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Synthesis of 1α,25-Dihydroxyvitamin D_2, Its 24 Epimer and Related Isomers, and Their Binding Affinity for the 1,25-Dihydroxyvitamin D_3 Receptor

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The synthesis of 1α-25-dihydroxyvitamin D_2 and of several stereoisomers (5,6-*trans* and 1β-hydroxy isomers and the 24*R*-epimers of these compounds) was reported. Synthesis was accomplished from two different starting materials, 25-hydroxyvitamin D_2 and 25,25-ethylenedioxy-26-norvitamin D_2, and involved C-1-hydroxylation via 3,5-cyclovitamin D intermediates. Synthetic 1α,25-dihydroxyvitamin D_2 was found to be identical with the biologically generated natural product. An analysis of the binding affinity of the synthetic products for the 1α,25-dihydroxyvitamin D_3 receptor protein showed that isomerization of the 5,6 double bond from *cis* to *trans*, or epimerization of the 24-methyl group from *S* to *R*, reduced ligand binding to the receptor only slightly, while both changes together led to a 100-fold reduction of binding affinity. The epimerization of the 1-hydroxy function from 1α to 1β attenuated binding dramatically (ca. 1000-fold).

The isolation and identification of the first biologically potent metabolite of vitamin D_3, i.e., 25-hydroxyvitamin D_3 (25-OH-D_3) (*1*) stimulated great interest in vitamin D_3 metabolism and mechanism of action. Extensive studies (*2, 3*) led to the discovery of the most potent and hormonal form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (*4*), and to the isolation and identification of several other metabolites (*3*). Parallel studies on vitamin D_2 metabolism led, in 1969, to the isolation and characterization of 25-OH-D_2 by Suda et al. (*5*). Later, 1α,25-$(OH)_2D_2$ (*6*) was isolated in pure form from an *in vitro* chick kidney mitochondrial system and its structure was established by means of its mass spectrum, uv absorption, and chemical modifications. The formation of 1,25-$(OH)_2D_2$ *in vivo* in rats and chicks was also shown. It is, therefore, clear that 1α,25-$(OH)_2D_2$ represents the active form of vitamin D_2. Limited biological testing has indicated that its biological activity is similar to the 1,25-$(OH)_2D_3$ in mammals (*6*) whereas in birds it is about one-fifth to one-tenth as active as the corresponding vitamin D_3 compound (*7*). For more detailed studies of the biological activity of 1α,25-$(OH)_2D_2$, synthetic material is required and, having prepared previously 25-OH-

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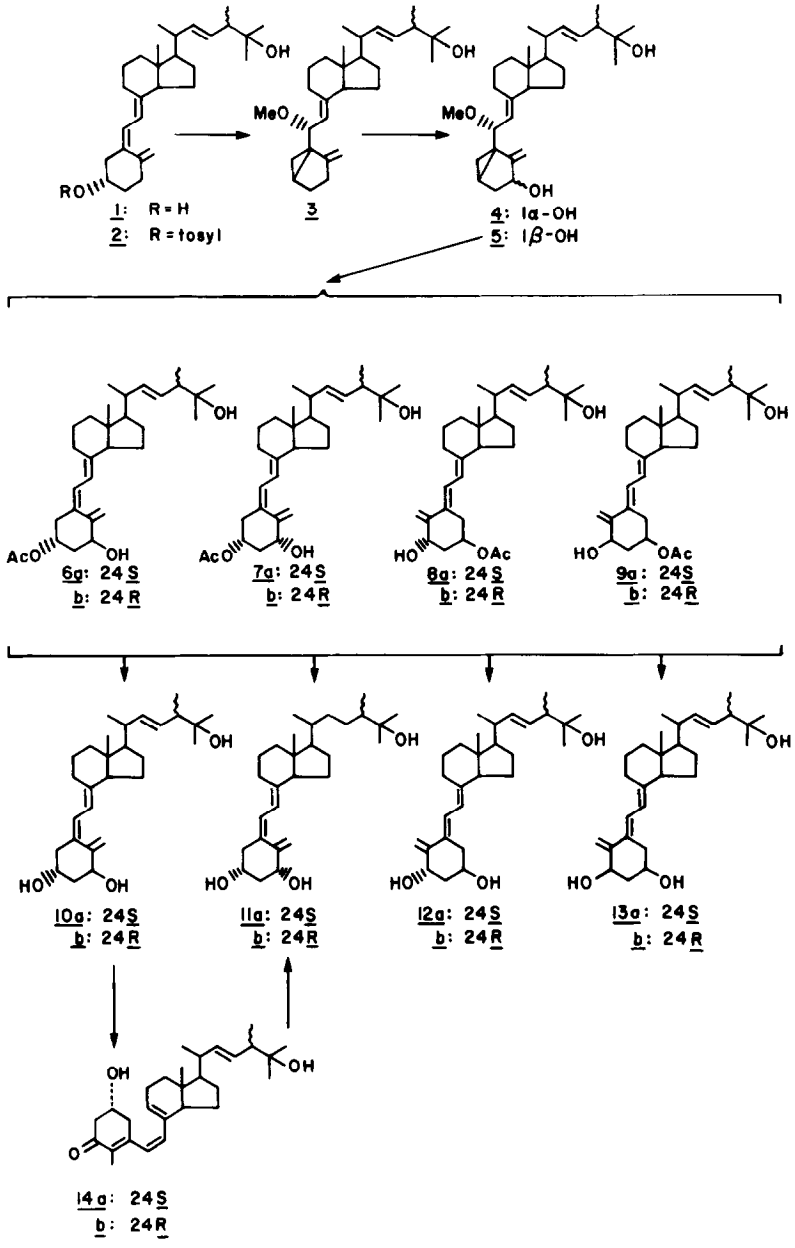
D_2 and its C-24-epimer (*8*), we extended our work to the preparation of the corresponding 1α-hydroxy derivatives. We also prepared several related analogs in this series (5,6-*trans* and 1β-hydroxy isomers) and report a comparative analysis of the relative binding affinity to the intracellular 1,25-$(OH)_2D_3$-receptor protein for all compounds.

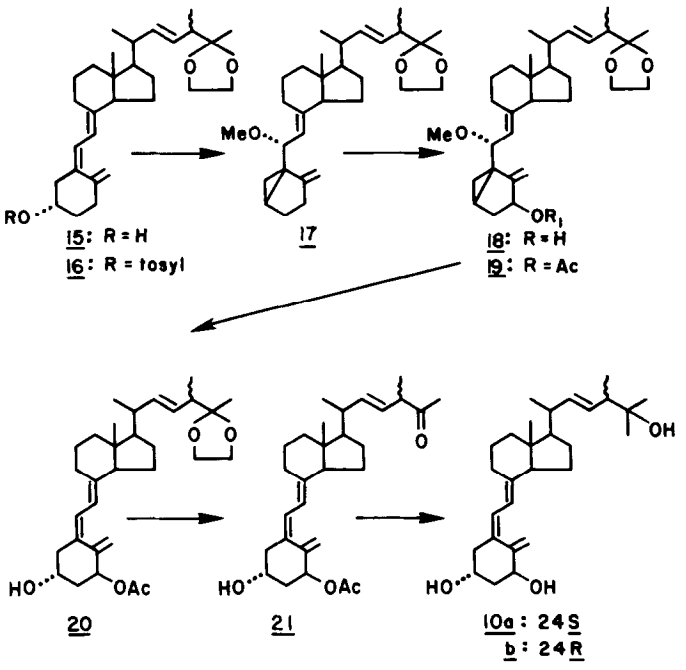
SYNTHESIS

We prepared 1α,25-$(OH)_2D_2$ and its isomers by two different, but related routes. The first, depicted in Scheme 1, involves the conversion of **1**, prepared previously (*8*), to C-1-hydroxylated compounds via 3,5-cyclovitamin intermediates (*9*). Since we were interested in obtaining both 1α,25-$(OH)_2D_2$ and its 24*R*-epimer, it was convenient to use the 24*R*/*S* mixture of **1** as starting material, with epimer separation being accomplished at a later stage. The C-3-tosylate, **2**, was prepared from **1** using conventional tosylation procedures. Bicarbonate-buffered methanolysis of **2** yielded the 3,5-cyclovitamin, **3**, which was, in turn, subjected to allylic oxidation with selenium dioxide and *tert*-butyl hydroperoxide. The reaction was carefully monitored by TLC in an attempt to minimize the loss of desired product due to subsequent oxidation processes leading to the 1-oxo compound. The isolated 1-hydroxy product was apparently homogeneous by TLC and its spectral data were consistent with the expected structure, **4**. However, the detection of 1β-hydroxy derivatives after acid-catalyzed solvolysis clearly indicates that **4** was contaminated with minor quantities of 1β-hydroxycyclovitamin, **5**. Formation of the 1β-hydroxy isomer, **5**, during allylic hydroxylation was unexpected, since in previous work (*9–11*) only the 1α-hydroxy products had been isolated. Evidently, the oxidant approach to the β-face of the A-ring is not completely prevented. Treatment of this 1-hydroxycyclovitamin product mixture, **4** and **5**, with glacial acetic acid provided, as a mixture, the 3β-acetates of the 5,6-*cis*- and 5,6-*trans*-1α,25-$(OH)_2D_2$ compounds (**6a/b** and **8a/b**) and the 1β-hydroxy epimers, **7a/b**, as well as their corresponding 5,6-*trans* isomers **9a/b.** Initial separation of this mixture by HPLC afforded a less polar fraction, containing **6a** (24*S* configuration was established after the hydrolysis step) and both 24 epimers of **7**, and a more polar fraction that contained **6b** and both 24 epimers of **8**. Although the 5,6-*trans* compounds, **9a/b**, were also detected in the solvolysis mixture, their abundance was too low to achieve effective purification. Mild base hydrolysis of each fraction and subsequent further separation and purification of products by HPLC afforded all compounds **10a, 10b, 11a, 11b, 12a,** and **12b** in pure form. The spectral properties of the isolated products were similar to those of analogous 1α- (*10*) and 1β-hydroxyvitamin D_3 compounds (*12*). Compound **10a** comigrated with biologically produced 1α,25-$(OH)_2D_2$, and its mass spectrum was essentially identical with that of the polar metabolite isolated by Jones *et al.* (*6*). The well-known method (*13*) of iodine-catalyzed isomerization of 5,6-*cis*-vitamin D to its 5,6-*trans* form was then used to correlate the C-24-epimers of **10** and **12**. Thus, isomerization of **10a** produced the 5,6-*trans* compound, **12a**, and isomerization of **10b** gave **12b.** This conversion was also used for preparing the corresponding 5,6-*trans* isomers, **13a**



SCHEME 1.

and **13b** (**11a** → **13a**; **11b** → **13b**). As in the case of 5,6-*cis*-1β-hydroxy compounds, **11**, the 5,6-*trans* derivatives, **13**, have an ultraviolet absorption maximum slightly shifted to the shorter wavelengths in comparison with 1α-hydroxy analogs. In order to establish securely the C-24 stereochemistry of 1β,25-$(OH)_2D_2$ compounds, these 1β isomers were chemically correlated with the 1α-hydroxy com-



SCHEME 2.

pounds by the following three-step process (*12*). Treatment of 1α,25-$(OH)_2D_2$, **10a**, with manganese dioxide gave the corresponding 1-oxo-25-hydroxy previtamin D_2 compound, **14a**. Upon reduction of **14a** with $LiAlH_4$, and subsequent thermal isomerization of the previtamin chromophore to the vitamin D triene system, there was obtained 1β,25-$(OH)_2D_2$, **11a**. In an analogous fashion, 1α,25-dihydroxy-24-epivitamin D_2, **10b**, gave ketone **14b** and then 1β,25-dihydroxy-24-epivitamin D_2, **11b**. These interconversions, and the *cis* to *trans* conversions, **11a**, **b** to **13a**, **b**, described earlier, correlate all four 1β,25-$(OH)_2D$ compounds with the corresponding 1α-hydroxy isomers and thus establish the C-24 stereochemistry for all compounds. It has been found in all cases examined that compounds with 24*S* configuration elute from silica HPLC after their respective 24*R* epimers.

The synthetic route described above provided adequate amounts of 1α,25-$(OH)_2D_2$ and its isomers for an evaluation of their biological activity. However, studies on tissue localization of the vitamin, and of its further metabolism, also require the availability of radiolabeled material. Therefore, a second synthetic route was developed which allows for the preparation of both unlabeled and radiolabeled 1α,25-$(OH)_2D_2$ of high specific activity. A suitable substrate for this purpose was the vitamin D-ketal derivative, **15** (Scheme 2), previously synthesized in our laboratory (*8*) as a mixture of 24*R* and 24*S* epimers. Compound **15** was converted to the 1α-hydroxy-3,5-cyclovitamin derivative, **18**, by solvolysis of the corresponding tosylate **16** in methanol followed by allylic oxidation of intermediate **17** with selenium dioxide and *tert*-butyl hydroperoxide. The acid-labile hydroxyl group at C-1 in **18** was then acetylated in a conventional manner, and the

resultant 1-acetoxy derivative was subjected to acid-catalyzed solvolysis. Careful purification by HPLC led to separation of the 5,6-*cis*-1α-acetoxyvitamin, **20**, from other isomers (which were not isolated). The next step, the removal of the ketal protecting group in **20**, was accomplished successfully by careful hydrolysis at moderate temperature using *p*-toluenesulfonic acid as a catalyst. It was advantageous to stop the reaction prior to completion and recover the unreacted substrate, **20**. Subsequent reaction of the isolated 25-ketone, **21**, with a methylmagnesium bromide then provided the expected mixture of 1α,25-$(OH)_2D_2$, **10a**, and 24-epi-1α,25-$(OH)_2D_2$, **10b**. Separation by HPLC yielded both epimers in pure form. They were found identical in all respects with those previously obtained.

It is evident that this alternative method of synthesis of 1α,25-$(OH)_2D_2$ also represents a convenient route to the radioactive compound. Advantageously, the introduction of the labeled fragment of the side chain might be performed at the last step of the synthesis by treatment of the keto intermediate, **21**, with an isotopically labeled Grignard reagent, e.g., C^3H_3MgBr or $^{14}CH_3MgBr$.

RECEPTOR BINDING STUDY

The noncovalent binding of 1α,25-$(OH)_2D_3$ to an intracellular receptor protein in target tissues is a key step in the mechanism of action of the hormone (*14*). The eight compounds prepared in this study provided an opportunity to investigate the effect of side chain methyl orientation and of ring-A and 1,3-diol-stereochemistry on binding affinity for the receptor protein, and all eight compounds were therefore subjected to a competitive binding analysis. The results, shown in Fig. 1,

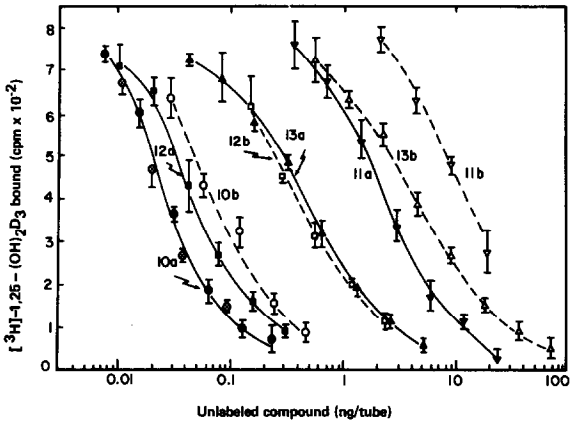


FIG. 1. Displacement of 1,25-$(OH)_2$[26,27-^{3}H]D_3 from chick intestinal cytosol receptor protein for 1,25-$(OH)_2D_3$ by 1α,25-$(OH)_2D_2$ (**10a**) and related synthetic isomers. Chick intestinal cytosol was incubated with 1,25-$(OH)_2$[26,27-^{3}H]D_3 and various concentrations of unlabeled compounds (**10a/b, 11a/b, 12a/b, 13a/b**) dissolved in 50 μl of 95% ethanol at 6°C for 18 h as described by Shepard *et al.* (*18*). Each point represents the mean value of triplicate determinations with standard deviation of the mean. Points (×) represent (24*S*)-1,25-$(OH)_2D_2$ biologically produced *in vitro* from (24*S*)-25-OH-D_2 as described by Jones *et al.* (*7*).

establish, not surprisingly, that 1α,25-$(OH)_2D_2$, compound **10a**, featuring the natural 24*S*-methyl and 1α-hydroxy stereochemistry, exhibits highest binding affinity; **10a** is, in fact, equivalent to the natural hormone, 1α,25-$(OH)_2D_3$, in receptor binding, indicating that the protein binding site can accommodate the moderate steric bulk of the 24-methyl group. Inversion of C-24-methyl stereochemistry (compounds **10b**) and *cis* to *trans* isomerization of the 5,6-double double bond (which, formally, is equivalent to a transposition of the exocyclic methylene group from the right to the left side of ring A; cf., **10a** vs **12a**), has only a small effect, reducing binding by about five- and twofold, respectively. Both changes together, however (as in the 5,6-*trans*-24*R*-compound, **12b**) diminish binding by a factor of ca. 100. Inversion of the C-1-hydroxy stereochemistry from 1α to 1β leads to a pronounced attenuation of binding affinity (ca. 1000-fold reduction; see compound **11a**, in Fig. 1), and the additional epimerization of the C-24-methyl group from the *S* to *R* (as in compound **11b**) reduces binding by ca. another order of magnitude. This result confirms the key role of the 1-hydroxy group in the binding interaction; the effect was not unexpected since 1β,25-$(OH)_2D_3$ and its 25-deoxy analog, 1β-OH-D_3, show a similarly dramatic loss of affinity for the receptor compared to their respective 1α-hydroxy isomers (*12, 15*). Another illustration of the importance of the 1α-hydroxy function is provided by the comparison of the relative binding affinities of the 1β-hydroxy analog, **11a**, and its 5,6-*trans*-isomer **13a** (see Fig. 1). The substantially improved binding of the latter is explained by the fact that **13a** does have the required "1α-hydroxy" group (i.e., the original 3β-hydroxyl, by virtue of double bond isomerization, occupies the position of a 1α-hydroxy group). The same relationship holds for the 24*R* pair, **11b** and **13b**, and these compounds show an analogous pattern of relative binding potency.

The finding that the exact stereochemistry of the 24-methyl group has but a modest influence on binding affinity (see **10a** vs. **10b** in Fig. 1) is noteworthy since it suggests that the side chain binding pocket of the protein is not overly demanding with respect to the exact structure of the ligand (unlike the ring A–1α-hydroxy binding region). This result is in accord with previous data, which show, for example, that analogs possessing either C-24- or C-25-hydroxy groups exhibit essentially equal affinity for the receptor (*16*). From a more practical point of view, one may speculate that some of these 1,25-$(OH)_2D_2$ analogs, by virtue of their high receptor binding potency, may have considerable therapeutic potential.

EXPERIMENTAL PROCEDURES

General. NMR spectra were taken on a Bruker WH-270 FT spectrometer ($CDCl_3$ solution, tetramethylsilane as internal standard). Ultraviolet (UV) spectra were recorded in ethanol with an Hitachi Model 100-60 spectrophotometer. Electron impact mass spectra (MS) were obtained at 110–120°C above ambient temperature, at 70 eV, with an AEI MS-9 mass spectrometer. High-performance liquid chromatography (HPLC) was performed on a Waters Associates Model ALC/GPC 204 (Milford, Mass.) using the following Zorbax-SIL (Dupont, Wilmington, Pa.) columns: 4.6 mm × 25 cm (2 ml/min, 800 psi), 6.2 mm × 25 cm

(4 ml/min, 1500 psi), and 9.6 mm × 25 cm (4 ml/min, 1000 psi). All reactions were performed under an argon atmosphere.

Preparation of 24R/S-1,25-dihydroxy-3,5-cyclovitamin D_2 (compounds 4 and 5)

Freshly recrystallized *p*-toluenesulfonyl chloride (50 mg) was added to a solution of 33 mg of (24*R/S*)-25-hydroxyvitamin D_2 **1** (*8*), in dry pyridine (300 μl). The reaction mixture was allowed to stand for 30 h at 4°C, poured into ice/saturated $NaHCO_3$ with stirring, and extracted with benzene. The combined extracts were washed with $NaHCO_3$, water, aqueous $CuSO_4$ solution, and water, and then dried over $MgSO_4$. Removal of solvent under reduced pressure gave a crude tosylate, **2**, which can be used directly for the next reaction.

A mixture of product, **2**, and $NaHCO_3$ (150 mg) in 10 ml of anhydrous methanol was heated at 55°C for 8.5 h with stirring, concentrated to ca. 2 ml, and diluted with 80 ml of benzene. The benzene solution was washed with water, dried over $MgSO_4$, and evaporated. The oily product, **3**, was sufficiently pure for the following oxidation step.

To a stirred suspension of 4 mg of SeO_2 in 5 ml of dry CH_2Cl_2 was added 13.2 μl of *t*-BuOOH. After 0.5 h, 40 μl of anhydrous pyridine was added and the mixture was stirred at room temperature until homogeneous. Dichloromethane (3 ml) was then added, the solution was cooled to 0°C, and the cyclovitamin product, **3**, was added in 4.5 ml of CH_2Cl_2. After 15 min, the reaction was allowed to warm slowly to room temperature, and continued until almost all starting material was consumed (ca. 30 min). The mixture was transferred to a separating funnel and shaken with 30 ml of 10% NaOH. Ether (150 ml) was added and the phases were separated. The etheral phase was washed with 10% NaOH, water, dried over $MgSO_4$ and evaporated *in vacuo*. The oily residue was purified by preparative TLC (developed with 6:4 *n*-hexane:ethyl acetate). The isolated product (12 mg) represents a mixture containing 1α-hydroxycyclovitamin, **4**, and a small amount of the corresponding 1β-hydroxy derivative, **5**, and is characterized by the following physical data: mass spectrum, *m/e* 442 (M^+, 13), 424 (8), 410 (9), 392 (26), 352 (15), 269 (27), 135 (88), 59 (100); NMR ($CDCl_3$) δ 0.55 (3H, s, 18-H_3), 0.63 (1H, m, 3-H), 1.00 (3H, d, J = 6.5 Hz, 28-H_3), 1.05 (3H, d, J = 6.5 Hz, 21-H_3), 1.13 and 1.18 (6H, each s, 26-H_3 and 27-H_3), 3.26 (3H, s, 6-OCH_3), 4.19 (1H, d, J = 9.5 Hz, 6-H), ~4.2 (1H, m, 1-H), 4.95 (1H, d, J = 9.5 Hz, 7-H), 5.17 and 5.24 (2H, each m, 19-H_2), 5.35 (2H, broad m, 22-H and 23-H).

*Preparation of 1,25-Dihydroxyvitamin D_2 Compounds **10a/b, 11a/b, 12a/b, and 13a,b***

(*a*) *Cycloreversion of 1-hydroxycyclovitamins **4** and **5**.* A solution containing a mixture of 1-hydroxycyclovitamins **4** and **5** (6 mg) in glacial acetic acid (0.5 ml) was heated at 55°C for 15 min, cooled, and poured carefully over ice-saturated $NaHCO_3$. The mixture was extracted with benzene and ether, and the combined extracts were washed with saturated $NaHCO_3$ and water. The residue was chromatographed on a HPLC column (6.2 mm × 25 cm Zorbax-Sil) using 3% of 2-propanol in hexane as eluant. Chromatography yielded a fraction (1.4 mg) contain-

ing 1α,25-dihydroxyvitamin D_2 3-acetate (**6a**) and a lesser amount of both 24-epimers of the corresponding 1β,25-dihydroxy derivative, **7** (peak collected at 90 ml), as well as a fraction (2.5 mg) containing (24*R*)-1α,25-dihydroxyvitamin D_2 3-acetate, **6b**, and both 24-epimers of the 5,6-*trans*-1α,25-dihydroxyvitamin, **8** (peak collected at 97 ml; 1 : 1 ratio of **6b** and **8** was established by NMR).

Mixture of **6a** and **7**: UV (EtOH) λ_{max} 265 nm, λ_{min} 227.5 nm; NMR ($CDCl_3$), δ 6.02 and 6.34 (2H, each d, J = 11 Hz, 7-H and 6-H from **6a**).

Mixture of **6b** and **8**: UV (EtOH) λ_{max} 269 nm, λ_{min} 227.5 nm; NMR ($CDCl_3$), δ 6.01 and 6.34 (2H, each d, J = 11 Hz, 7-H and 6-H from **6b**), 5.81 and 6.57 (2H, each d, J = 11.5 Hz, 7-H and 6-H from **8**).

(*b*) *Hydrolysis of 3β-acetoxy group*. A solution of the mixture containing **6a** and **7** (1.1 mg) as obtained above in 10% methanolic NaOH (1 ml) was heated at 55°C for 1 h, and then poured into the water and extracted with benzene, ether, and methylene chloride. The organic extracts were washed with water, dried, combined, and evaporated. HPLC of the residue (10% 2-propanol : hexane, 6.2 × 25 cm Zorbax-Sil column) afforded a mixture of 24-epimers of 1β,25-dihydroxyvitamin D_2, **11** (0.15 mg, peak collected at 52 ml), and pure 1α,25-dihydroxyvitamin D_2, **10a** (0.6 mg, peak collected at 59 ml).

Mixture of **11a** and **11b**: UV (EtOH) λ_{max} 263 nm, λ_{min} 227 nm; NMR, ($CDCl_3$), δ 0.57, (3H, s, 18-H_3), 1.00 (3H, d, J = 6.5 Hz, 28-H_3), 1.04 (3H, d, J = 6.5 Hz, 21-H_3), 1.14 and 1.18 (6H, each s, 26-H_3 and 27-H_3), 4.12 (1H, m, 3-H), 4.38 (1H, m, 1-H), 5.01 (1H, narrow m, 19-H), 5.34 (3H, broad m, 19-H, 22-H and 23-H), 6.06 (1H, d, J =11.5 Hz, 7-H), 6.45 (1H, d, J = 11.5 Hz, 6-H).

Rechromatography of **11** was performed on HPLC (4.6 mm × 25 cm Zorbax-Sil column using 4% 2-propanol in hexane as an eluant). Pure compounds **11a** and **11b** were collected at 90 and 97 ml.

By analogous treatment of the mixture containing **6b** and **8** (2.4 mg) as obtained above with NaOH, a mixture of (24*R*)-1α,25-dihydroxyvitamin D_2, **10b**, and 24-epimers of 5,6-*trans*-1α,25-dihydroxyvitamin, **12** (1.7 mg, 1 : 1 ratio of **10b** and **12**) was obtained.

Mixture of **10b** and **12**: UV (EtOH) λ_{max} 270.5 nm, λ_{min} 228 nm; NMR ($CDCl_3$) 6.02 and 6.39 (2H, each d, J = 11 Hz, 7-H and 6-H from **10b**), 5.89 and 6.59 (2H, each d, J = 11.5 Hz, 7-H and 6-H from **12**).

Separation of the isomers was achieved on HPLC (4.6 × 25 cm Zorbax-Sil column, 6% 2-propanol : hexane). The chromatographic peaks for (24*S*)-5,6-*trans*-1α,25-dihydroxyvitamin D_2 (**12a**), (24*R*)-1α,25-dihydroxyvitamin D_2 (**10b**), and (24*R*)-5,6-*trans*-1α,25-dihydroxyvitamin D_2 (**12b**) partially overlapped (57, 59, and 62 ml, respectively) but recycling afforded pure compounds **10b, 12a,** and **12b**.

(*c*) *cis to trans Isomerization*. The 5,6-*cis* form of the 1,25-dihydroxyvitamin D compounds thus obtained were converted to the *trans* compounds by treatment with iodine. Thus, treatment of compound **10a** in ether (0.001% solution) with a catalytic amount of iodine (2% of the amount of **10a**), while keeping the solution under diffuse daylight for 1 h, results in *cis* to *trans* isomerization, and after HPLC purification (Zorbax-Sil column, 4.6 × 25 cm; 6% 2-propanol : hexane), the 5,6-*trans* isomer, **12a**, was obtained. Under like conditions, compound **10b** is isomerized to **12b**. Similarly, compound **11a**, upon treatment with iodine under the above

conditions, provided a mixture of the 5,6-*cis* and 5,6-*trans* isomers (**11a, 13a**), which, when separated by HPLC (Zorbax-Sil, 9.6 × 25 cm, 10% 2-propanol/hexane), gave **13a** in pure form, and treatment of compound **11b** under the same conditions gave the 5,6-*trans* compound, **13b**.

1α,25-Dihydroxyvitamin D_2 (**10a**): UV (EtOH) λ_{max} 265.5 nm, λ_{min} 227.5 nm; mass spectrum, *m/e* 428 (M^+, 6), 410 (4), 352 (4), 287 (6), 269 (10), 251 (10), 152 (42), 134 (100), 59 (99); NMR ($CDCl_3$) δ 0.56 (3H, s, 18-H_3), 1.01 (3H, d, J = 6.5 Hz, 28-H_3), 1.04 (3H, d, J = 6.5 Hz, 21-H_3), 1.14 and 1.18 (6H, each s, 26-H_3 and 27-H_3), 4.24 (1H, m, 3-H), 4.43 (1H, m, 1-H), 5.01 (1H, narrow m, 19-H), 5.34 (3H, broad m, 19-H, 22-H and 23-H), 6.02 (1H, d, J = 11 Hz, 7-H), 6.39 (1H, d, J = 11 Hz, 6-H).

1α,25-Dihydroxy-24-epivitamin D_2 (**10b**): UV (EtOH) λ_{max} 265.5 nm, λ_{min} 227.5 nm; mass spectrum, *m/e* 428 (M^+, 13), 410 (9), 352 (7), 287 (11), 269 (15), 251 (13), 152 (52), 134 (100), 59 (97).

1β,25-Dihydroxyvitamin D_2 (**11a**): UV (EtOH) λ_{max} 263.5 nm, λ_{min} 227 nm; mass spectrum, *m/e* 428 (M^+, 9), 410 (27), 392 (12), 352 (8), 334 (7), 269 (12), 251 (15), 152 (48), 135 (68), 134 (53), 59 (100).

1β,25-Dihydroxy-24-epivitamin D_2 (**11b**): UV (EtOH) λ_{max} 263.5 nm, λ_{min} 227 nm; mass spectrum, *m/e* 428 (M^+, 10), 410 (29), 392 (13), 352 (9), 334 (7), 269 (13), 251 (16), 152 (58), 135 (76), 134 (59), 59 (100).

5,6-*trans*-1α,25-Dihydroxyvitamin D_2 (**12a**): UV (EtOH) λ_{max} 273.5 nm, λ_{min} 230 nm; mass spectrum, *m/e* 428 (M^+, 8), 410 (3), 287 (3), 269 (7), 251 (7), 152 (34), 134 (100), 59 (78).

5,6-*trans*-1α,25-Dihydroxy-24-epivitamin D_2 (**12b**): UV (EtOH) λ_{max} 273.5 nm, λ_{min} 230 nm; mass spectrum, *m/e* 428 (M^+, 10), 410 (4), 352 (4), 287 (5), 269 (9), 251 (8), 152 (37), 134 (100), 59 (82).

5,6-*trans*-1β,25-Dihydroxyvitamin D_2 (**13a**): UV (EtOH) λ_{max} 270 nm; λ_{min} 229.5 nm; mass spectrum, *m/e* 428 (11), 410 (5), 351 (4), 287 (4), 269 (12), 251 (11), 152 (67), 135 (100), 134 (75), 59 (72).

5,6-*trans*-1β,25-Dihydroxy-24-epivitamin D_2 (**13b**): UV (EtOH) λ_{max} 270 nm, λ_{min} 229.5 nm; mass spectrum, *m/e* 428 (M^+, 11), 410 (4), 351 (4), 287 (4), 269 (13), 251 (12), 152 (64), 135 (100), 134 (70), 59 (63).

*Conversion of 1α,25-Dihydroxyvitamin D_2 (**10a, b**) to 1β,25-Dihydroxyvitamin D_2 Analogs (**11a, b**)*

A solution of 1α,25-$(OH)_2D_2$ **10a** (40 μg) in anhydrous ether (1 ml) was treated with activated MnO_2 (6 mg) for 5 h at room temperature. The mixture was filtered (Celite) and evaporated, and the residue was chromatographed by HPLC (6.2 mm × 25 cm Zorbax-Sil) using 10% iPrOH : hexane mixture as an eluant. 1-oxo-Previtamin, **14a** [UV (EtOH) λ_{max} 233.5, 297 nm] was collected at 26 ml. Compound **14a** was then reduced with $LiALH_4$ in anhydrous ether (−23°C, 40 min). Excess reagent was decomposed with water, anhydrous $MgSO_4$ was added, and inorganic material was removed by filtration. The solvent was removed under vacuum, and the organic residue was dissolved in EtOH and refluxed for 2.5 h under argon atmosphere. Products were separated by HPLC (9.4 mm × 25 cm Zorbax-Sil

column) using 15% iPrOH : hexane mixture. Pure 1β,25-$(OH)_2D_2$ (**11a**) was collected at 55 ml and 1α,25-$(OH)_2D_2$ (**10a**) at 63 ml (3 : 1 ratio of **11a** and **10a**).

The analogous reaction sequence, performed with 1α,25-dihydroxy-24-epivitamin D_2 (**10b**), resulted in the formation of 1β,25-dihydroxy-24-epivitamin D_2 (**11b**).

25,25-*Ethylenedioxy*-1α-*hydroxy-3,5-cyclovitamin* ***18.*** Compound **15** (50 mg) was converted to **18** in a manner analogous to that described for 25-hydroxyvitamin **1**. The crude cyclovitamin **17** resulting from methanolysis of the tosyl derivative, **16**, was oxidized with SeO_2 (5.05 mg) and *t*-BuOOH (16.5 μl) in CH_2Cl_2 (12.5 ml) containing pyridine (50 μl). Work-up of the reaction mixture and preparative TLC (20 × 20-cm plates; AcOEt : hexane 4 : 6) yielded 20 mg of 1α-hydroxy derivative, **18**: mass spectrum, *m/e* 470 (M^+, 5), 428 (20), 87 (100); NMR ($CDCl_3$) δ 0.53 (3H, s, 18-H_3), 0.63 (1H, m, 3-H), 1.24 (3H, s, 26-H_3), 3.26 (3H, s, 6-OCH_3), 3.59 (4H, m, -OCH_2CH_2O-), 4.19 (1H, d, J = 9.5 Hz, 6-H), 4.2 (1H, m, 1-H), 4.95 (1H, d, J = 9.5 Hz, 7-H), 5.17 and 5.25 (2H, each m, 19-H_2), 5.35 (2H, m, 22-H and 23-H).

Acetylation of compound ***18.*** A solution of cyclovitamin **18** (18 mg) in pyridine (1 ml) and acetic anhydride (0.33 ml) was heated at 55°C for 2 h. The mixture was poured into ice-cold saturated $NaHCO_3$ and extracted with benzene and ether. The combined organic extracts were washed with water, saturated $CuSO_4$, and aqueous $NaHCO_3$ solutions, dried, and evaporated to give 1-acetoxy derivative, **19** (19 mg): mass spectrum, *m/e*: 512 (M^+, 5), 420 (5), 87 (100); NMR ($CDCl_3$) δ 0.53 (3H, s, 18-H_3), 1.23 (3H, s, 26-H_3), 2.10 (3H, s, 1-$OCOCH_3$), 3.25 (3H, s, 6-OCH_3), 3.94 (4H, m, -OCH_2CH_2O-), 4.18 (1H, d, J = 9.5 Hz, 6-H), 4.97 (2H, m, 7-H and 19-H), 5.24 (2H, m, 1-H and 19-H), 5.35 (2H, m, 22-H and 23-H).

Solvolysis of ***19*** *to* ***20.*** A solution of cyclovitamin **19** (4.5 mg) in 3 : 1 mixture of dioxane/H_2O (1.5 ml) was heated at 55°C. *p*-Toluenesulfonic acid (1 mg in 20 μl of H_2O) was then added and heating was continued for 15 min. The mixture was poured into saturated $NaHCO_3$ on ice, and extracted with benzene and ether. The organic phases were washed with $NaHCO_3$ and water and dried over $MgSO_4$. Evaporation of solvents gave a residue which was chromatographed on HPLC (6.2 mm × 25 cm Zorbax-Sil) using 2% 2-propanol in hexane as solvent and eluant.

Product **20**, eluting at 117 and 119 ml (mixture of epimers) was contaminated with a 5,6-*trans* isomer but rechromatography afforded 1.2 mg of the pure 1α-acetoxy compound, **20:** UV (EtOH) λ_{max} 266 nm, 250 nm, λ_{min} 224.5 nm; mass spectrum, *m/e* 498 (M^+, 7), 134 (11), 87 (100); NMR δ 0.52 (3H, s, 18-H_3), 1.23 (3H, s, 26-H_3), 2.03 (3H, s, 1-$OCOCH_3$), 3.95 (4H, m, -OCH_2CH_2O-), 4.19 (1H, m, 3-H), 5.03 (1H, narrow m, 19-H), 5.33 (3H, m, 19-H, 22-H and 23-H), 5.49 (1H, m, 1-H), 5.92 (1H, d, J = 11 Hz, 7-H), 6.37 (1H, d, J = 11 Hz, 6-H).

Hydrolysis of Compound ***20*** *to ketone* ***21***

To the solution of ketal **20** (1.35 mg) in ethanol (1.5 ml), *p*-toluenesulfonic acid (0.34 mg in 45 μL of H_2) was added and the mixture was heated under reflux for 30 min. The reaction mixture was poured into dilute $NaHCO_3$ solution and extracted with benzene and ether. The combined organic extract was washed in water, dried

over $MgSO_4$ and evaporated. High-pressure liquid chromatography of the crude mixture (4% 2-propanol : hexane; 6.2 mm × 25 cm Zorbax-Sil) afforded some unreacted ketal, **20** (0.12 mg, collected at 48 ml) and desired ketone, **21** (0.36 mg, collected at 52 ml), characterized by the following data: UV (EtOH) λ_{max} 266 nm, 250 nm, λ_{min} 225 nm; mass spectrum, *m/e*: 454 (M^+, 9), 394 (17), 376 (10), 134 (23), 43 (100); NMR ($CDCl_3$) δ 0.53 (3H, s, 18-H_3), 1.03 (3H, d, J = 6.5 Hz, 21-H), 1.13 (3H, d, J = 7.0 Hz, 28-H_3), 2.03 (3H, s, 1-$OCOCH_3$), 2.12 (3H, s, 26-H_3), 4.19 (1H, m, 3-H), 5.03 (1H, narrow m, 19-H), 5.33 (3H, broad m, 19-H, 22-H and 23-H), 5.49 (1H, m, 1-H), 5.93 (1H, d, J = 11 Hz, 7-H), 6.37 (1H, d, J = 11 Hz, 6-H).

*Reaction of ketone **21** with methylmagnesium bromide.* Ketone **21** in anhydrous ether was treated with excess CH_3MgBr (2.85 M solution in ether). The reaction mixture was stirred at room temperature for 30 min, quenched with aqueous NH_4Cl, and extracted with benzene, ether, and CH_2Cl_2. The organic phases were washed with dilute $NaHCO_3$, dried over $MgSO_4$, and evaporated. The mixture of **10a** and **10b** thus obtained was separated by HPLC (6% 2-propanol : hexane; 4.6 mm × 25 cm Zorbax-Sil) to yield pure epimers **10a** and **10b** collected at 54 and 59 ml.

Receptor Binding of Analogs

Day-old chicks were obtained from Northern Hatcheries (Beaver Dam, Wisc.) and fed a rachitogenic diet for 4 weeks (*17*). They were killed by cervical dislocation and whole duodenal mucosal extracts were prepared as described previously. The extracts were incubated at 6°C with 1,25-$(OH)_2$[26,27-^{3}H]D_3 (160 Ci/mmol) and various amounts of unlabeled compounds to be tested for 18 h until equilibrium was reached. Free or unbound 1,25-$(OH)_2$[26,27-^{3}H]D_3 was removed with dextran-coated charcoal, and specific binding of the 1,25-$(OH)_2$[26,27-^{3}H]D_3 was calculated as previously described (*18*). The resulting competitive binding curves, showing displacement of [^{3}H]-1,25-$(OH)_2D_3$ from the receptor protein as a function of the concentration of analog, are plotted in Fig. 1.

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