was 35—40%. Cholesta-5,7-diene-2α,3β-diol (3a) was irradiated in ether and the products were again separated by chromatography on Sephadez LH-20 to give the 2α-hydroxyprevitamin D (4a), in *ca.* 35% yield based on consumed previtamin D (3a). Storage of (4a) in ether under argon in the dark at room temperature for 2 weeks followed by chromatography on Sephadex LH-20 afforded pure 2α-OH-D_3 (5), characterized by its ^{1}H n.m.r., mass, and u.v. spectra.

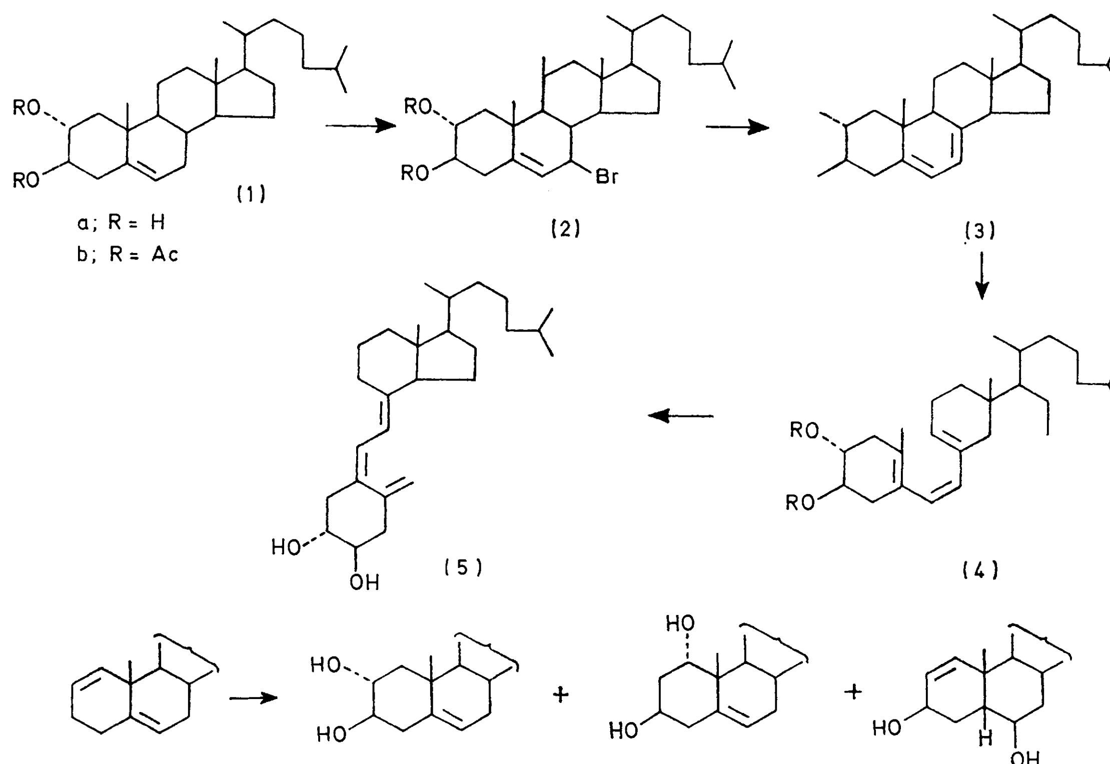
2α-Hydroxycholesterol (1a) has been prepared previously in *ca.* 20% yield by hydroboration of cholesta-1,5-dien-3β-ol (6) followed by oxidation with alkaline hydrogen peroxide. However, two other hydroxycholesterols [1α-hydroxycholesterol (7) and an isomeric

† The synthetic study was carried out in the Research Institute for Medical Engineering, and the biological tests in the Department of Biochemistry.

1 M. F. Holick, H. K. Schnoes, H. F. DeLuca, T. Suda, and R. J. Cousins, *Biochemistry*, 1971, **10**, 2799.
2 D. E. M. Lawson, D. R. Fraser, E. Kodicek, H. R. Morris, and D. H. Williams, *Nature*, 1971, **230**, 228.
3 A. W. Norman, J. F. Myrtle, R. J. Midgett, H. G. Norwicki, V. Williams, and G. Popjak, *Science*, 1971, **173**, 51.
4 M. F. Holick, E. J. Semmler, H. K. Schnoes, and H. F. DeLuca, *Science*, 1973, **180**, 190.
5 D. H. R. Barton, R. H. Hesse, M. M. Pechet, and E. Rizzardo, *J. Amer. Chem. Soc.*, 1973, **95**, 2748.
6 C. Kaneko, S. Yamada, A. Sugimoto, Y. Eguchi, M. Ishikawa, T. Suda, M. Suzuki, and S. Sasaki, *Steroids*, 1974, **23**, 75.
7 M. F. Holick, M. Garabedian, and H. F. DeLuca, *Biochemistry*, 1972, **14**, 2715.
8 R. B. Hallick and H. F. DeLuca, *J. Biol. Chem.*, 1972, **247**, 91.
9 C. Kaneko, S. Yamada, A. Sugimoto, M. Ishikawa, T. Suda, and S. Sasaki, *Tetrahedron Letters*, 1973, 2339; *Chem. and Pharm. Bull. (Japan)*, 1974, **22**, 2101.

diol* (8)] and a mixture of triols are also formed in this reaction in appreciable amounts. We now report that hydroboration with 9BBN (9-borabicyclo[3.3.1]nonane),[11] which is far more bulky than diborane gives (1a) in 70—80% yield. The selectivity of formation of the 2α-hydroxy-derivative could be due to steric hindrance in the addition steps leading to the other products.

Biological Activity.—For intestinal calcium transport and bone mineral mobilization measurements, male weanling rats of the Wistar strain were fed for 3 weeks with a vitamin D deficient-low calcium (0·003%) diet.[12] At the end of the third week, groups of rats which were either intact or bilaterally nephrectomized, received intrajugularly the appropriate dose of 2α-OH-D_3, 1α-OH-D_3, or vitamin D_3 in 95% ethanol (0·05 ml). Twelve hours later the animals were decapitated, and the blood and the duodena were collected. The everted duodenal sacs were prepared according to the procedure of Martin and DeLuca [13] for measuring intestinal calcium transport activity, and were incubated under oxygen for 90 min in 10 ml of a solution consisting of sodium chloride (125 mM), fructose (10 mM), [^{45}Ca]calcium chloride (0·25 mM; 0·2 μCi in 10 ml), and tris(hydroxymethyl)aminomethane buffer (30 mM pH 7·4). After the incubation, samples from inside (serosa) and outside (mucosa) the sacs were taken out and their radioactivities were measured in a liquid scintillation spectrometer (Packard model 3385). The data are expressed as ratios of ^{45}Ca inside the sac to that outside.



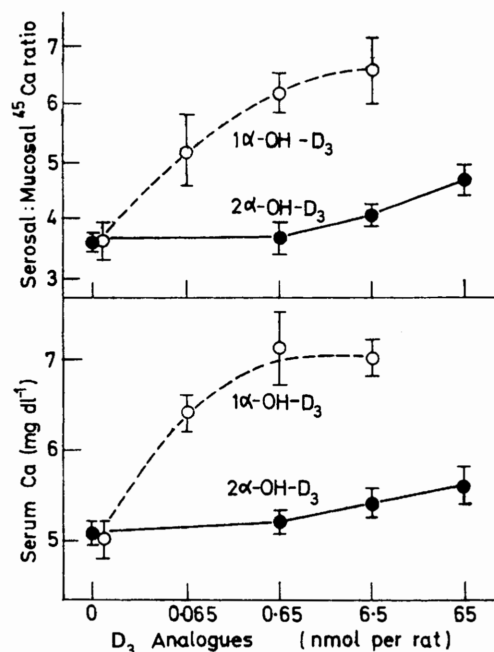
For the assay of bone mineral mobilization activity, serum calcium concentrations in rats were determined with a Perkin-Elmer 403 atomic absorption spectrometer. The rise in serum calcium concentration in rats on this low calcium diet reflects increased mobilization of bone mineral.

The Figure shows the comparative effectiveness of 2α-OH-D_3 and 1α-OH-D_3 in intestinal calcium transport as well as in the bone mineral mobilization activity of intact rats. Unlike 1α-OH-D_3, 2α-OH-D_3 did not elicit significant response in either activity. Although a high dose (65 nmol) of 2α-OH-D_3 was able slightly to stimulate intestinal calcium transport and bone mineral mobilization activity, as little as 0·065 nmol of 1α-OH-D_3 elicited higher response in both activities.[6] The biological activity of 2α-OH-D_3 was estimated to be less than 1/1000th of that induced by 1α-OH-D_3. Of great interest is that vitamin D_3 itself is much more potent than 2α-OH-D_3 in inducing intestinal calcium transport activity (Table). Six and a half nmol of vitamin D_3 elicited higher response than that induced by 65 nmol of 2α-OH-D_3 in intestinal calcium transport activity, suggesting that the presence of 2α-hydroxy-function is inhibitory to the initiation of the biological activity induced by the

* The product (8) was identified as 5β-cholest-1-ene-3β,6β-diol on the basis of (i) the n.m.r. spectrum [τ 4·3 (s, *cis*-CH=CH) and 5·9 and 6·2 (each m, $W_{\frac{1}{2}}$ 5 Hz, *CH*·OH)] and (ii) the m.p. of the dihydro-derivative, which agreed well with that of 5β-cholestane-3β,6β-diol.[10]

[10] W. J. Wechter, *Chem. and Ind.*, 1959, 294.
[11] H. C. Brown, E. F. Knight, and R. A. Coleman, *J. Amer. Chem. Soc.*, 1969, **91**, 2144.
[12] T. Suda, H. F. DeLuca, and Y. Tanaka, *J. Nutrition*, 1970, **100**, 1049.
[13] D. L. Martin and H. F. DeLuca, *Amer. J. Physiol.*, 1969, **217**, 1351.

vitamin D_3 administration. The Table also shows that a high dose (65 nmol) of 2α-OH-D_3 is capable of similar stimulation of intestinal calcium transport activity in intact as well as in anephric rats, while the same amount



Comparative effectiveness of 2α-OH-D_3 (solid line) and 1α-OH-D_3 (dotted line) in intestinal calcium transport and bone mineral mobilization activity of intact rats. The vertical bars represent standard errors of the means. Five (for the 1α-OH-D_3 experiment) and eight (for the 2α-OH-D_3 experiment) rats were used for each determination

of vitamin D_3 is unable to stimulate intestinal calcium transport activity in anephric rats.

Intestinal calcium transport response to 2α-OH-D_3, 1α-OH-D_3, and vitamin D_3 in intact and in anephric rats

Dose	nmol (I.U.)	Serosal : Mucosal ^{45}Ca ratio: Intact	Anephric *
Control (ethanol 0·05 ml)		3·6 ± 0·3 (10)	4·2 ± 0·5 (4)
2α-OH-D_3	6·5	4·1 ± 0·2 (8)	
	65	4·7 ± 0·3 (8)	5·5 ± 1·0 (5)
1α-OH-D_3	0·65	6·2 ± 0·5 (5)	5·2 ± 0·5 (4)
	6·5	6·6 ± 0·7 (5)	6·7 ± 0·5 (4)
D_3	6·5 (100)	5·8 ± 0·9 (5)	
	65 (1000)		3·6 ± 0·5 (5)

* Rats were bilaterally nephrectomized and immediately after the surgery given the indicated dose intrajugularly. Twelve h later they were sacrificed for the determination of intestinal calcium transport and bone mineral mobilization activity. Data show the mean values ± standard error (number of animals).

These biological data suggest two important points. First, the 1α-hydroxy-function of cholecalciferol seems to be essential for inducing intestinal calcium transport and bone mineral mobilization activity, as already suggested by many laboratories, and the 2α-hydroxy-function does not mimic the 1α-hydroxy-function, though a high dose of 2α-OH-D_3 elicited a slight stimulation in both activities in intact as well as in anephric rats. Secondly, the presence of the 2α-hydroxy-function seems to be inhibitory to the metabolism of cholecalciferol. Since 2α-OH-D_3 contains a hydroxy-function in the nearest possible position to a 1α-hydroxy-group, the 1α-hydroxylation reaction of 2α-OH-D_3 may be inhibited in the kidney. These possibilities are undergoing a more detailed investigation.

EXPERIMENTAL

^{1}H N.m.r. spectra were determined for solutions in deuteriochloroform at 100 MHz. Mass spectra were run on a Hitachi model RMU-7M double-focusing spectrometer, operating at an accelerating voltage of 8 kV and an electron beam energy of 70 eV.

2α-Hydroxycholesterol (1a).—(*a*) According to the procedure described in our previous paper,[6] three diols [(1), (7), and (8)] were obtained. The identification of the diols (1) and (7) has been described in detail.[6,9] Compound (8) (100 mg) was hydrogenated in methanol (50 ml) over 10% palladium–charcoal (*ca.* 50 mg) at room temperature under atmospheric pressure. Catalyst and solvent were removed and the residue was chromatographed on alumina. Elution with benzene gave an oily substance (37 mg), *m/e* 388, 371, and 370, whose structure is now under investigation. Elution with 2% methanol–methylene chloride gave a crystalline diol (42 mg), m.p. 194—196° (from acetone), *m/e* 404, 387, 386, and 371, τ 5·95 (1H, m) and 6·3 (1H, m). The m.p. was in good accord with that of 5β-cholestane-3β,6β-diol.[10]

(*b*) To a solution of cholesta-1,5-dien-3β-ol (6) (300 mg) in tetrahydrofuran (3 ml) was added 0·5M-9-borabicyclo-[3.3.1]nonane in tetrahydrofuran (3·9 ml, 2·5 mol. equiv.) and the whole was heated at 65° for 20 h in a sealed tube. After the reaction, ethanol (3 ml), aqueous 6N-sodium hydroxide (1 ml), and aqueous 30% hydrogen peroxide (2 ml) were added, and the mixture was again heated at 50° for 1 h. After cooling, the mixture was dissolved in ethyl acetate (100 ml) and stored over potassium carbonate at room temperature overnight. The solution was filtered and the filtrate evaporated to dryness *in vacuo*. Recrystallization of the residue from acetone gave pure 2α-hydroxycholesterol (1a) (175 mg), m.p. 191—194°, identical with material obtained by method (*a*). Chromatography of the mother liquor on alumina gave the starting material (*ca.* 10 mg) (eluted with chloroform) and more of the diol (1a) (42 mg) (eluted with 2% methanol–chloroform).

Cholesta-5,7-diene-2α,3β-diol (*2α-Hydroxyprovitamin* D_3) (3a).—2α,3β-Diacetoxycholest-5-ene (1b) (300 mg) was dissolved in light petroleum (5 ml) and benzene (5 ml). After addition of 1,3-dibromo-5,5-dimethylhydantoin (100 mg), the mixture was warmed at 70—72° for 15 min. After cooling in an ice-bath, the crystalline precipitate was filtered off and washed with a small amount of ice-cooled petroleum. The combined solution was evaporated below 40° *in vacuo*. The crude brominated compound (2b) was then dissolved in xylene (6 ml). After addition of trimethyl phosphite (0·6 ml), the mixture was refluxed gently for 90 min. The residue obtained after evaporation (below 60° *in vacuo*) was taken up in dry ether (10 ml) and was reduced with lithium aluminum hydride (100 mg in 25 ml of ether) for 2 h at room temperature. The residue obtained after the usual work-up was chromatographed over Sephadex LH-20 (15 g) with chloroform–hexane (65 : 35 v/v). The fractions showing a typical homoannular diene u.v. absorption were collected and recrystallized from methanol

to give the pure 5,7-diene (3a) (95 mg), m.p. 207—210°, $\lambda_{max.}$ (EtOH) 262, 271, 282, and 293·5 nm (log 3·52, 3·97, 4·02, and 3·78), *m/e* 400 (M^+), 367, 325, 157, 149, and 145. Further elution with the same solvent afforded a fraction (35 mg) having a typical heteroannular diene u.v. absorption. Recrystallization from methanol gave pure cholesta-4,6-diene-2α,3β-diol, m.p. 162—165°, $\lambda_{max.}$ (EtOH) 233, 239, and 247 nm, *m/e* 400 (M^+), 382, and 376.

Irradiation of Cholesta-5,7-*diene*-2α,3β-*diol* (3a).—A solution of 2α-hydroxyprovitamin D_3 (3a) (50 mg) in ether (600 ml) was irradiated with a 200 W Hanovia high-pressure immersion mercury lamp (354A-36) with a Vycor filter. During the irradiation (20 min), the solution was agitated gently with a stream of argon. Evaporation to dryness *in vacuo* below 25° left a residue which was chromatographed on Sephadex LH-20 (20 g) with chloroform–hexane (65 : 35 v/v) as eluant. The fractions corresponding to the hydroxylated previtamin, tachysterol, and the starting material were eluted in this order. The combined previtamin fraction amounted to *ca.* 13·5 mg and those of the tachysterol and the starting material 8·5 and 8 mg, respectively. Storage of the previtamin (4a) in ether (100 ml) in the dark for 10 days at room temperature completed the thermal 1,7-antarafacial hydrogen migration from (4a) to 2α-hydroxyvitamin D_3 (the reaction was followed by u.v. spectroscopy). The residue obtained by evaporation was again chromatographed on Sephadex LH-20 (15 g) with the same solvent as above and pure 2α-hydroxyvitamin D_3 (5) (9·8 mg) was obtained; $\lambda_{max.}$ (ether) 266 nm (ε 18,000 taken as standard for calculation),[14] $\lambda_{min.}$ 228 nm; τ ($CDCl_3$) 3·73 (1H, d, *J* 11 Hz) and 4·03 (1H, d, *J* 11 Hz) (6- and 7-H), 4·88br (1H, s) and 5·10br (1H, s) (19-H_2), and *ca.* 6·45 (2H, m, 2- and 3-H); *m/e* 400 (M^+), 367, 364, 287, 251, 159, 152, 149, and 134.

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[14] L. F. Fieser and M. Fieser, ' Steroids,' Reinhold, New York, 1959, p. 148.