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5,6-Trans-25-Hydroxycholecalciferol: Vitamin D Analog Effective on Intestine of Anephric Rats

Abstract. *A new compound, 5,6-*trans*-25-hydroxycholecalciferol, has been synthesized and tested for its biological activity. Like 1,25-dihydroxycholecalciferol, it stimulates intestinal calcium transport in anephric rats, whereas 25-hydroxycholecalciferol does not. But this analog has little if any activity in stimulating mobilization of calcium from the bone of anephric rats.*

The first serious attempt to synthesize analogs of vitamin D_2 was made by Windaus and co-workers. They explored the possibility of producing antirachitic substances from $\Delta^{5,7}$ sterols having differing side chains. Two analogs of ergosterol, 22-dihydroergosterol and 7-dehydrocholesterol, upon irradiation yielded two new biologically active compounds known as vitamin D_4 (*1*) and vitamin D_3 (*2*), respectively. Since these initial experiments, vitamin D–modified chemicals have been tested for biological activity in various ways. One of the most important group of analogs is the dihydrotachysterols, which have little antirachitic activity (180 I.U./mg) but display preferential activity in mobilizing calcium from bone (*3*).

Verloop *et al.* (*4*) were the first to report the synthesis of 5,6-*trans*-ergocalciferol [9,10-*seco*-(5*E*,7*E*,22*E*)-5,7,-10(19,22)-ergostatetraene-3β-ol] by iodine-catalyzed isomerization of vitamin D_2 under neutral conditions in a nonpolar solvent. They reported that the ultraviolet absorption maximum shifted from 265 nm for vitamin D_2 (5,6-*cis*-ergocalciferol) to 272 nm for the 5,6-*trans* isomer. Similarly, Inhoffen *et al.* (*5*) studied this isomerization and reported the synthesis of 5,6-*trans*-vitamin D_3.

In light of the finding that 25-hydroxycholecalciferol (25-OHD_3) must be hydroxylated at C-1 by the kidney (*6*, *7*) to 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] before it can induce either intestinal calcium transport (*8*) or bone calcium mobilization (*9*), it was of interest to investigate the biopotency of the 5,6-*trans* isomer of 25-OHD_3 because it was similar in appearance to 1,25-$(OH)_2D_3$ (Fig. 1). Compared to 25-OHD_3, the 5,6-*trans* isomer has its A ring rotated 180°, with the 3β-hydroxyl function in the same geometrical position as the 1-hydroxyl of 1,25-$(OH)_2D_3$. It seemed possible, therefore, that the 5,6-*trans*-25-OHD_3 may well be able to substitute for 1,25-$(OH)_2D_3$.

Ten milligrams of 25-OHD_3 was dissolved in 10 ml of a mixture of Skellysolve B (a light petroleum fraction redistilled at 67° to 69°C) and diethyl ether (9 : 1, by volume) and 50 μl of a solution of iodine in Skellysolve B (0.1 mg/1 ml) was added. After 1 hour at 25°C, the reaction was terminated with solid $Na_2S_2O_3$ and washed with water, and the product in the Skellysolve B–ether phase was dried over anhydrous Na_2SO_4, according to procedure of Verloop *et al.* (*4*). The solvent was evaporated under nitrogen, and the product was redissolved in 1 ml of a mixture of Skellysolve B and diethyl ether (7 : 3, by volume). The sample was applied to a multibore silicic acid (15 g) column, measuring stepwise in diameter 1.2, 0.8, and 0.4 cm (*10*). The column was eluted with a hyperbolic gradient generated by having 230 ml of Skellysolve B and diethyl ether (7 : 3, by volume) in a mixing chamber and 400 ml of Skellysolve B and diethyl ether (3: 7, by volume) in a holding chamber. Diethyl ether (300 ml) was added to the holding chamber after it emptied. One hundred fractions, 5 ml each, were collected, and the ultraviolet absorption spectrum of each tube was taken to determine the elution position of the 5,6-*trans*-25-OHD_3. The isomer was collected and chromatographed once again on the multibore silicic acid column.

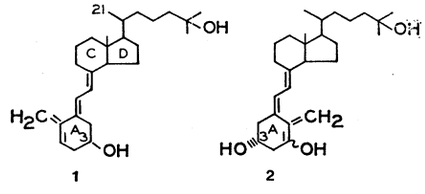

Fig. 1. Structure **1** is 5,6-*trans*-25-OHD_3; structure **2** is 1,25-$(OH)_2D_3$.

The ultraviolet spectrum of the isomer showed the characteristic maximum at 273.5 nm and a minimum at 232 nm (*4*, *5*) for the 5,6-*trans* triene system. The mass spectrum of the analog showed a molecular ion at *m/e* 400 similar to 25-OHD_3 and fragments at *m/e* 271 and 253 (271 − H_2O) which are characteristic for loss of the side chain, and at *m/e* 136 and 118 (136 − H_2O) which are characteristic for the A ring plus C-6 and C-7. Gas-liquid chromatography of the 5,6-*trans*-25-OHD_3 showed only one component, which is consistent with a previous report for 5,6-*trans*-vitamin D_3 (*11*).

Antirachitic activity for the 5,6-*trans*-25-OHD_3 was measured by the antirachitic line test assay method described in *The United States Pharmacopeia* (*12*) and was found to be one-tenth that of vitamin D_3.

For intestinal calcium transport measurements, weanling male albino rats (Holtzman, Madison, Wis.) were housed individually in hanging wire cages and given free access to food and water. They were fed for 3 weeks on a purified diet deficient in vitamin D and low in calcium (0.02 percent) (*13*) and then divided into five groups. One group received only the ethanol

Table 1. Intestinal calcium transport response to 5,6-*trans*-25-OHD_3.

Compound	Amount (μg)	Animal		^{45}Ca serosal/ ^{45}Ca mucosal (mean ± S.E.)
		Condition	No.	
None	0	Normal	5	1.8 ± 0.2
5,6-*trans*-25-OHD_3	25	Sham-operated	5	4.4 ± 0.3
5,6-*trans*-25-OHD_3	25	Anephric	6	3.3 ± 0.3
25-OHD_3	25	Anephric	5	1.9 ± 0.3
25-OHD_3	25	Sham-operated	5	4.5 ± 0.8

Table 2. Calcium mobilization from bone in response to 5,6-*trans*-25-OHD_3.

Compound	Amount (μg)	Animal		Milligrams of Ca/ 100 ml of serum (mean ± S.E.)
		Condition	No.	
None	0	Anephric	6	4.3 ± 0.1
5,6-*trans*-25-OHD_3	25	Anephric	6	4.9 ± 0.1
25-OHD_3	0.25	Anephric	6	4.5 ± 0.1
1,25-$(OH)_2D_3$	0.25	Anephric	6	6.1 ± 0.1

vehicle. Two other groups (either sham-operated or bilaterally nephrectomized) received either 25 μg of 5,6-*trans*-25-OHD_3 or 25 μg of 25-OHD_3 intrajugularly in 0.05 ml of 95 percent ethanol. Sixteen hours later the rats were decapitated. The small intestines were removed for the measurement of intestinal calcium transport by the everted gut sac technique described by Martin and DeLuca (*14*).

For the measurement of calcium mobilization from bone, male weanling Holtzman rats were fed for 2 weeks with a diet adequate in calcium and phosphorus and deficient in vitamin D (*15*), and then a low calcium (0.02 percent) vitamin D–deficient diet for another 10 days (*13*). In some groups the rats were bilaterally nephrectomized and immediately after surgery were injected with either 25 μg of 5,6-*trans*-25-OHD_3, 0.25 μg of 25-OHD_3, or 0.25 μg of 1,25-$(OH)_2D_3$ dissolved in 0.05 ml of 95 percent ethanol. Controls received 0.05 ml of 95 percent ethanol vehicle. Twenty-four hours after the administration of the dose, the animals were killed by decapitation, and the blood serum was collected.

Serum calcium was determined with an atomic absorption spectrophotometer (Perkin-Elmer model 214). For this determination, serum samples (0.10 ml) were diluted with 1.9 ml of 0.1 percent $LaCl_3$.

The results in Table 1 demonstrate that 5,6-*trans*-25-OHD_3 is effective in stimulating calcium transport in the duodenum of vitamin D–deficient rats. More important, however, is the observation that the 5,6-*trans*-25-OHD_3, like 1,25-$(OH)_2D_3$ (*7*), is more active than 25-OHD_3 in stimulating intestinal calcium transport in bilaterally nephrectomized rats.

The major difference in the biological activity between 5,6-*trans*-25-OHD_3 and 1,25-$(OH)_2D_3$ is shown in Table 2. Like 25-OHD_3, the 5,6-*trans* analog has little effect in the mobilization of calcium from bone in anephric rats, as is demonstrated by only a small rise in the serum calcium, whereas 1,25-$(OH)_2D_3$ elicited a marked response. Furthermore, this *trans* analog showed little if any potential to induce bone resorption in fetal rat bone tissue culture (*16*), whereas 1,25-$(OH)_2D_3$ was extremely effective (*17*).

These unusual properties make the 5,6-*trans*-25-OHD_3 a promising drug for the treatment of calcium abnormalities associated with chronic renal failure. Furthermore, the biological activity of this analog provides additional evidence that a hydroxyl function must be present on C-1 of vitamin D compounds for the stimulation of intestinal calcium transport.

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Development of Sensitivity to Tetrodotoxin in Beating Chick Embryo Hearts, Single Cells, and Aggregates

Abstract. *The spontaneous activity of intact embryonic heart becomes progressively more sensitive to tetrodotoxin block with increasing age of the embryo. The activity of isolated single heart cells in culture was relatively insensitive, independent of embryo age. Aggregates formed from single cells responded to tetrodotoxin in the same manner as intact hearts; aggregated cells from older hearts were sensitive.*

Tetrodotoxin (TTX) specifically blocks inward sodium current in many excitable tissues (*1*), including heart (*2*), and abolishes spontaneous activity in cells whose action potential is dependent on a transient increase in sodium conductance (*3*). Such a mechanism underlies action potential generation in the embryonic chick heart (*4*); the fast inward sodium current in adult heart tissue is blocked by TTX (*5*, *6*). We have examined the effect of TTX on the spontaneous beating of chick embryo heart preparations, namely, whole hearts, single isolated cells, and aggregates of dissociated cells. We have