Relationship of 25-Hydroxyvitamin D₃ Side Chain Structure to Biological Activity*

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SUMMARY

27-nor-25-Hydroxyvitamin D₃, 26,27-bisnor-25-hydroxyvitamin D₃, and 22-27-hexanor-20-hydroxyvitamin D₃ and the corresponding 5,6-trans isomers have been synthesized. All compounds were tested for their ability to induce intestinal calcium transport and bone calcium mobilization in normal and anephric rats. The 27-nor- and 26,27-bisnor-25-hydroxyvitamin D₃ analogs are capable of stimulating intestinal calcium transport and bone calcium mobilization in normal rats but are 10 to 100 times less active than 25-hydroxyvitamin D₃. Although these analogs are inactive in anephric rats, their corresponding 5,6-trans isomers are capable of stimulating both intestine and bone activity in these animals. The 22-27-hexanor-20-hydroxyvitamin D₃ and its corresponding 5,6-trans isomer are incapable of stimulating either intestinal calcium transport or bone calcium mobilization. These results suggest that minor alterations in the side chain significantly decrease the biopotency of 25-hydroxyvitamin D₃. Since these analogs are biologically active in normal but not in anephric animals, it appears that the kidney 1α-hydroxylation is necessary for activity. Since 22-27-hexanor-20-hydroxyvitamin D₃ and its corresponding 5,6-trans analog are biologically inactive, it is likely that at least part of the side chain is necessary for 25-hydroxyvitamin D₃ to stimulate intestinal calcium transport and bone calcium mobilization.

In 1935 Windaus and co-workers (1) reported the synthesis of antirachitic compounds with the 5,6-cis-triene system of vitamin D₃ with differing side chain structures. They successfully synthesized an analog of ergosterol which had a cholesteral side chain and reported that one of its irradiation products (vitamin D₂) possessed about the same antirachitic potency as vitamin D₃. Since this initial effort to make biologically active analogs of vitamin D, various side chain analogs have been synthesized. Reduction of the vitamin D₂ side chain (22,23-dihydrovitamin D₂) decreased its biological effectiveness by 25% (2) and, when the vitamin had side chains of stigmasterol (3) and sitosterol (4), they only had about 5 to 10% of the antirachitic activity of vitamin D₃ in rats. However, when the side chain was replaced with a hydroxyl group (5) or a bile acid side chain (6), the compounds were reported to be devoid of vitamin D activity. Thus it appeared that almost any alteration in the side chain greatly reduced antirachitic effectiveness when compared with vitamin D₃.

The recent advances in the metabolism, chemistry, and function of vitamin D have reopened the question of biological activity of vitamin D analogs. It seems well established that vitamin D must be hydroxylated on carbon 25 in the liver before it can proceed to the kidney to be hydroxylated on carbon 1 and thereby become a hormone responsible for inducing intestinal calcium transport, bone calcium mobilization, and the elevation of serum phosphorus concentration (7). It is apparent that vitamin D analogs may be ineffective because they may not be hydroxylated on either carbon 25 or carbon 1. Alternatively, discrimination may be at the level of the target organ itself.

To gain insight into this problem we have made analogs of 25-hydroxyvitamin D₃ (25-OH-D₃) which are lacking carbon 26 or carbon atoms 26 and 27 or the entire side chain. These analogs were tested for their ability to stimulate intestinal calcium transport and bone calcium mobilization in normal and anephric rats and their ability to heal rachitic lesions. Each of the compounds was then converted to its corresponding 5,6-trans isomer to provide a pseudo 1α-hydroxy analog of the corresponding side chain derivative. The isomers were also tested for their ability to induce intestinal calcium transport and bone calcium mobilization in anephric rats.

EXPERIMENTAL PROCEDURE

Ultraviolet absorption spectra were determined in diethyl ether with a Beckman DB-G spectrophotometer, and mass spectrometric determinations were carried out with an AEI MS-9 mass spectrometer, using direct probe inlet temperatures of 110 to 130° above ambient. Gas-liquid partition chromatography was performed in an F & M model 462 gas chromatograph, using a glass column (4 feet X 0.25 inch) packed with 3% SE-50 on Gas-Chrom Z (100 to 120 mesh) (Applied Science Laboratories Inc., State College, Pa.). The column temperature was 250° and an outlet flow rate of 80 ml per min was obtained. All solvents used were of reagent grade. Radioactive determinations were carried out

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with a Packard Tri-Carb model 3375 liquid scintillation counter equipped with an automatic external standardization system. Samples were counted in 10 ml of toluene counting solution consisting of 2 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per liter of toluene.

**Synthesis**

*Acetylation*—Each of the analogs was prepared in a similar fashion, as illustrated in Fig. 1. One hundred milligrams each of A'-pregnalone, 27-nor-5-cholen-25-one, and homocholenic acid methyl ester (kindly supplied by the Upjohn Co.) were acetylated separately in 5 ml of acetic anhydride with 0.1 ml of pyridine at 80° for 24 hours. The reaction mixtures were extracted with diethyl ether and water (pH 4). The ether phase was collected and the aqueous phase was extracted twice with EtO. The ether phases were combined, the solvent was removed by flash evaporation, and the residue was redissolved in 1 ml of 1:1 Skellysolve B-EtO and applied to a glass column (2 X 30 cm) containing 10 g of silicic acid (Mallinckrodt) in the same solvent. The column was eluted with 150 ml of the same solvent. The solvent was removed by flash evaporation in preparation for the next step.

*Bromination and Dehydrohalogenation*—The acetylated compounds (II) were dissolved in 4 ml of dry 1:1 benzene-Skellysolve B and brought to a temperature of 72° in a water bath. N, N'-Dibromomethylhydantoin (0.62 to 0.56 mol) was then added according to the procedure of Blunt and Deluca (8). After 15 min, the reaction vessel was placed on ice for 10 min and then the reaction mixture was filtered to remove the precipitate (dimethylhydantoin). The filtrate was dried under nitrogen, dissolved in 0.2 ml of dry xylene, and added dropwise to 0.2 ml of xylene and 0.1 ml of trimethylphosphite solution which was brought to 135° in an oil bath. The dehydrohalogenation was continued at 135° for an additional 90 min. The solvent was flash-evaporated, and the residue was dissolved in 2 ml of 1:4 EtO-Skellysolve B and applied to a multibore silicic acid column prepared from 14 g of heat-activated silicic acid. The column was eluted with a convex gradient obtained by running approximately 2 mg of 26,27-bisnor-25-OH-Da, 27-nor-25-OH-Da, and 22-27-hexanor-20-hydroxycalciferol (pregcalciferol) were recovered (V).

Each of the vitamin D analogs had an ultraviolet absorption spectrum of \( \lambda_{max} \) 265 nm and \( \lambda_{min} \) 228 nm characteristic of the 5,6-cis-triene chromophore (8, 10). The mass spectra also demonstrated fragments \( m/e \) 136 and \( m/e \) 118, representing cleavage at the bridge carbon atoms 6 and 7, and the C and D rings (8, 11). The molecular ion peaks of 386, 372, and 316 were found in the mass spectra of 27-nor-25-OH-Da. The ultraviolet absorption spectra of each analog had a \( \lambda_{max} \) at 294, 282, and 272 nm and was homogeneous on the basis of gas-liquid chromatography.

**Reduction to Remove Acetyl Groups**—The acetylated 5,7-diene analogs (20 mg) were dissolved in 10 ml of dry EtO. An excess of lithium aluminum hydride (30 to 40 mg) was added to the ether solution. The ultraviolet absorption spectra of each had a \( \lambda_{max} \) at 294, 282, and 272 nm and was homogeneous on the basis of gas-liquid chromatography.

**Irradiation**—Ten milligrams of each compound (IV) were individually irradiated in 400 ml of EtO in a jacketed, water-cooled quartz well for 80 s, as previously described (9). Each product was applied in 1 ml of 1:1 EtO-Skellysolve B to a multibore silicic acid column prepared from 14 g of heat-activated silicic acid. The column was eluted with a convex gradient obtained by running 100% EtO into a 250-ml Erlenmeyer constant volume mixing chamber initially filled with EtO-Skellysolve B (1:1). Approximately 2 mg of 26,27-bisnor-25-OH-Da, 27-nor-25-OH-Da, and 22-27-hexanor-20-hydroxycalciferol (pregcalciferol) were recovered (V).

**Biological Assays**

Weanling male rats (Holtzman Co., Madison, Wis.) were housed in overhanging wire cages and were fed the adequate calcium and...
Intestinal calcium transport and bone calcium mobilization response to various analogs of 25-OH-D₃ in normal and anephric rats

Rats were fed a 0.3% phosphorus and 0.47% calcium diet for 2 weeks and then fed the low calcium, vitamin D-deficient diet for 1 week. All anephric animals were bilaterally nephrectomized and both these and normal rats were given the indicated dose intravenously. Twenty-four hours later they were killed for the bioassays.

### TABLE I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Condition of animal</th>
<th>(^{45})Ca Serosal/(^{45})Ca mucosal</th>
<th>Serum calcium (mg%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µl 95% EtOH</td>
<td>Normal</td>
<td>1.5 ± 0.2</td>
<td>4.3 ± 0.1 (6)</td>
</tr>
<tr>
<td>50 µl 95% EtOH</td>
<td>Anephric</td>
<td>1.5 ± 0.2</td>
<td>4.1 ± 0.2 (6)</td>
</tr>
<tr>
<td>0.25 µg 25-OH-D₃</td>
<td>Normal</td>
<td>3.0 ± 0.2</td>
<td>7.4 ± 0.2 (6)</td>
</tr>
<tr>
<td>0.25 µg pregcalciferol</td>
<td>Normal</td>
<td>1.0 ± 0.2</td>
<td>4.5 ± 0.1 (6)</td>
</tr>
<tr>
<td>25 µg 5,6-trans-pregcalciferol</td>
<td>Normal</td>
<td>2.0 ± 0.2</td>
<td>4.5 ± 0.2 (6)</td>
</tr>
<tr>
<td>25 µg 5,6-trans-pregcalciferol</td>
<td>Anephric</td>
<td>1.8 ± 0.3</td>
<td>4.4 ± 0.2 (6)</td>
</tr>
<tr>
<td>0.25 µg 27-nor-25-OH-D₃</td>
<td>Normal</td>
<td>1.5 ± 0.3</td>
<td>4.7 ± 0.2 (6)</td>
</tr>
<tr>
<td>2.5 µg 27-nor-25-OH-D₃</td>
<td>Normal</td>
<td>2.9 ± 0.2</td>
<td>5.1 ± 0.1 (6)</td>
</tr>
<tr>
<td>25 µg 27-nor-25-OH-D₃</td>
<td>Normal</td>
<td>3.2 ± 0.2</td>
<td>7.8 ± 0.3 (6)</td>
</tr>
<tr>
<td>25 µg 27-nor-25-OH-D₃</td>
<td>Anephric</td>
<td>1.8 ± 0.3</td>
<td>4.0 ± 0.2 (6)</td>
</tr>
<tr>
<td>25 µg 5,6-trans-27-nor-25-OH-D₃</td>
<td>Anephric</td>
<td>2.6 ± 0.2</td>
<td>4.7 ± 0.2 (6)</td>
</tr>
<tr>
<td>0.25 µg 26,27-bisnor-25-OH-D₃</td>
<td>Normal</td>
<td>1.5 ± 0.2</td>
<td>4.6 ± 0.2 (6)</td>
</tr>
<tr>
<td>2.5 µg 26,27-bisnor-25-OH-D₃</td>
<td>Normal</td>
<td>3.0 ± 0.2</td>
<td>5.4 ± 0.1 (6)</td>
</tr>
<tr>
<td>25 µg 26,27-bisnor-25-OH-D₃</td>
<td>Normal</td>
<td>3.1 ± 0.3</td>
<td>7.4 ± 0.2 (6)</td>
</tr>
<tr>
<td>25 µg 26,27-bisnor-25-OH-D₃</td>
<td>Anephric</td>
<td>1.7 ± 0.3</td>
<td>4.2 ± 0.2 (6)</td>
</tr>
<tr>
<td>25 µg 5,6-trans-26,27-bisnor-25-OH-D₃</td>
<td>Anephric</td>
<td>2.4 ± 0.2</td>
<td>4.6 ± 0.2 (6)</td>
</tr>
</tbody>
</table>

* Plus or minus S.E. The numbers in parentheses represent the number of rats in each group.

The results in Table I demonstrate that omission of carbon 27 or carbon atoms 26 and 27 from 25-OH-D₃ (Fig. 3) decreased intestinal calcium transport activity by a factor of 10 and bone calcium mobilization activity by a factor of 100 (Table I). Replacement of the side chain with a hydroxyl resulted in virtually a total loss of activity in either the intestine or the bone. Nephrectomy prevented the bone and intestinal response to 25 µg of 27-nor-25-OH-D₃ and 26,27-bisnor-25-OH-D₃. However, the 5,6-trans isomers of the 27-nor and 26,27-bisnor analogs did show some activity in the intestine and bone of nephrectomized rats. Table II demonstrates that 2.5 µg of 27-nor-25-OH-D₃ and 2.5 µg of 26,27-bisnor-25-OH-D₃ were incapable of calcifying bone in rachitic rats, despite the fact that these compounds at those doses do stimulate intestinal calcium transport and bone calcium mobilization.
The realization that vitamin D₃ must undergo a sequential carbon 25 hydroxylation in the liver and a carbon 1 hydroxylation in the kidney as a necessary prerequisite before it can act as a calcium homeostatic hormone has stimulated new interest in synthesizing biologically active analogs of vitamin D. The most interesting are analogs of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), a metabolically active form of vitamin D₃. When vitamin D₃ is treated with iodine to cause a cis-trans isomerization, the rotation of the A ring places the 3β-hydroxyl in a pseudo 1α-hydroxy position, resulting in an analog of 1,25-(OH)₂D₃. Similarly, dihydrotachysterol also has its A ring rotated 180° and also has its 3β-hydroxyl in a pseudo 1α-hydroxy position. Both of these classes of analogs, although 10 to 100 times less active than 1,25-(OH)₂D₃, are capable of stimulating intestinal calcium transport and bone calcium mobilization in anephric rats (16, 17). The most potent analog of 1,25-(OH)₂D₃, however, is 1α-hydroxyvitamin D₃ (1α-OH-D₃) (18–20).

In this paper some of the structural activity relationships of the various side chain analogs of 25-OH-D₃ were examined. By making the 25-hydroxy derivatives, the necessary 25-hydroxylation system of the liver was bypassed and the kidney was provided with 25 hydroxy derivatives that had either carbon 26, or carbon atoms 26 and 27 (or the entire side chain) missing. The 27-nor-25-OH-D₃ and 26,27-bisnor-25-OH-D₃ are capable of stimulating both intestinal calcium transport and bone calcium mobilization. However, these analogs are biologically inactive at a 25-μg dosage in anephric animals, suggesting that these analogs were recognized by the kidney 25-OH-D₃-1-hydroxylase. Additional evidence that this hydroxylation occurred is suggested by the fact that rotation of the A rings (180°) of the 27-nor-25-OH-D₃ and 26,27-bisnor-25-OH-D₃, which places the 3β-hydroxyl in the pseudo 1α-hydroxy position (Fig. 3), partially restores the intestinal calcium transport and bone calcium mobilization activity in anephric rats. When the side chain is replaced with a hydroxyl (pregcalciferol), no biological activity in either intestine or bone is found. In order to rule out that the lack of activity might be due to the inability of the kidney to cause hydroxylation of this analog in the 1α position, the 5,6-trans isomer was made. 5,6-trans-Pregcalciferol was also found to be biologically inactive in normal and anephric rats. Therefore, these results, combined with previous reports concerning biologically active analogs of vitamin D₃, suggest the following structural activity relationships for vitamin D₃: (a) 5,6-cis-Triene and the carbon 19 methylene are not absolute requirements for recognition in both intestine and bone, since both 5,6-trans-vitamin D₃ and dihydrotachysterol are biologically active in both systems. (b) The 3β-hydroxyl is not required for vitamin D to be biologically active once there is a 1α-hy-
droxyl or a hydroxyl in a pseudo 1a position, since neither 5,6-
trans-vitamin D₃ nor dihydrotachysterol has a hydroxyl in the
3β position when compared with 1,25-(OH)₂D₃. (c) All 27
carbons in the vitamin D structure are not absolutely necessary
for the vitamin's activity. The side chain analog, 14 nor-25
OH-D₃, and 27-nor-25-OH-D₃, are capable of stimulating intestinal calcium transport and bone calcium mobilization. However, removal of 1 or 2 carbon atoms from the side chain drastically reduces the biological potency. (d) At least part of the side chain is needed in order for vitamin D to retain some of its biological activity. Replacement of the side chain with a hydroxyl completely eliminates intestinal calcium transport and bone calcium mobilization activity.

The present results suggest that the intestine may be less specific for side chain structure than is bone. Although 2.5 µg of 27-nor-25-OH-D₃ and 26,27-bisnor-25-OH-D₃ are capable of eliciting an intestinal response similar to that elicited with 0.25 µg of 25-OH-D₃, 10 times that amount is needed to achieve a bone calcium mobilization response comparable to that obtained with 25-OH-D₃.

Recently Crump et al. (21) reported the synthesis of 22-hydroxyvitamin D₃, and Bontekoe et al. (22) prepared 27-nor-25-OH-D₃ and 26,27-bisnor-25-OH-D₃ and noted that these analogs were biologically inactive on the basis of tests for antirachitic activity. Our results confirm the lack of antirachitic activity for both 27-nor- and 26,27-bisnor-25-OH-D₃ at a 2.5-µg dose. However, it has been clearly demonstrated that these analogs are biologically active on intestinal calcium transport and bone calcium mobilization, suggesting the need to test various analogs for both 27-nor- and 26,27-bisnor-25-OH-D₃. In addition, it has been demonstrated that these analogs are capable of stimulating intestinal calcium transport and bone calcium mobilization before an analog is considered biologically inactive.

Although the data presented show that 27-nor- and 26,27-
bisnor-25-OH-D₃ are less active than 25-OH-D₃, it is not clear why. One possibility is that these analogs are not transported as effectively on the plasma 25-OH-D₃-binding protein to the kidney for 1α-hydroxylation. Belsey et al. (23) have shown that 24-nor-25-OH-D₃, 27-nor-25-OH-D₃, and 26,27-bisnor-25-OH-D₃ bind less effectively than 25-OH-D₃ to the rat plasma 25-OH-D₃-binding protein. Another possibility is that these various side chain analogs are rapidly metabolized and degraded in comparison to 25-OH-D₃. Poor binding to, or poor function in, the target organs is of course the final possibility. The availability of these analogs will, therefore, provide additional tools for investigation of their sites of function.

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