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Ring A-Stereoisomers of 1-Hydroxyvitamin D_3 and Their Relative Binding Affinities for the Intestinal 1α,25-Dihydroxyvitamin D_3 Receptor Protein

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A set of eight 1-hydroxyvitamin D_3 compounds comprising the four possible (5*Z*)-1,3-diol stereoisomers and the corresponding (5*E*)-double bond isomers, has been prepared in order to assess the effect of 1,3-diol stereochemistry and 5,6-double bond geometry on binding affinity for the intestinal 1,25-$(OH)_2D_3$-receptor protein. The compounds were synthesized from either vitamin D_3 or 3-epivitamin D_3 via 3,5-cyclovitamin D intermediates. Competitive receptor binding assays establish that all changes from the natural ring A-configuration (1*S*, 3*R*, 5*Z*) lead to decreased binding affinity, and confirm the importance of the 1-hydroxy function since the conversion of stereochemistry at that center from 1α(*S*) to 1β(*R*) has the most pronounced effect on binding affinity (attenuation by more than three orders of magnitude). Other modifications (i.e., conversion at C-3, or *cis* to *trans* isomerization of the 5,6-double bond) decrease binding affinity by more moderate (ca. 10-fold) but cumulative factors.

The expression of "vitamin D" activity in the intestine (i.e., stimulation of intestinal calcium transport) is believed to be mediated, at least in part, by a classical steroid–hormone-type mechanism in which the active metabolite, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), binds with high affinity and specificity to an intracellular receptor protein present in low concentrations (*1–3*). The formation of this protein–ligand complex then initiates the transcription of genetic information in the nucleus to produce the functional components of the intestinal calcium transport system. In addition to its natural ligand, the 1,25-$(OH)_2D_3$-receptor protein also binds structurally related vitamin D metabolites and analogs, and competitive binding studies (*4*) with a variety of analogs have defined at least the gross structural requirements for effective binding of the ligand. In general, any significant departure from the seco-steroid carbon skeleton and 1,3,25-hydroxy substitution pattern embodied by 1,25-$(OH)_2D_3$ results in substantial reduction of binding affinity. All three hydroxy groups contribute to hormone–receptor binding, but those at C-1 and C-25 are known to be most important, since their deletion attenuates binding by two to three orders of magnitude, whereas the removal of the C-3-

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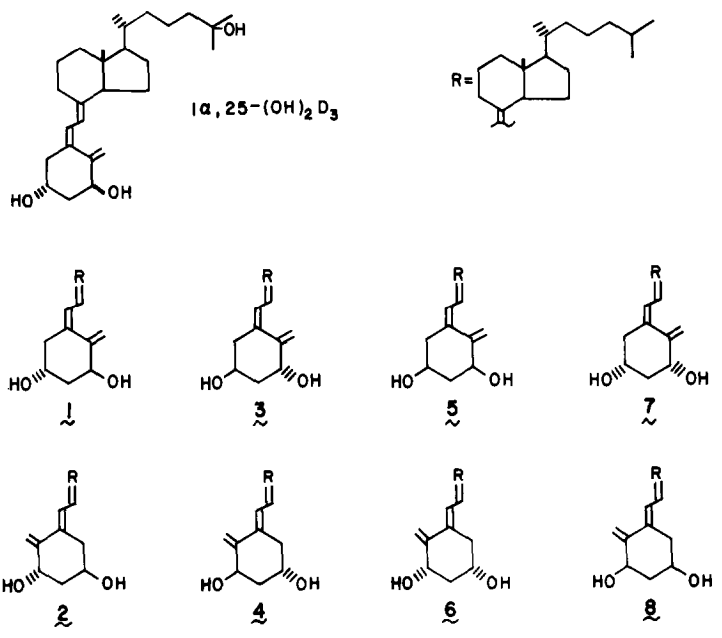
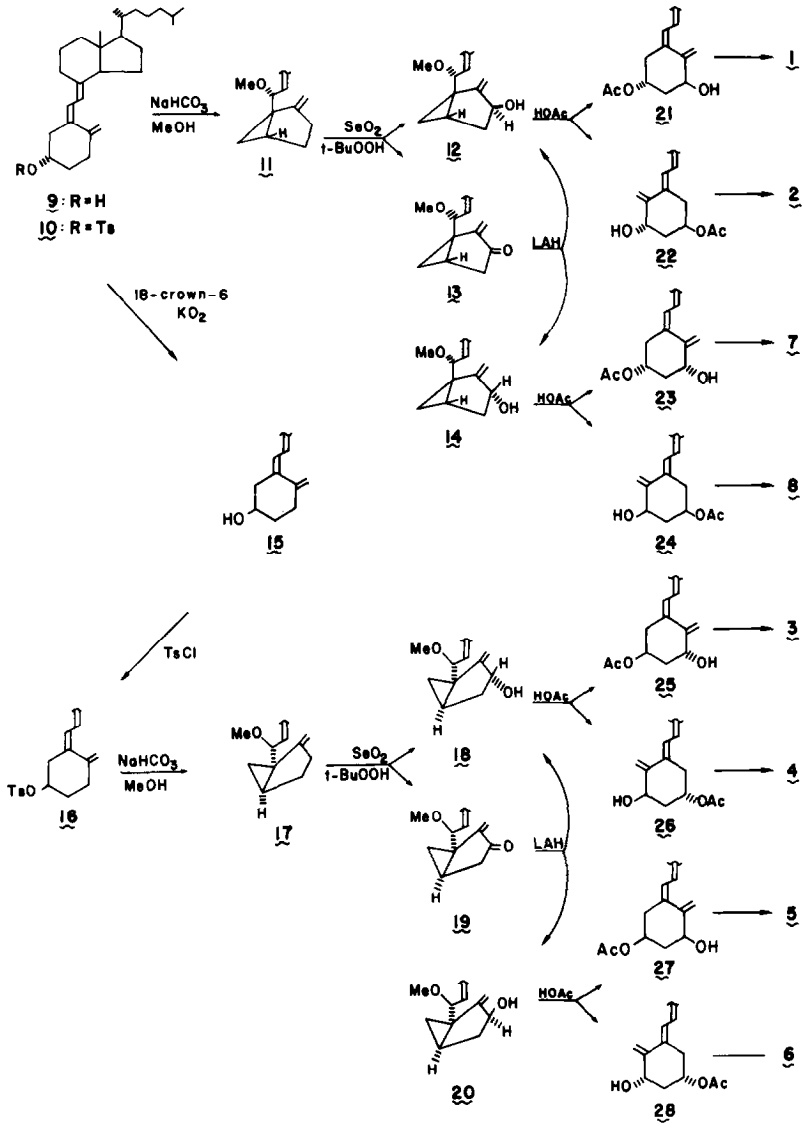


SCHEME I.

hydroxy group results in a more modest (ca. 10-fold) reduction in binding affinity (*4*). Given the evident key role of the 1α-hydroxy function, it was of interest to explore in more detail the extent to which ring A stereochemical modification influences binding affinity. To this end, we prepared a set of eight compounds, comprising the four possible 1,3-diol stereoisomers (**1–4**) (Scheme I), as well as their corrresponding 5,6-*trans* isomers (**5–8**), which, in a purely formal sense, can be considered as the analogs of compounds **1** to **4** in which the exocyclic methylene is transposed from the right to the left side of ring A. The relative binding affinity of these eight isomers for the 1,25-$(OH)_2D_3$-receptor protein was then determined by standard competitive binding assays to gauge the consequences of each of the three structural modifications—1α vs 1β and 3α vs 3β-hydroxy stereochemistry, and *cis* vs *trans* 5,6-double bond orientation.

SYNTHESIS

A preparative route to all eight cases from vitamin D_3 is outlined in Scheme II. Vitamin D_3, **9**, was tosylated to **10** (TsCl, pyr, 5°C) and solvolyzed (CH_3OH, $NaHCO_3$, reflux, 6.0 hr) to yield the cyclovitamin derivative **11** in 75% yield (*5*). Treatment of the cyclovitamin with 0.5 eq SeO_2 and 2.0 eq *t*-BuOOH (*6*), in CH_2Cl_2, at 10°C for 45 min afforded the 1(*S*)-hydroxycyclovitamin **12** (55% yield) along with a 20% yield of the 1-oxo analog **13** (*7*). The stereoselectivity of the oxidation can be attributed to the angular orientation of the cyclopropyl ring of the [3.1.0] bicyclic A-ring and the 6*R* configuration of the 6-methoxyl functionality,



SCHEME II.

both of which direct oxidant approach to the least hindered side of the bicyclic ring system. Reduction of ketone **13** ($LiAlH_4$, inverse addition, 13°C) produced a mixture of the alcohols **12** and **14** (1 : 5 ratio), that were separated on HPLC to obtain pure 1(*R*) epimer **14.**

The conformational flexibility of the A-ring of vitamin D_3 was exploited to extend the above C(1)-hydroxylation scheme to epi-vitamin D_3 (**15**),[3] which was

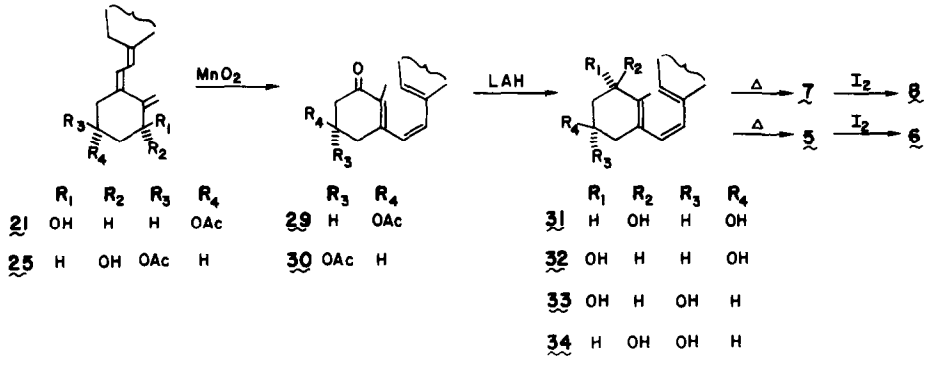
[3] Although spectrally indistinguishable (i.e. MS, NMR, uv) from vitamin D_3, the epivitamin was found to produce only one thermal isomer when subjected to gas chromatography, whereas vitamin D_3 produces both the pyro and isopyro isomers.

obtained in 40% yield by reacting vitamin D_3 tosylate (**10**) with KO_2 in DMSO in the presence of 18-crown-6 (*8*). Tosylation and buffered solvolysis of **15** produced the epi-cyclovitamin **17** in 70% yield. Oxidation with SeO_2 and *t*-BuOOH gave the expected 1(*R*)-hydroxy-epi-cyclovitamin **18** and the corresponding 1-oxo derivative **19** in 45 and 25% yields, respectively. Reduction of the ketone ($LiAlH_4$, ether) gave a mixture containing **18** and **20** in a ratio of 1 : 2, from which the desired 1(*R*) epimer **20** was purified by HPLC.

Solvolysis of the four 1-hydroxycyclovitamin D_3 isomers **12, 14, 18,** and **20** in glacial acetic acid (55°C, 15 min) (*7*) led in each case to the (5*Z*) and (5*E*) pair of the vitamin D_3-diol 3-mono-acetates, namely, **21/22, 23/24, 25/26,** and **27** and **28** (see Scheme I). These *cis* and *trans* pairs were separated by HPLC and hydrolytic or reductive removal of the acetate then furnished the set of eight isomers **1–8.**

An alternative approach to the eight-isomer set involves preparation of the four 1,3-*cis* diols, **5–8,** from the 3-monoacetates corresponding to 1,3-*trans* diols **1** and **3.** Thus, as shown in Scheme III, oxidation of 3-acetate **21** with 10 eq of activated MnO_2 in CH_2Cl_3 produced in about 70% yield the previtamin ketone analog **29** (*9*). Subsequent reduction of **29** with $LiALH_4$ at 0°C gave a 3 : 1 mixture of *cis* : *trans*-1,3-diol previtamins, **31** and **32,** which were easily separated by HPLC. The stereoselectivity observed in this reduction can be accounted for by assuming that metal atom coordination with the hydroxy-ketone yields a conformationally fixed bidentate ligand that forces metal-hydride attack from the less-hindered side of the pre-vitamin A-ring (*10*). Thermal isomerization (80°C, 3.0 hr, EtOH) of **31** yielded the 1,3-*cis*-diol vitamin derivative **7** and from **7,** in turn, the corresponding (5*E*) isomer **8** was obtained by I_2-catalyzed *cis/trans* isomerization (*11*). An analogous sequence performed on acetate **25** led first to ketone **30,** then to the diol mixture **33** and **34,** and from **33** to *cis*-diol **5** and its 5(*E*) isomer **6.**

The 1,3-*cis*-diol vitamins **5** and **7** are readily distinguished by mass spectral analysis from their 1,3-*trans*-diol counterparts, **1** and **3.** Characteristic triene/A-ring fragments at *m/z* 152 and *m/z* 134 (*m/z* 152-H_2O) occur in the mass spectra of all four isomers; however, the *trans*-diol isomers show a strong tendency to lose the elements of water from the fragment of *m/z* 152, which results in a 134 : 152 peak intensity ratio always greater than 2 : 1, while the *cis*-diol isomers are less



SCHEME III.

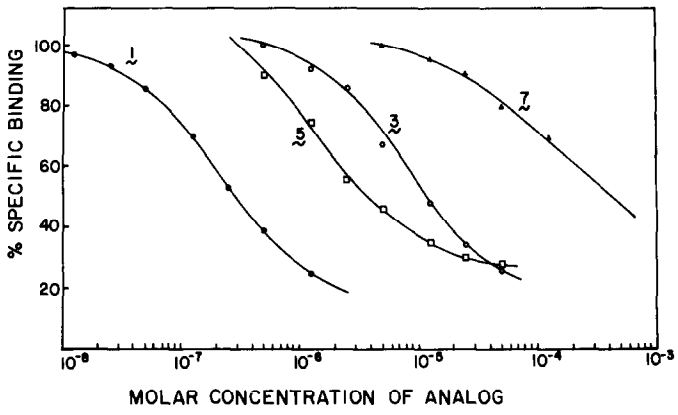


FIG. 1. Competitive binding analysis of the (5*Z*)-1-hydroxyvitamin D_3 analogs **1, 3, 5,** and **7.**

prone to eliminate water and show intensity ratios close to 1 : 1 for the *m/z* 152/134 fragment ions.

RECEPTOR BINDING STUDIES

The efficacy of each analog to compete with 1α,25-dihydroxyvitamin D_3 for the high affinity, ($K_d \sim 10^{-11}$ M) binding site of the cytosolic receptor protein from chick intestine was determined by the standard competitive binding assay, which measures the progressive displacement from the receptor of bound tritiated 1,25-$(OH)_2D_3$ by increasing concentrations of analog. The competitive binding curves thus obtained for each of the analogs are shown in Figs. 1 and 2, and Table 1 summarizes the results in terms of the concentration of each analog required to effect 50% displacement of [^{3}H]-1α,25-dihydroxyvitamin D_3. Normalized to that

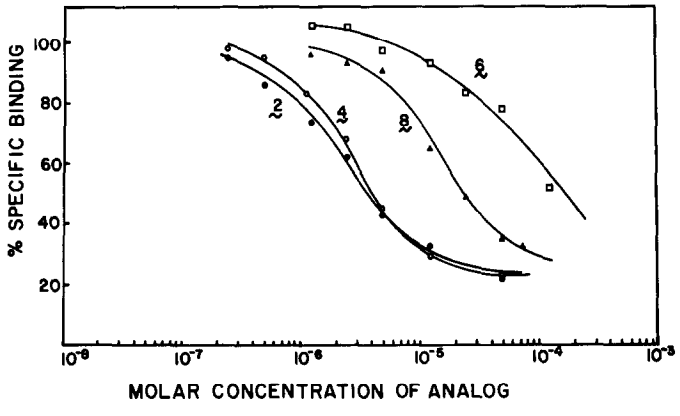


FIG. 2. Competitive binding analysis of the (5*E*)-1-hydroxyvitamin D_3 analogs **2, 4, 6,** and **8.**

TABLE 1

RELATIVE EFFICACY OF 1,3-DIOL STEREOISOMERS **1–8** IN COMPETING WITH 1,25-$(OH)_2$-[26,27-^{3}H]D_3 FOR BINDING TO THE RECEPTOR PROTEIN

Compound	Molar concentration[a] (50% specific binding)	Binding ratio[b] (relative binding affinity)
1	2.8×10^{-7}	1
2	3.6×10^{-6}	13
3	1.2×10^{-5}	43
4	4.0×10^{-6}	15
5	3.8×10^{-6}	14
6	1.7×10^{-4}	610
7	4.0×10^{-4}	1,400
8	2.4×10^{-5}	86

[a] Concentration of analog required to displace 50% of bound 1,25-$(OH)_2$-[26,27-^{3}H]D_3 from receptor protein.

[b] Ratio = [analog]/[**1**].

of the most effective synthetic ligand, 1α-hydroxyvitamin D_3 (compound **1**), these concentration values yield a binding ratio, representing a measure of the relative binding affinity of the analogs for the receptor protein. These binding ratios are listed in column 3 of Table 1, and are plotted for convenient comparison in Fig. 3. The values vary over three orders of magnitude; compound **1**, featuring the ring A stereochemistry of the natural hormone, shows the highest and its C(1)-hydroxy epimer **7** exhibits the lowest binding affinity. The data of Table 1 and Fig. 3 clearly show that, among the ring A substituents, the C(1)-hydroxy functionality is the dominant factor in receptor binding, with the C(3)-hydroxyl group and the 10(19)-exocyclic methylene group being lesser and approximately equal contributors. The single change from C(1)-(*S*) to C(*1*)-(*R*) stereochemistry (**1** → **7**) converts the best to the poorest ligand (1400-fold lower binding affinity). The analogous epimerization of the C(3)-hydroxy group (**1** → **5**) has a much more modest effect (14-fold reduction), roughly equal to that produced by transposition of the exocyclic methylene unit (**1** → **2**, 13-fold). As shown in Fig. 3, the eight isomers can be grouped into two sets of four compounds, depending on the orientation of the "C(1)"-hydroxy group, i.e., the set featuring the natural stereochemistry (compounds **1, 2, 5,** and **8**) and the "C(1)" epimeric set comprising compounds **3, 4, 6,** and **7**. Ranked in order of decreasing binding affinity, it can be seen that in both sets roughly two orders of magnitude separate the best from the worst ligand (1 to 86 for **1** vs **8**, and 15 to 1400 for **4** to **7**) and, given a constant "C(1)"-hydroxy stereochemistry, other ring A-modifications result in an apparently regular pattern of affinity differences in both sets. Thus for the group comprising compounds **1, 2, 5** and **8**, a single change (**1** → **2** or **1** → **5**) lowers binding affinity by roughly one, and a double modification (**1** → **8**) by roughly two orders of magnitude (Fig. 3). A similar order is observed for the set characterized by the inverted "C(1)"-hydroxy stereochemistry (compounds **3, 4, 6,** and **7**). The transposition of the exocyclic methylene, e.g., **4** → **3** or **6** → **7** leads to 2- to 3-fold decrease in binding affinity in

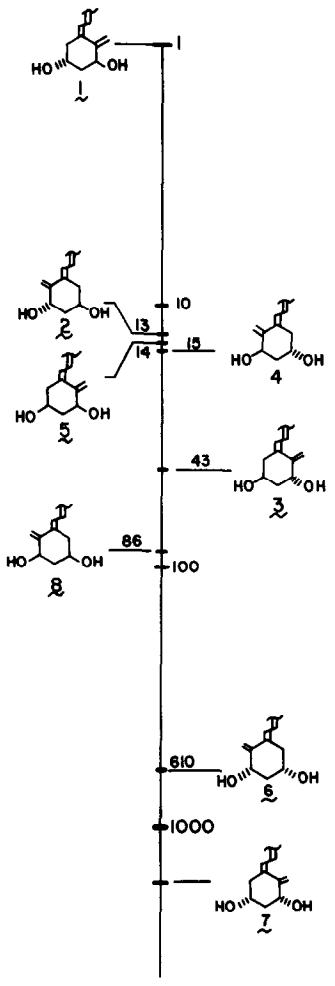


FIG. 3. Plot of the relative binding affinities of the 1,3-diol isomers **1–8**.

both cases, while inversion of the C-3-hydroxy group (**4** → **6** and **3** → **7**) leads to a 30- to 40-fold change (3), and both alterations (**4–7**) result in a ca. 100-fold decrease in binding affinity (Table 1, Fig. 3). In both sets the rank order from best to poorest ligand results from the same type of structural modification: Transposition of the exocyclic methylene group converts the best (compounds **1** and **4**) to the second best ligand (compounds **2** and **3**) of each group, epimerization at C(3) produces the third ranking member of the set (compounds **5** and **6**), and both transformations result in the analogs of lowest affinity (compounds **8** and **7**).

The above results show that all modifications of the natural A-ring stereochemistry as represented by structure **1** lead to substantial decreases in binding affinity for the receptor. The single inversion of the C(1)-hydroxy group has the most profound effect, and it is interesting to note that once that structural change has been made, any further alteration actually improves binding affinity (see pro-

gression from **7** → **6** → **3** → **4**), whereas the analogous transformations in compounds featuring the natural "C(1)" stereochemistry progressively decreases receptor binding (see **1** → **2** → **5** → **8**).

EXPERIMENTAL PROCEDURES

General Methods

UV spectra were taken in absolute ethanol on a Beckman Model 24 spectrophotometer. Mass spectra (EI) were obtained by direct probe introduction, using an MS9 mass spectrometer operated at 70 eV ionization potential. Proton NMR spectra were recorded on samples dissolved in $CDCl_3$, with $CHCl_3$ as an internal standard, using a Bruker WH-270 pulse Fourier transform instrument.

Epivitamin D_3

To 3.0 ml of a 1 M solution of 18-crown-6 in DMSO was added 9.0 mmol of finely divided KO_2, and the mixture was rapidly stirred for 30 min at room temperature. A solution of 1 mmol vitamin D_3 tosylate (**10**) in 2.0 ml benzene was added dropwise and the reaction was continued for an additional 30 min. At the end of this time, saturated NaCl was added dropwise and the quenched reaction was diluted with Et_2O. The organic extracts were washed four times with saturated NaCl, and were taken to dryness *in vacuo*. The crude oil was chromatographed in 15 : 85 ethylacetate : hexane (20 × 20 cm, 750-μm silica gel plate) to yield 40% of (15).

Cyclovitamin Formation

To a stirring solution of 50 ml anhydrous methanol was added 500 mg of finely divided $NaHCO_3$ and 2.0 mmol of the respective vitamin D_3-tosylates (**10** or **16**). The solution was brought to reflux for 6–8 hr, cooled, and concentrated *in vacuo*. The crude reaction was diluted with Et_2O, washed with H_2O, dried over $MgSO_4$, and taken to an oil. Silica gel TLC (20 × 20 cm, 750 μm) in 1 : 9 ethylacetate : hexane gave a 70% yield of the 3,5-cyclovitamin (**11**) or (**17**). (**11**): TLC R_f = 0.50; mass spectrum, *m/e* 398 (M^+, 20), 366 (100), 253 (45), 247 (30), 135 (50), 118 (60); NMR δ 0.53 (3H, s, 18-H_3), 0.73 (1H, m, 3-H), 3.26 (3H, s, 6-OCH_3), 4.17 (1H, d, J = 9.4 Hz, 6-H), 4.88 (1H, m(sharp), 19(Z)-H), 4.99 (1H, d, J = 9.4 Hz, 7-H), 5.05 (1H, m(sharp), 19(E)-H).

(**17**): TLC R_f 0.50; mass spectrum, *m/e* 398 (M^+, 20), 366 (100), 253 (75), 247 (50), 135 (80), 118 (92); NMR δ 0.61 (3H, s, 18-H_3), 0.86 (1H, m, 3-H), 3.27 (3H, s, 6-OCH_3), 4.34 (1H, d, J = 9.2 Hz, 6-H), 4.82 (1H, d, J = 9.2 Hz, 7-H), 4.86 (2H, degenerate m(sharp), 19(Z)-H and 19(E)-H).

SeO_2/t-BuOOH Oxidation of Cyclovitamin D Analogs

To a stirred suspension of 0.1 mmol SeO_2 in 10 ml CH_2Cl_2 was added 0.4 mmol *tert*-butylhydroperoxide (bp 34°C at 25 mm). After stirring for 30 min, the reaction

was diluted with 40 ml CH_2Cl_2 and cooled to 15°C. A solution of 0.2 mmol cyclovitamin D (**11**) or (**17**) in 10 ml CH_2Cl_2 was added, and the reaction was continued for 30–45 min. At the end of this time the mixture was transferred to a separatory funnel, quenched with 10% NaOH, and diluted with Et_2O, and the organic layer was isolated. This phase was washed with 10% NaOH, water, dried over $MgSO_4$, and taken to dryness *in vacuo*. Preparative TLC (20 × 20 cm, 750-μm silica gel) in 3 : 7 ethylacetate : hexane gave 50% of the 1-hydroxycyclovitamin (**12**) or (**18**) and 20% of the corresponding 1-keto-cyclovitamin (**13**) or (**19**).

(**12**): TLC R_f 0.25; mass spectrum, *m/e* 414 (M^+, 30), 382 (75), 341 (45), 269 (20), 247 (55), 135 (65), 69 (100); NMR, δ, 0.53 (3H, s, 18-H_3), 0.61 (1H, m, 3-H), 3.26 (3H, s, 6-OCH_3), 4.19 (1H, d, J = 9.5 Hz, 6-H), 4.26 (1H, m, 1-H), 4.96 (1H, d, J = 9.5 Hz, 7-H), 5.17 (1H, m(sharp), 19(Z)-H), 5.25 (1H, m(sharp), 19(E)-H).

(**13**): TLC R_f 0.50; uv λ_{max} 253 nm; mass spectrum, *m/e* 412 (M^+, 35), 380 (40), 247 (20), 241 (30), 135 (50), 133 (100); NMR, δ, 049 (3H, s, 18-H_3), 0.58 (1H, m, 3-H), 3.30 (3H, s, 6-OCH_3), 4.07 (1H, d, J = 9.2 Hz, 6-H), 5.02 (1 H, d, J = 9.2 Hz, 7-H), 5.62 (1 H, m(sharp), 19(Z)-H), 6.04 (1H, m(sharp), 19(E) H).

(**18**): TLC R_f 0.25; mass spectrum, *m/e* 414 (M^+, 30), 382 (60), 341 (25), 269 (15), 247 (40), 135 (100), 69 (50); NMR δ 0.61 (3H, s, 18-H_3), 0.50 (1H, m, 3-H), 3.27 (3H, s, 6-OCH_3), 4.19 (1H, m, 1-H), 4.34 (1H, d, J = 9.2 Hz, 6-H), 4.79 (1H, d, J = 9.2 Hz, 7-H), 5.06 (1H, m(sharp), 19(Z)-H), 5.13 (1H, m(sharp), 19(E)-H).

(**19**): TLC R_f 0.45; uv λ_{max} 253 nm; mass spectrum, *m/e* 412 (M^+, 20), 380 (25), 247 (20), 241 (10), 135 (45), 133 (100); NMR δ 0.61 (3 H, s, 18-H_3), 0.44 (1 H, m, 3-H), 3.30 (3 H, s, 6-OCH_3), 4.19 (1 H, d, J = 9.2, 6-H), 4.91 (1 H, d, J = 9.2, 7-H), 5.43 (1 H, m(sharp), 19(Z)-H), 5.98 (1H, m(sharp), 19(E)-H).

$LiAlH_4$ Reduction of 1-Keto-cyclovitamins

To 2.5 ml of anhydrous Et_2O at room temperature was added 0.05 mmol of either 1-keto-cyclovitamin (**13**) or (**19**). This rapidly stirring solution was then cooled to 2°C and 0.5 ml of a saturated solution of $LiAlH_4$ in Et_2O was added dropwise. After 10 min the reaction was carefully quenched by the dropwise addition of 2% NaOH, dried over $MgSO_4$, and concentrated to an oil *in vacuo*. Preparative HPLC (7.5 × 300-mm μ-Porosil, 1.2% *i*-propanol : hexanes) yielded the 1-hydroxy epimers (**12**) and (**14**) in a 1 : 5 ratio from (**13**), while (**18**) and (**20**) were produced in a 1 : 2 ratio from the reduction of (**19**).

(**12**): HPLC V_e = 73 ml; for spectral data see preceding experiment.

(**14**): HPLC V_e = 98 ml; mass spectrum, *m/e* 414 (M^+, 20), 382 (45), 341 (20), 269 (10), 247 (30), 135 (100), 69 (60); NMR δ 0.52 (3 H, s, 118-H_3), 0.90 (1 H, m, 3-H), 3.27 (3 H, s, 6-OCH_3), 4.05 (1 H, d, J = 9.2 Hz, 6-H), 4.52 (1 H, m, 1-H), 4.98 (1 H, d, J = 9.2 Hz, 7-H), 5.21 (1 H, m(sharp), 19(Z)-H), 5.33 (1 H, m(sharp), 19(E)-H).

(**18**): HPLC V_e = 68 ml; for spectral data, see preceding experiment.

(**20**): HPLC V_e = 94 ml; mass spectrum, *m/e* 414 (M^+, 25), 382 (50), 341 (30), 269 (15), 247 (40), 135 (100), 69 (60); NMR δ 0.61 (e H, s, 18-H_3), 0.86 (1 H, m, 3-H), 3.28 (3 H, s, 6-OCH_3), 4.25 (1 H, d, J = 9.5 Hz, 6-H), 4.52 (1 H, m, 1-H), 4.81 (1 H, d, J = 9.5 Hz, 7-H), 5.16 (1 H, m(sharp), 19(Z)-H), 5.19 (1 H, m(sharp), 19(E)-H).

Glacial Acetic Acid-Catalyzed Cycloreversion

A solution of 0.1 mmol of a 1-hydroxy cyclovitamin D analog (**12, 14, 18,** or **20**) in 1.0 ml glacial acetic acid was heated to 55°C for 15 min. The reaction mixture was then cooled, carefully quenched with saturated $NaHCO_3$, and extracted with Et_2O. The organic extracts were washed with water, dried over $MgSO_4$, and taken to an oil *in vacuo*. Preparative HPLC (system A: 6.25 × 250-mm Zorbax-Sil, 1.5% *i*-propanol/hexanes; system B: 7.5 × 300-mm μ-Porosil, 2.0% *i*-propanol/hexanes) consistently gave the (5*Z*)- and (5*E*)-3-acetoxy-1-hydroxyvitamin analogs in ~2 : 1 ratios and approx 70% overall yield.

21: HPLC (B) V_e = 53 ml; uv λ_{max} 264 nm; NMR δ 0.54 (3 H, s, 18-H_3), 2.03 (3 H, s, 3-$OCOCH_3$), 4.41 (1 H, m, 1-H), 5.21 (1 H, m, 3-H), 5.02 (1 H, m, 19(Z)-H), 5.34 (1 H, m, 19(E)-H), 6.02 (1 H, d, J = 11.1 Hz, 7-H), 6.34 (1 H, d, J = 11.1 Hz, 6-H).

22: HPLC (B) V_e = 62 ml; uv λ_{max} 270 nm; NMR δ 0.57 (3 H, s, 18-H_3), 2.03 (3 H, s, 3-$OCOCH_3$), 4.49 (1 H, m, 1-H), 5.25 (1 H, m, 3-H), 4.99 (1 H, m, 19(Z)-H), 5.13 (1 H, m, 19(E)-H), 5.81 (1 H, d, J = 11.4, 7-H), 6.57 (1 H, d, J = 11.4, 6-H).

23: HPLC (A) V_e = 36 ml; uv λ_{max} 264 nm; NMR δ 0.55 (3 H, s, 18-H_3), 2.06 (3 H, s, 3-$OCOCH_3$), 4.19 (1 H, m, 1-H), 4.98 (1 H, m, 3-H), 5.00 (1 H, m, 19(Z)-H), 5.36 (1 H, m, 19(E)-H), 6.01 (1 H, d, J = 11.2 Hz, 7-H), 6.37 (1 H, d, J = 11.2 Hz, 6-H).

24: HPLC (A) V_e = 46 ml; uv λ_{max} 271 nm; NMR δ 0.57 (3 H, s, 18-H_3), 2.06 (3 H, s, 3-$OCOCH_3$), 4.24 (1 H, m, 1-H), 4.97 (1 H, m, 3-H), 5.04 (1 H, m, 19(Z)-H), 5.14 (1 H, m, 19(E)-H), 5.80 (1 H, d, J = 11.6 Hz, 7-H), 6.59 (1 H, d, J = 11.6 Hz, 6-H).

25: HPLC (B) V_e = 51 ml; uv λ_{max} 264 nm; NMR δ 0.54 (3 H, s, 18-H_3), 2.05 (3 H, s, 3-$OCOCH_3$), 4.43 (1 H, m, 1-H), 5.20 (1 H, m, 3-H), 5.02 (1 H, m, 19(Z)-H), 5.33 (1 H, m, 19(E)-H), 6.00 (1 H, d, J = 11.0 Hz, 7-H), 6.37 (1 H, d, J = 11.0 Hz, 6-H).

26: HPLC (B) V_e = 59 ml; uv λ_{max} 273 nm; NMR δ 0.57 (3 H, s, 18-H_3), 2.04 (3 H, s, 3-$OCOCH_3$), 4.48 (1 H, m, 1-H), 5.28 (1 H, m, 3-H), 4.98 (1 H, m, 19(Z)-H), 5.13 (1 H, m, 19(E)-H), 5.81 (1 H, d, J = 11.4, 7-H), 6.59 (1 H, d, J = 11.4, 6-H).

27: HPLC (A) V_e = 40 ml; uv λ_{max} 264 nm; NMR δ 0.55 (3 H, s, 18-H_3), 2.06 (3 H, s, 3-$OCOCH_3$), 4.13 (1 H, m, 1-H), 4.90 (1 H, m, 3-H), 5.03 (1 H, m, 19(Z)-H), 5.39 (1 H, m, 19(E)-H), 6.01 (1 H, d, J = 11.4 Hz, 7-H), 6.36 (1 H, d, J = 11.4 Hz, 7-H).

28: HPLC (A) V_e = 44 ml; uv λ_{max} 272 nm; NMR δ 0.57 (3 H, s, 18-H_3), 2.07 (3 H, s, 3-$OCOCH_3$), 4.25 (1 H, m, 1-H), 4.99 (1 H, m, 3-H), 5.04 (1 H, m, 19(Z)-H), 5.13 (1 H, m, 19(E)-H), 5.80 (1 H, d, J = 11.0 Hz, 7-H), 6.60 (1 H, d, J = 11.0 Hz, 6-H).

The purified 1-hydroxyvitamin-3-acetates were then subjected to either reductive ($LiAlH_4$) or hydrolytic (NaOH, CH_3OH) deacetylation, producing the set of eight 1,3-dihydroxyvitamins.

(1): uv λ_{max} 265 nm; mass spectrum, *m/e* 400 (M^+, 15), 382 (10), 364 (8), 152 (30), 134 (100); NMR δ 0.55 (3 H, s, 18-H_3), 4.23 (1 H, m, 3-H), 4.43 (1 H, m, 1-H), 5.01

(1 H, m, 19(Z)-H), 5.32 (1 H, m, 19(E)-H), 6.02 (1 H, d, J = 11.4 7-H), 6.38 (1 H, d, J = 11.4, 6-H).

(**2**): uv λ_{max} 273 nm; mass spectrum, *m/e* 400 (M^+, 15), 382 (5), 364 (1), 152 (40), 134 (100); NMR δ 0.57 (3 H, s, 18-H_3), 4.24 (1 H, m, 3-H), 4.50 (1 H, m, 1-H), 4.97 (1 H, m, 19(Z)-H), 5.12 (1 H, m, 19(E)-H), 5.89 (1 H, d, J = 11.6 Hz, 7-H), 6.58 (1 H, d, J = 11.6 Hz, 6-H).

(**3**): uv λ_{max} 264 nm; mass spectrum, *m/e* 400 (M^+, 25), 382 (20), 364 (10), 152 (55), 134 (100); NMR δ 0.55 (3 H, m, 18-H_3), 4.21 (1 H, m, 3-H), 4.44 (1 H, m, 1-H), 5.01 (1 H, M, 19(Z)-H), 5.32 (1 H, m, 19(E)-H), 6.00 (1 H, d, J = 11.4 Hz, 7-H), 6.39 (1 H, d, J = 11.4 Hz, 6-H).

(**4**): uv λ_{max} 273 nm; mass spectrum, *m/e* 400 (M^+, 10), 382 (6), 364 (1), 152 (35), 134 (100); NMR δ 0.57 (3 H, m, 18-H_3), 4.25 (1 H, m, 3-H), 4.50 (1 H, m, 1-H), 4.97 (1 H, m, 19(Z)-H), 5.12 (1 H, m, 19(E)-H), 5.88 (1 H, d, J = 00.4 Hz, 7-H), 6.58 (1 H, d, 11.4 Hz, 6-H).

(**5**): uv λ_{max} 263 nm; mass spectrum, *m/e* 400 (M^+, 20), 382 (35), 364 (15), 152 (100), 134 (90); NMR δ 0.54 (3 H, s, 18-H_3), 405 (1 H, m, 3-H), 4.30 (1 H, m, 1-H), 5.00 (1 H, m, 19(Z)-H), 5.30 (1 H, m, 19(E)-H), 6.02 (1 H, d, J = 11.4 Hz, 7-H), 6.44 (1 H, d, J = 11.4, 6-H).

(**6**): uv λ_{max} 271 nm; mass spectrum, *m/e* 400 (M^+, 20), 382 (5), 364 (1), 152 (100), 134 (70); NMR δ 0.57 (eH, s, 18-H_3), 4.13 (1H, m, 3-H), 4.38 (1H, m, 1-H), 4.96 (1 H, m, 19(Z)-H), 5.12 (1H, m, 19(E)-H), 5.90 (1H, d, J = 11.4 Hz, 7-H), 6.64 (1H, d, J = 11.4 Hz, 6-H).

(**7**): uv λ_{max} 263 nm; mass spectrum, *m/e* 400 (M^+, 15), 382 (25), 364 (10), 152 (100), 134 (80); NMR δ 0.55 (3H, s, 18-H_3), 4.10 (1H, m, 3-H), 4.37 (1H, m, 1-H), 5.02 (1H, m, 19(Z)-H), 5.29 (1H, m, 19(E)-H), 6.06 (1H, d, J = 11.4 Hz, 7-H), 6.46 (1H, d, J = 11.4 Hz, 6-H).

(**8**): uv λ_{max} 271 nm; mass spectrum, *m/e* 400 (M^+, 15), 382 (5), 364 (2), 152 (100), 134 (80); NMR δ 0.57 (3H, s, 18-H_3), 4.11 (1H, m, 3-H), 4.38 (1H, m, 1-H), 4.96 (1H, m, 19(Z)-H), 5.12 (1H, m, 19(E)-H), 5.90 (1H, d, J = 11.4 Hz, 7-H), 6.64 (1H, d, J = 11.4 Hz, 6-H).

MnO_2 Oxidation of 1-Hydroxyvitamin D-acetates

To a stirring solution of 0.025 mmol of the 1-hydroxyvitamin-3-acetates (**21** or **25**) in 3.0 ml CH_2Cl_2 was added 100 mg of finely divided activated MnO_2 in two 50-mg portions at 0 and 30 min time points. After 1.0 hr the reaction mixture was filtered through celite and concentrated *in vacuo* to an oil. Preparative HPLC (7.5 × 300-mm μ-Porosil, 2% *i*-propanol : hexanes) gave **29** (from **21**) or **30** (from **25**) in ~70% yield.

(**29**): HPLC V_e = 22 ml; uv λ_{max} 300, 240 nm; mass spectrum, *m/e* 440 (M^+, 20), 380 (100), 365 (25), 267 (25) 172 (60), 157 (60); NMR δ 0.69 (3H, s, 18-H_3), 1.81 (3H, s, 19-H_3), 2.03 (3H, s, 3-$OCOCH_3$), 5.21 (1H, m, 3-H), 5.49 (1H, m, 9-H), 6.04 (1H, d, J = 12.1 Hz, 7-H), 6.11 (1H, d, J = 12.1 Hz, 6-H).

(**30**): HPLC V_e = 22 ml; uv λ_{max} 300, 240 nm; mass spectrum, *m/e* 440 (M^+, 20), 380 (100), 365 (35), 267 (25), 172 (40), 157 (40); NMR δ 0.69 (3H, s, 18-H_3), 1.81

(3H, s, 19-H_3), 2.03 (3H, s, 3-$OCOCH_3$), 5.24 (1H, m, 3-H), 5.50 (1H, m, 9-H), 6.03 (1H, d, J = 12.2 Hz, 7-H), 6.10 (1H, d, J = 12.2 Hz, 6-H).

$LiAlH_4$ Reduction of 1-Keto-previtamin-acetates

A solution of 0.02 mmol of either (**29**) or (**30**) in Et_2O was cooled at 0°C and treated with 0.1 ml $LiAlH_4$-saturated Et_2O solution. After 10 min the reaction was quenched with 2% NaOH and, after the usual work-up, preparative HPLC (7.5 × 300-mm μ-Porosil, 7% *i*-propanol : hexanes) afforded a 3 : 1 mixture of **31** and **32** (from **29**) and **33** and **34** (from **30**) in 80% overall yield.

(**31**): HPLC V_e = 53 ml; UV λ_{max} 256–258 nm; mass spectrum identical to (**7**); nmr δ 0.71 (3H, s, 18-H_3), 1.81 (3H, s, 19-H_3), 4.02 (1H, m, 3-H), 4.25 (1H, m, 1-H), 5.54 (1H, m, 9-H), 5.79 (1H, d, J = 12.1 Hz, 7-H), 5.98 (1H, d, J = 12.1 Hz, 6-H).

(**32**): HPLC V_e = 93 ml; uv λ_{max} 256–258 nm; mass spectrum identical to (**1**): NMR δ 0.71 (3H, s, 18-H_3), 1.78 (3H, s, 19-H_3), 4.06 (1H, m, 3-H), 4.20 (1H, m, 1-H), 5.51 (1H, m, 9-H), 5.79 (1H, d, J = 12.1 Hz, 7-H), 5.92 (1H, d, J = 12.1 Hz, 6-H).

(**33**): HPLC V_e = 50 ml; uv λ_{max} 256–258 nm; mass spectrum identical to (**5**); NMR δ 0.69 (3H, s, 18-H_3), 1.81 (3H, s, 19-H_3), 4.01 (1H, m, 3-H), 4.24 (1H, m, 1-H), 5.53 (1H, m, 9-H), 5.79 (1H, d, J = 12.2 Hz, 7-H), 5.97 (1H, d, J = 12.2 Hz, 6-H).

(**34**): HPLC V_e = 90 ml; uv λ_{max} 256–258 nm; mass spectrum identical to (**3**); NMR δ 0.71 (3H, s, 18-H_3), 1.78 (3H, s, 19-H_3), 4.06 (1H, m, 3-H), 4.20 (1H, m, 1-H), 5.49 (1H, m, 9-H), 5.79 (1H, d, J = 12.2, 7-H), 5.97 (1H, d, J = 12.2 Hz, 6-H). Thermal isomerization (EtOH, 70–80°C, 2 hr) converted previtamin **31** to **7**, and **33** to **5**.

I_2-Catalyzed (5Z) to (5E)-Vitamin Conversion

To a solution of 0.01 mmol of a (5*Z*)-vitamin analog (**5**) or (**7**) in 1.0 ml Et_2O was added 10 μl pyridine and 20 μl 1.0 mg/ml solution of I_2 in hexane. After 5.0 min at room temperature the reaction was terminated by the addition of 2.0 ml 0.02 M $Na_2S_2O_3$. The organic phase was separated, washed with water, dried over $MgSO_4$, and taken to an oil *in vacuo*. Preparative HPLC (7.5 × 300-mm μ-Porosil, 6% *i*-propanol/hexanes) separated *trans* isomer (**6**) from (**5**) and *trans* isomer (**8**) from (**7**) in ca. 60% yield. HPLC elution volumes were **5**, 90 ml; **6**, 94 ml; **7**, 100 ml; and **8**, 108 ml.

Competitive Binding Assay

Chemicals. 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (80 Ci/mmol) was prepared enzymatically from 25-OH-[26,27-^{3}H]D_3 (*12*) by the method of Frolik and DeLuca (*13*), and was used at a purity of greater than 96% as determined by high-performance liquid chromatography.

Preparation of cytosol binding protein. One-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, Wisc.) were fed a vitamin D-deficient soy

protein diet (*14*) and received water *ad libitum* for 4–6 weeks. All animals were maintained in a vivarium at 25–26°C on an alternating 12-hr light and dark cycle using incandescent lighting.

Chickens were fasted for 16–20 hr, after which time they were killed by cervical dislocation, and the duodenal loop was excised and freed of pancreas. Unless otherwise stated, all procedures were carried out between 0 and 4°C. Mucosa was scraped free of serosa and washed three times in several volumes (w/v) of buffer as previously described (*15*). Washed mucosa was homogenized in 2 vol buffer (50 mM Tris–HCl, 300 mM KCl, 1.5 mM EDTA, 5.0 mM DTT, pH 7.4) by using a Polytron, Type PT-20 (Brinkman Instruments, Westbury, W.V). Cytosol was the supernatant fraction (without the fluffy lipid layer) obtained by centrifuging the homogenate at 78,000*g* for 90 min in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) using a Type 30 rotor. The cytosol was lyophilized and stored at −70°C until use. Cytosol protein concentration was determined by the method of Bradford (*16*), using crystalline bovine serum albumin as a standard.

Competitive binding assay. Various concentrations of C(1)-hydroxylated vitamin D analogs and 0.25 nM 1,25-$(OH)_2$[^{2}H]D_3 were added to glass tubes (0.12 × 0.75 cm) in 50 μl absolute ethanol. To the sterols were added cytosol protein (0.4 mg) and sufficient homogenization buffer (except that the concentration of DTT was 0.5 mM) to make a final incubation volume of 0.5 ml. The assay tubes were incubated in a shaking water bath for 1 hr at 25°C and then placed on ice. Subsequently, the unbound sterol was removed from the cytosol by adsorption to 0.25 ml of a slurry of dextran-coated charcoal (0.05 and 0.5% w/v, respectively) for 10 min at 0–4°C. The charcoal was removed by centrifugation at 23,000*g* for 5 min in a Sorvall LR-5 refrigerated centrifuge using an HS4 rotor (Dupont Instruments, Newtown, Conn.). A 0.5-ml aliquot of the supernatant was removed and added to 3.5 ml of a scintillation fluid mixture consisting of 1.32 liters Triton X-100, 16 g 2,5-diphenyloxazole, and 0.2 g of 1,4-bis[2-(5-phenyloxazole)]benzene per 4 liters of toluene. The readioactivity was determined by liquid scintillation spectrometry with a Beckman LS-100 C spectrometer having an efficiency for tritium of 36%. Quench correction was carried out by means of automatic external standardization.

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