# STUDIES ON THE MODE OF ACTION OF CALCIFEROL. X. 24-NOR-25-HYDROXYVITAMIN $D_3$, AN ANALOG OF 25-HYDROXYVITAMIN $D_3$ HAVING "ANTI-VITAMIN" ACTIVITY

R. Lorne Johnson*, William H. Okamura+ and Anthony W. Norman*

Departments of Biochemistry* and Chemistry+
University of California
Riverside, California 92502

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Summary

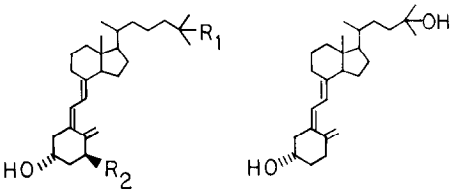
24-Nor-25-hydroxyvitamin $D_3$, an analog of 25-hydroxyvitamin $D_3$, has been chemically synthesized in six steps. This steroid was tested in chicks, in vivo, for its ability to generate the classic vitamin D mediated responses of stimulation of intestinal calcium transport and bone calcium mobilization. Although the 24-nor-25-OH-vitamin $D_3$ itself exhibited no biological activity in these assays, the analog was found to inhibit the normal responses produced by a physiological dose of vitamin $D_3$. These results suggest that 24-nor-25-OH-vitamin $D_3$ may satisfy certain requirements expected of a calciferol "anti-vitamin."

It is well established that the biologically significant form of vitamin $D_3$ is $1^{\alpha}$,25-dihydroxyvitamin $D_3$[1] (1, 2). This form of the vitamin is made by successive obligatory hydroxylations of vitamin D; first by the liver at the C-25 position to give 25-hydroxyvitamin $D_3$ (3) and second by the kidney at the C-1$\alpha$ position (4, 5). (See Figure 1)

It is apparent that in the complex metabolic pathway or endocrine system required to produce 1$\alpha$,25-$(OH)_2$-$D_3$, that there are many sites of steroid-protein(enzymes, carrier proteins and ultimately receptor)interaction.

In our continuing efforts to elucidate the minimal and optimal structural features of the vitamin D molecule required for biological activity, we have initiated a program of synthesis of a series of side chain analogs of 25-OH-$D_3$ in which the 8 carbon side chain of vitamin $D_3$ is extended by one carbon or shortened by 1,2,3 or 5 carbons, but always with retention of the tertiary

[1]Abbreviations employed are: 25-hydroxyvitamin $D_3$ [25-OH-$D_3$]; 1$\alpha$,25-dihydroxyvitamin $D_3$ [1$\alpha$,25-$(OH)_2$-$D_3$]; 24-nor-25-hydroxyvitamin $D_3$ [24-nor-25-OH-$D_3$]; 24,25-dihydroxyvitamin $D_3$ [24,25-$(OH)_2$-$D_3$].



1, $R_1 = R_2 = H$
2, $R_1 = OH$; $R_2 = H$
3, $R_1 = R_2 = OH$

4

Figure 1. Vitamin D and related compounds. For vitamin $D_3$, 1, (when $R_1 = R_2 = H$); 25-OH-$D_3$, 2, (when $R_1 = OH$, $R_2 = H$); 1α,25-$(OH)_2$-$D_3$, 3 (when $R_1 = R_2 = OH$); For 4 steroid shown is 24-nor-25-OH-$D_3$.

hydroxyl group (6). In this communication we wish to report the improved chemical synthesis and previously unrecognized biological properties of the analog 24-nor-25-OH-$D_3$ in which the side chain has been shortened by one methylene unit.

## Experimental

In Figure 2 is outlined the synthetic sequence utilized in the synthesis of 24-nor-25-OH-$D_3$, 4.

Cholenic acid acetate, 5, was converted to its methyl ester, 6, in 94% yield [mp 154-56°; lit (7) 154-56°] using excess diazomethane generated by the action of base on bis-(N-methyl-N-nitroso)terepthalamide (8). Allylic bromination of 6 (1,3-dibromo-5,5-dimethylhydantoin in refluxing benzene/hexane, 1:1) followed by dehydrobromination (refluxing s-collidine, 30 min) (9) afforded after chromatography (10% $AgNO_3$-silica gel, petroleum ether-ether) a 46% yield of the 5,7-diene, 7 [mp 124-27°; lit (7) 125-27°; uv λ max 293 (ε6300), 282 (ε11,000), 271 (ε10,300) nm]. Alkylation of the latter (r.t., 24 hrs) in tetrahydrofuran with methyl lithium (1.8 M in ether) yielded after crystallization (methanol) the provitamin diene-diol, 8 (97%) [uv λmax 293 (ε5860), 282 (ε9700), 271 (ε9100); nmr τ 4.40 and 4.62 ($H_{6,7}$, ABq, $J_{AB} \approx 6H_z$), 6.13-6.75 ($H_{3\alpha}$, br m), 6.52 ($C_{25}OH$, s), 8.79 ($C_{26,27}CH_3$, s), 9.02 ($C_{21}CH_3$, d, $J \simeq 6H_z$), 9.04 ($C_{19}CH_3$,s), 9.35 ($C_{18}CH_3$,s); anal. calcd. for $C_{26}H_{42}O_2 \cdot H_2O$: C, 78.93; H, 11.21. Found: C, 78.55; H, 11.10], which was converted to the desired vitamin 4 by ultraviolet irradiation on a 0.5 mg/ml solution of the provitamin (EtOH) using a Hanovia 450 medium pressure mercury lamp (4 min at 0°). Following repeated chromatography over 10% $AgNO_3$ impregnated silica gel ('Baker-Analyzed', 60-200 mesh; 1 x 80 cm column), the vitamin (colorless, 31% yield) was found [$uv_{EtOH}$ λmax 264 nm, λmin 227; nmr ($CDCl_3$) τ 3.72 and 3.99 ($H_{6,7}$, ABq, $J_{AB} \approx 11$ $H_z$), 4.96 and 5.20 ($H_{19Z}$ and $H_{19E}$, m and d), 5.79-6.37 ($H_{3\alpha}$, br m), 8.80 ($C_{26,27}$ $CH_3$, s), 9.46 ($C_{18}CH_3$,s); ms, m/e 386 ($M^+$)] to be homogeneous in several TLC systems. The overall yield of 24-nor-25-OH-$D_3$ was 13% from 5.

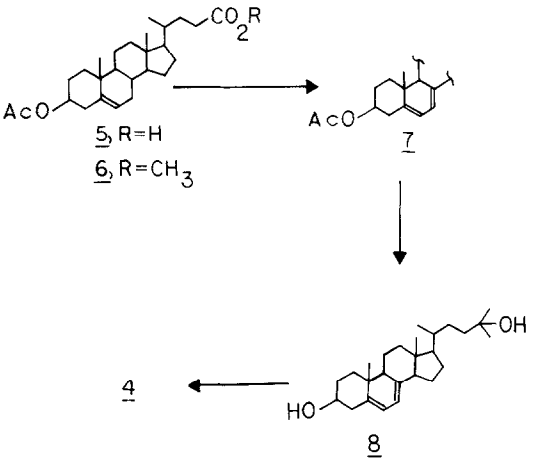



Figure 2. Pathway for the chemical synthesis of 24-nor-25-OH-$D_3$, 4 from cholenic acid acetate, 5.

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The analog was assayed along with vitamin $D_3$ and 25-OH-$D_3$ according to the procedure of Hibberd and Norman (10) for their ability to stimulate intestinal calcium transport and bone calcium mobilization in vitamin D deficient (rachitic) chicks. White Leghorn cockerels were raised for three weeks on a standard rachitogenic, low calcium diet (11) without vitamin D supplement. Three days before assay the chicks were placed on a zero calcium diet. The vitamin D elicited responses were determined by published procedures (6) as follows: the duodenum of lightly ether anaesthetized chicks was surgically exposed and 5 μCi $^{45}Ca^{2+}$ + 4 mg nonradioactive $^{40}Ca^{2+}$ carrier was placed into the small intestine. Thirty minutes later the animal was sacrificed by decapitation and the blood collected and allowed to clot. Intestinal calcium absorption was assessed by determining the amount of radioactivity present in a 0.2 ml aliquot of serum. Bone calcium mobilization response was directly quantitated via atomic absorption spectrometry on appropriately diluted samples of the serum. The results were expressed as mg $Ca^{2+}$/100 ml serum.

## Results and Discussion

The results of the bioassays are shown in Tables 1 and 2.

From the data expressed in Table 1 it is quite evident that in the chick there is neither an intestinal calcium absorption nor bone calcium mobilization response elicited by the new analog 24-nor-25-OH-$D_3$. However, a very interesting observation was made when birds were dosed with vitamin D after a predose with 24-nor-25-OH-$D_3$. As shown in Table 2, animals receiving a predose of the nor analog had a significantly lowered response to vitamin $D_3$. Both intestinal calcium transport and bone calcium mobilization were affected. It

Table 1

Intestinal Calcium Absorption and Bone Calcium Mobilization Activity of 24-Nor-25-Hydroxyvitamin $D_3$

| Compound | Dose[a] | n | Intestinal $Ca^{2+}$ Absorption Assay[b] | Bone $Ca^{2+}$ Mobilization Assay[b] |
|---|---|---|---|---|
| | (nmoles) | | CPM $^{45}Ca^{2+}$/ 0.2 ml serum ± SD | Serum $Ca^{2+}$ (mg/100 ml) ± SD |
| None | -- | (19) | 330 ± 130 | 5.4 ± 0.7 |
| 24-nor-25-OH-$D_3$ | 97.5 | (9) | 385 ± 100 | 5.4 ± 0.5 |
| Vitamin $D_3$ | 97.5 | (9) | 1680 ± 360* | 7.3 ± 0.9* |
| 25-OH-$D_3$ | 97.5 | (8) | 1640 ± 250* | 7.8 ± 1.0* |

[a]Twenty-four hours before assay, birds received a single intraperitoneal dose of the indicated compound in 0.2 ml 1,3-propandiol/EtOH (3:1). Control birds received only 0.2 ml of the vehicle. At the time of assay the birds were killed and bone calcium mobilization and intestinal calcium tranport activities were determined as described in the Methods. Data are expressed as mean ± SD.

[b]Values indicated by a * are significantly different from the control (-D) at $p < 0.001$.

should also be noted that the onset of this "inhibitory" activity occurred when the analog was given as little as twelve hours before vitamin D and the duration of "inhibition" was for at least 36 hours. The fact that the analog did not impair the biological response of 25-OH-$D_3$ (Table 2, bottom line) but does block the response of vitamin D suggests that its site of action may be in the liver. Ongoing experiments will attempt to determine the reason for the observed decrease in vitamin D activity. Results from a preliminary experiment which employed radioactive vitamin $D_3$ indicate that in the presence of 24-nor-25-OH-$D_3$ the metabolism of vitamin $D_3$ to 25-OH-$D_3$ is greatly impaired.

The inhibitory effect of predosing with 24-nor-25-OH-$D_3$ on the biological response on the chick to vitamin D represents the first known instance of

Table 2

Inhibition of Vitamin $D_3$ Activity by Predosing with 24-Nor-25-OH-$D_3$[a]

| Compound | Time of Predose before $D_3$ | n | Intestinal $Ca^{2+}$ Absorption Assay[b] | Bone $Ca^{2+}$ Mobilization Assay[b] |
|---|---|---|---|---|
| | | | CPM $^{45}Ca^{2+}$/ 0.2 ml serum ± SD | Serum $Ca^{2+}$ (mg/100 ml) ± SD |
| None | -- | (12) | 180 ± 70* | 5.8 ± 0.4* |
| Vitamin $D_3$ | -- | (18) | 930 ± 380 | 8.1 ± 0.9 |
| NOR[c] + $D_3$ | 12 | (9) | 400 ± 190* | 6.4 ± 0.5* |
| NOR + $D_3$ | 36 | (9) | 470 ± 115* | 6.0 ± 0.4* |
| 25-OH-$D_3$ | -- | (14) | 1200 ± 180 | 7.1 ± 0.6 |
| NOR + 25-OH-$D_3$ | 24 | (10) | 1475 ± 150 | 8.5 ± 0.9 |

[a]This experiment was done exactly as for that shown in Table 1 except three groups received one intraperitoneal predose of 32.5 nmoles of 24-nor-25-OH-$D_3$ analog at the indicated time before a 6.5 nmole dose of vitamin $D_3$ or 25-OH-$D_3$. Control groups received only an IP dose of vehicle. Data are expressed as mean ± standard deviation.

[b]Values indicated by a * are significantly different from the vitamin $D_3$ treated group $p < 0.001$.

[c]NOR = 24-nor-25-OH-$D_3$

"anti-vitamin" activity in the calciferol field. It has been known for many years that the chick has an unusually stringent requirement with regard to allowable side chain structures. The 8 carbon side chain of cholesterol (as in vitamin $D_3$) is 10X more active than the 9 carbon branched side chain of ergosterol (as in vitamin $D_2$) (10). Further the chick can discriminate between the two epimers, 24R,25-$(OH)_2$-$D_3$ and 24S,25-$(OH)_2$-$D_3$ (11). Thus before the concept of an "anti-vitamin" is firmly established for 24-nor-25-OH-$D_3$, it will be important to assess its inhibitory effects in other animal systems; e.g. it is known that vitamin $D_2$ and $D_3$ are equivalently active in the rat.

In this respect Holick et al. (13) have obtained some preliminary data suggesting that in the rat, 24-nor-25-OH-$D_3$ (at a dose level of 25 μg or 65 nmoles) is active in stimulating bone calcium mobilization and is not significantly active in mediating intestinal calcium absorption. There are several disorders of vitamin D in man, e.g. vitamin D intoxication, and sarcoidosis where the availability of an anti-vitamin D compound might be of clinical utility. However much further work is necessary to validate thoroughly the concept of "anti-vitamin" in relation to the steroid 24-nor-25-OH-$D_3$ and to evaluate its potential usefulness.

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