14-Epi Stereoisomers of 25-Hydroxy- and 1α,25-Dihydroxyvitamin D_3: Synthesis, Isomerization to Previtamins, and Biological Studies[1a]

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The C-14 epimers of vitamin D, 14-epi-25-hydroxyvitamin D_3 (**4**) and 14-epi-1α,25-dihydroxyvitamin D_3 (**5**), were synthesized, and their isomerization via [1,7]-sigmatropic hydrogen shifts to the corresponding previtamin forms (**4′** and **5′**, respectively) was studied. The activation parameters of the [1,7]-sigmatropic hydrogen shifts were found to be similar to those of other vitamin D analogues, although epimerization at C-14 shifts the equilibrium of the triene chromophore to the previtamin form. The in vivo biological activities of **4**, **4′**, **5**, and **5′** in the chick in terms of their ability to elicit intestinal calcium absorption and bone calcium mobilization were determined. These vitamin D analogues, the first in the natural steroid series modified at the C-14 position, were essentially devoid of activity. The relative competitive indices (RCIs), derived in an in vitro assay reflecting the ability of these analogues to bind to the chick intestinal nuclear receptor, were determined. Analogues **4**, **4′**, **5**, and **5′** had RCI values of 0.08, 0.01, 15, and 1.6, respectively, in comparison to the natural ligand, 1α,25-dihydroxyvitamin D_3 (**3**), whose value is 100 by definition. Thus, the in vivo and in vitro data were somewhat at variance, particularly for **5**, which bound significantly to the chick intestinal receptor. In vitro binding studies with the human serum vitamin D binding protein (DBP) were also conducted. The RCI values for human DBP reflects the ability of an analogue to bind to this protein in comparison to the hormone **3**, whose value is 100. The measured RCI values for **4**, **4′**, **5**, and **5′** were 3450, 90, 12, and 2.2, respectively. It is noteworthy that analogue **4** binds ~35 times more effectively than the parent hormone **3**, but ~20 times less effectively than 25-hydroxyvitamin D_3 (**2**).

Introduction

The metabolic activation of vitamin D_3 (**1**, D_3) to 25-hydroxyvitamin D_3 (**2**, 25-OH-D_3) and then to 1α,25-dihydroxyvitamin D_3 (**3**, 1α,25-$(OH)_2$-D_3) has been well discussed in recent years (Scheme 1).[2] That 1α,25-$(OH)_2$-D_3 is a hormone, which manifests its principal biological functions predominantly via a genomic mechanism like other steroid or steroid like hormones (e.g., estradiol, testosterone, stanolone, progesterone, cortisol, aldosterone, retinoic acid, thyroxine), has also been discussed.[2,3] This hormone also displays some of its biological functions through novel nongenomic pathways.[4] Because of the emergence of both genomic and nongenomic effects that characterize the vitamin D endocrine system, and because of the emergence of the potential use of vitamin D in medical therapy (e.g., in the treatment of certain cancers,[5] skin diseases,[6] and osteoporosis[7] and in immunoregulation[8]), there is increasing interest in understanding structure–function relationships for vitamin D action. In connection with the latter, studies of stereoisomers of the natural metabolite 1α,25-$(OH)_2$-D_3 such as A-ring carbinol epimers[9] have provided valuable insight into the structural demands of vitamin D receptors. One analog, the C-1 epimer of 1α,25-$(OH)_2$-D_3, has even exhibited selective inhibitory properties toward one of the vitamin D receptors.[9b,c] Accordingly, stereoisomers of the natural metabolites **2** and **3**, such as **4** and **5**, respectively, became interesting targets for study (Scheme 2). Another area which has been less well discussed is the assessment of the biological function, if any, of the previtamin forms of D_3 (**1**),[10] 25-OH-D_3 (**2**),[11] and 1α,-25-$(OH)_2$-D_3 (**3**),[12] namely **1′**, **2′**, and **3′**, respectively.

It is clear that the principal metabolic forms of vitamin D, **1**, **2**, and **3**, can back-equilibrate to a small extent to the corresponding previtamin forms **1′**, **2′**, and **3′**, respectively. At 37 °C, the [1,7]-sigmatropic hydrogen shift of D_3 (or 25-OH-D_3) to pre-D_3 (or 25-OH-pre-D_3) occurs with a half-life of 6.3 days, and at equilibrium, there exists 92% of vitamin and 8% of previtamin.[10] Under similar conditions at 37 °C, the isomerization of 1α,25-$(OH)_2$-D_3 to 1α,25-$(OH)_2$-pre-D_3 occurs with a half-life of 9.3 days, and at equilibrium, there exists 94% of vitamin **3** and 6% of previtamin **3′**.[12] Biological studies of the "natural" previtamins will be ambiguous in that they can inevitably isomerize back to vitamin. Even though the isomerization of previtamin to vitamin is reasonably slow (and recent studies from our laboratory have led to the replacement of the C-19-methyl with a C-19-trideuteriomethyl to slow down this process even more as a consequence of the primary deuterium kinetic isotope effect, k_H/k_D is ~6),[10b,12] it is possible that this isomerization could be catalyzed by the biological matrix (e.g., possibly by some putative enzyme).[13] Thus, one cannot be certain that the observed biological activity is due to endogenously produced vitamin (rather than the previtamin). In an early biological study of previtamin D_3 itself, Rappoldt and co-workers[10a] found that the biological data obtained for previtamin D_3 could be explained by its prior isomerization to D_3.

One of the purposes of this article is to describe the synthesis of the analogues of 25-OH-pre-D_3 (**2′**) and of 1α,25-$(OH)_2$-pre-D_3 (**3′**), namely **4′** and **5′**, respectively, which because of *cis*-fusion of the CD ring were expected

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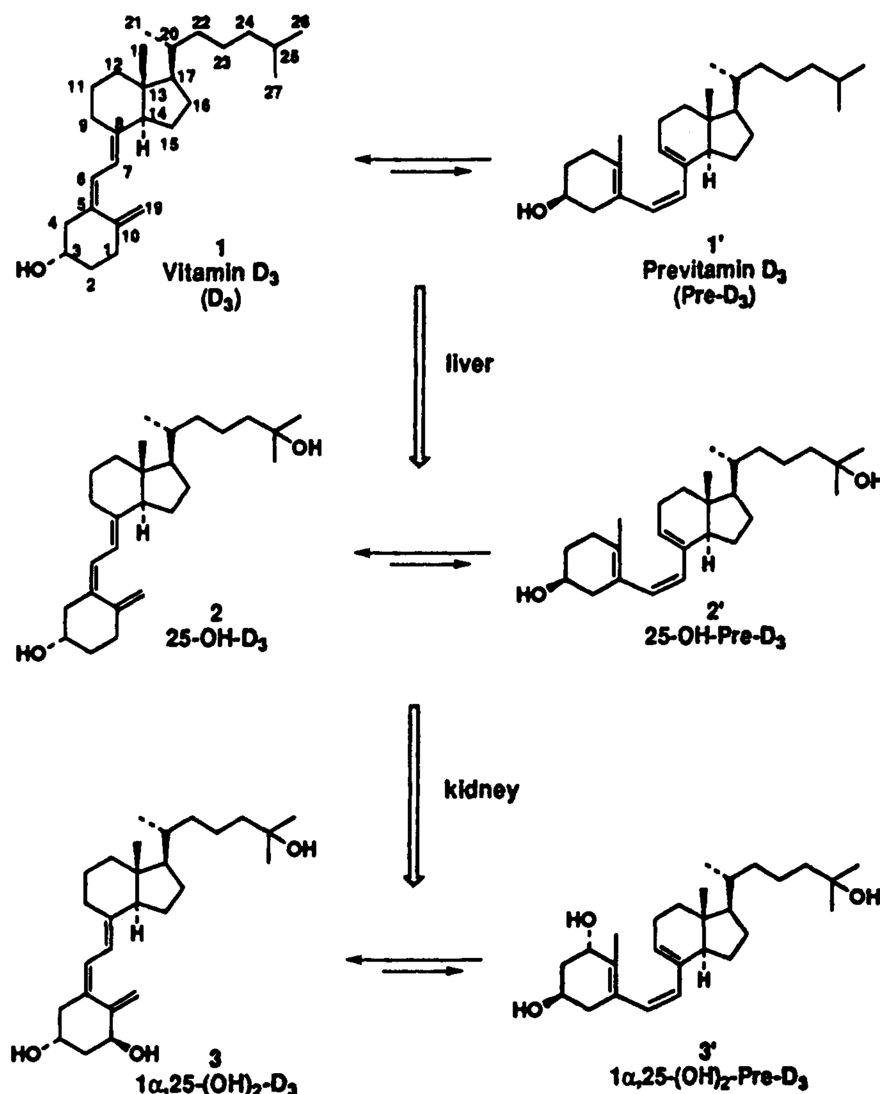
Scheme 1



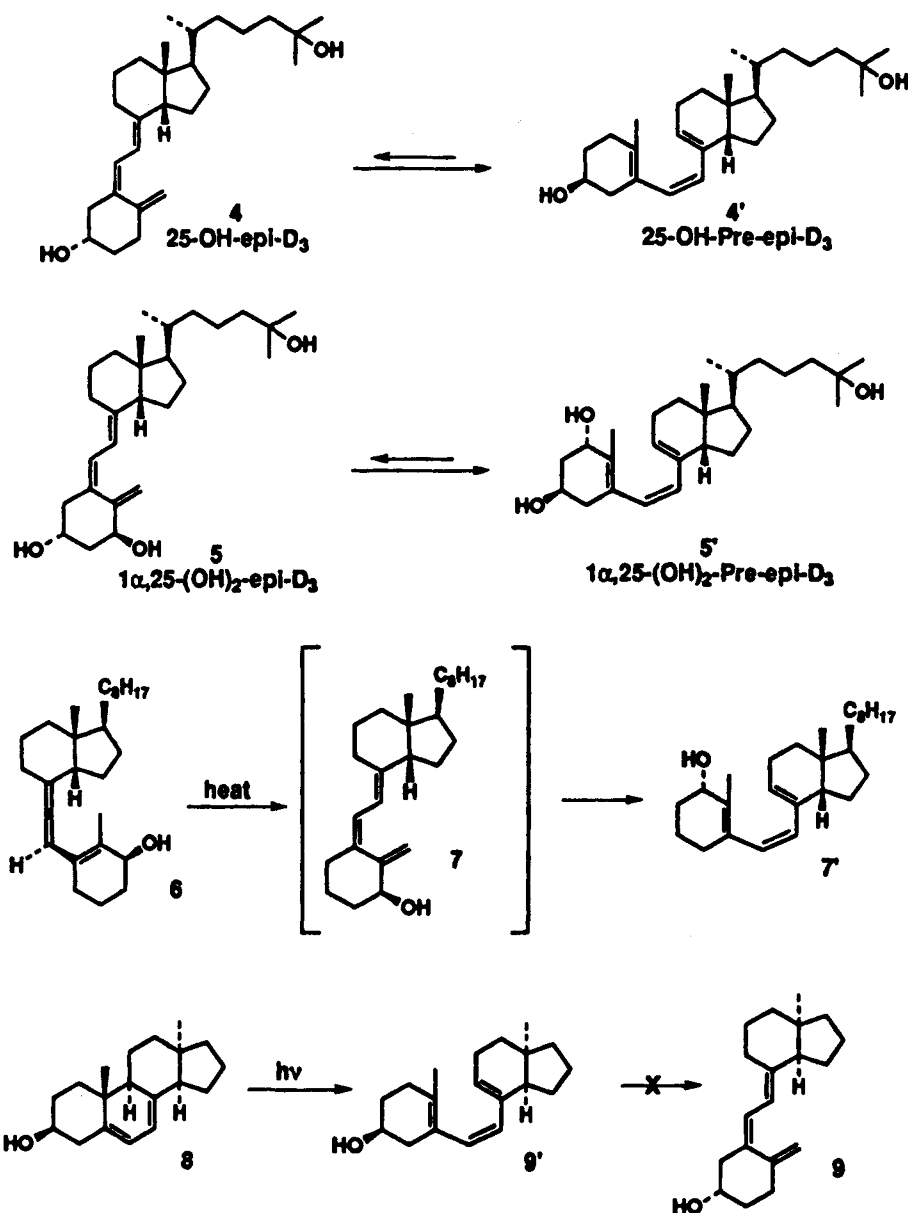
to be strongly biased in their previtamin forms (Scheme 2). This expectation is based on two earlier findings, also shown in Scheme 2. In the more recent study, thermolysis of the C-1-oxygenated vinylallene **6** afforded primarily the previtamin **7'**, which was considered to be produced via the intermediate vitamin **7**.[14] In a still earlier study,[15] photochemical irradiation of the B-ring diene **8** afforded only the previtamin **9'**, which exhibited no tendency to isomerize to the corresponding vitamin **9**. The actual characterization of the CD *cis*-fused vitamins **7** and **9** proved elusive because of the nature of their syntheses. Thus, a second purpose of this report is to describe the actual isolation and characterization of the CD *cis*-fused vitamin forms (i.e., **4** and **5**) and to quantitate their isomerization to the previtamins. Finally, another goal of this investigation was to obtain information concerning the initial biological profile of the analogues **4**, **5**, **4'**, and **5'**.

Results and Discussion

The desired vitamins **4** and **5** were prepared from the known A-ring phosphine oxides **10a**[16] and **10b**,[17] respectively, and the CD ketone **14** synthesized as shown in Scheme 3. Grundmann's ketone (**11**),[18] readily available from the ozonolysis of vitamin D_3, was selectively oxidized at C-25 to alcohol **12** as previously described.[19] Epimerization of the latter to the *cis*-fused hydrindanone **13** was accomplished with base. The crude mixture consisted of a 71/29 ratio of **13**/**12**, whereas a 49% yield (66% based on recovered **12**) of purified **13** was actually isolated by HPLC.[12,14,20] Protection of the C-25 hydroxyl as the TMS ether afforded CD fragment **14** in 89% yield. A-ring phosphine oxide **10a** was coupled to CD fragment 14, and the deprotection of the silyl ether with TBAF provided 14-epi-vitamin **4**. In a similar manner, the 1α-hydroxylated 14-epi-vitamin **5** was prepared. Both 14-epi analogues **4** and **5** underwent smooth thermal [1,7]-sigmatropic hydrogen shifts at 80 °C (benzene-d_6) to afford previtamins **4'** and **5'**, respectively.

For the kinetic studies, monitoring of the isomerization of vitamin **4** to previtamin **4'** was performed by ^{1}H-NMR analyses; using benzene-d_6 as solvent, integration of the H_7 signal of the vitamin **4** (steroid numbering system) in the ^{1}H-NMR spectrum provided a convenient means of monitoring the reaction and determining first-order rate constants for the transformation of **4** to **4'** over a ~30 °C temperature range. Activation parameters (80 °C) for the isomerization of **4** to **4'** are summarized in Table 1. The activation parameters for the isomerization of **4** most closely resemble those of

Scheme 2



the parent (*Z*,*Z*)-1,3,5-heptatriene.[21] Table 2 compares rate and equilibrium constants at 80 °C for the analogue **4** versus those for the natural metabolites **1**, **2**, and **3** (assuming the data for **1** and **2** are identical since they differ only in the side chain). In earlier studies of the interconversion between previtamin D and vitamin D, reactivity profiles were discussed in terms of the transformation of previtamin D going to vitamin D, since equilibration invariably favored the vitamin.[10,12,14,22] Here we compare the rates of transformation of vitamin to previtamin. From Table 2, the half-lives at 80 °C for the [1,7]-shift of **4** to **4′** and **5** to **5′** were calculated to be 13.7 and 11.7 min, respectively. As is apparent from the latter table, after prolonged heating, the vitamin/previtamin ratio assumes a relatively constant value of 5:95, in stark contrast to the 79:21 and 88:12 values obtained for the **1**/**1′** (or **2**/**2′**) and **3**/**3′** ratios, respectively.

With analogues **4**, **4′**, **5**, and **5′** in hand, intestinal calcium absorption (ICA) and bone calcium mobilization (BCM) were measured in vivo in comparison to 1α,25-$(OH)_2$-D_3 (**3**) in the vitamin D deficient chick system previously described.[23] The results in this standard rachitic chick assay can be reported as the percentage of activity observed for ICA and BCM in comparison to standard doses of 1α,25-$(OH)_2$-D_3.[24] With respect to the ICA determination, the four analogues **4**, **4′**, **5**, and **5′** exhibited $<0.1\%$, $<0.04\%$, 3.9%, and $<0.1\%$, respectively, of the activity as compared to 1α,25-$(OH)_2$-D_3. Similar results ($<1.0\%$, $<0.01\%$, $<0.01\%$ and 2%, respectively) were obtained with respect to the BCM determination. One interpretation of this result is that the seemingly simple stereochemical change at C-14 in **4** and **5** (*cis*-CD ring junction) as compared to the corresponding natural metabolites **2** and **3** (*trans*-CD ring junction), respectively, results in loss of in vivo calcemic activity (ICA and BCM). That the previtamins **4′** and **5′**, the rearrangement products of **4** and **5**, which possess topologies quite different from **2** and **3**, respectively, exhibit little calcemic activity is not surprising. An alternative explanation for the lack of calcemic activity of **4** and **5** is that under these in vivo bioassay conditions, they undergo isomerization to **4′** and **5′**, respectively, which are in themselves inactive. This is opposite the situation encountered by Rappoldt and co-

Scheme 3

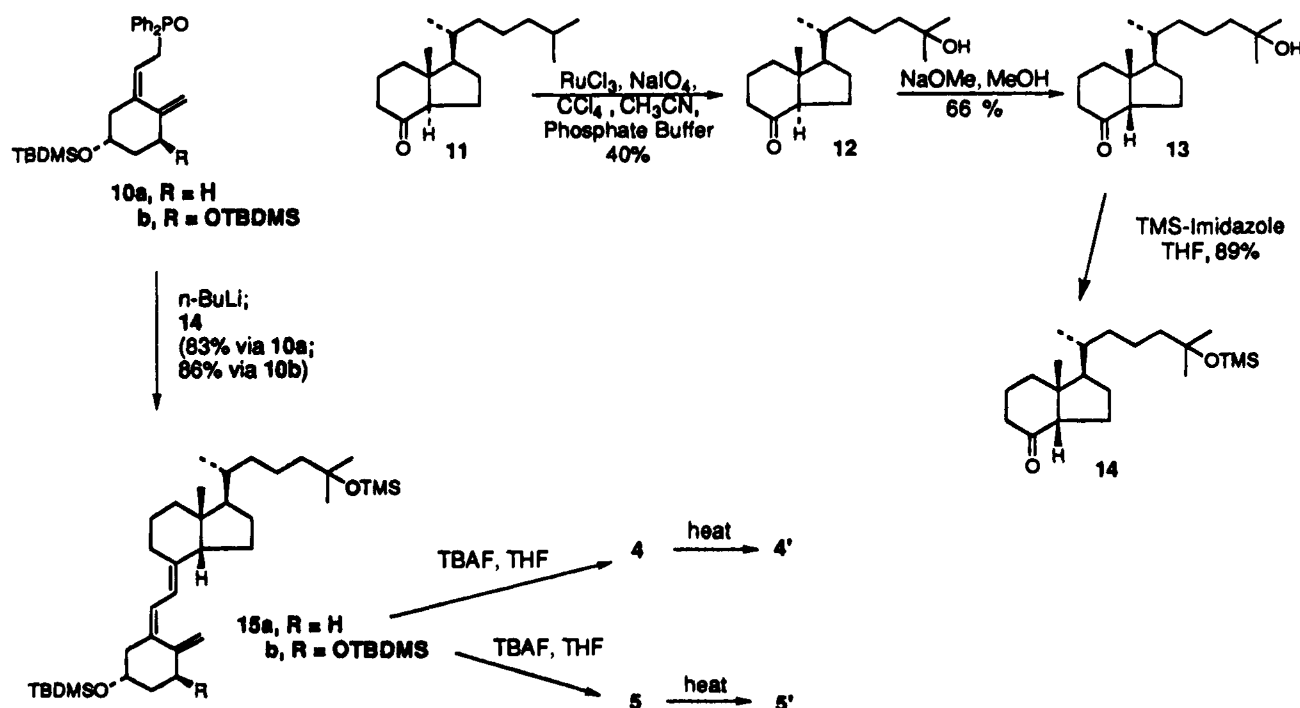


Table 1. Activation Parameters[a] for the Transformation of Vitamin to Previtamin

substrate	E_a [b]	log A [c]	$\Delta G^{\ddagger}$ [b]	$\Delta H^{\ddagger}$ [b]	$\Delta S^{\ddagger}$ [d]
4	18.7 (±0.7)	8.5 (±0.3)	25.8 (±1.0)	18.0 (±0.7)	−22.1 (±0.8)
1(2)[e]	23.6 (±0.2)	10.7 (±0.1)	27.0 (±0.3)	22.9 (±0.2)	−11.8 (±0.1)
3[f]	22.7 (±0.5)	9.9 (±0.1)	27.4 (±0.2)	22.0 (±0.2)	−15.5 (±0.1)
(Z,Z)-1,3,5-heptatriene[g]	20.7 (±0.8)	8.2 (±0.3)	28.8 (±1.1)	20.0 (±0.8)	−23.3 (±0.9)

[a] At 80 °C. Standard deviations are given in parentheses. [b] Units = kcal/mol. [c] A is given in s^{-1}. [d] Units = cal/mol K. [e] These parameters were calculated from data for the substrate without the side chain hydroxyl (ref 10 of the text). [f] These parameters were calculated from data given in ref 12a of the text. [g] See ref 21 of the text.

Table 2. Kinetic Data for the Transformation of Vitamin D to Previtamin D[a]

substrate	$k_1 \times 10^4$, s^{-1}	$k_{-1} \times 10^4$, s^{-1}	K_{eq}
4	0.492 (±0.022)	8.45 (±0.39)	0.058 (±0.003)
3	5.63 (±0.29)	0.760 (±0.040)	7.3 (±0.4)
2(1)	5.02 (±0.37)	1.35 (±0.06)	3.8 (±0.2)

[a] Rate constants were calculated from activation parameters at 80 °C. K_{eq} is defined as k_1/k_{-1} where the forward process is for the isomerization of previtamin D to vitamin D.

workers[10a] mentioned earlier wherein it was concluded that the calcemic activity observed for previtamin D_3 (1′) may be due to endogenously produced vitamin D_3 (1) (the assumption being that 1′ is inactive). Thus, in this study, the lack of activity observed for 4 and 5 may be because these analogues rearrange to the inactive previtamin analogues 4′ and 5′ under the in vivo bioassay conditions. It can be calculated that at 25–37 °C, the analogue 4 isomerizes to 4′ with a half-life of 33 to 10 h. The earliest time point recorded in acquiring calcemic bioassay data was less than 12 h, so some activity for 4 or 5 should have been detected. Accordingly, we conclude that neither 4 nor 5 exhibit calcemic action because of the stereochemical change at C-14.

In order to evaluate whether the *cis* ring junction renders these analogues inactive because of their inability to bind to receptors, which is a necessary step for eliciting the calcemic effect, the analogues were evaluated in vitro in terms of their ability to bind to the chick intestinal nuclear receptor. In this assay,[25] the analogues are evaluated in terms of their chick intestinal receptor relative competitive indices (RCIs) wherein the value for 1α,25-$(OH)_2$-D_3 is 100 by definition. The RCI values for 4, 4′, 5, and 5′ were 0.082 ± 0.018, 0.011 ± 0.008, 15.0 ± 2.0, and 1.6 ± 0.9, respectively. The lack of in vivo calcemic activity observed for 5 is somewhat at variance with its RCI value of 15. It is possible that 5 binds to the chick intestinal receptor without inducing its necessary activation, which is required of steroid hormone receptors prior to stimulation of transcription. This could render the 14-epi structural feature of 5 a useful parameter in designing an antogonist of the steroid hormone, 1α,25-$(OH)_2$-D_3.

Analogues 4, 4′, 5, and 5′ were also subjected to a second in vitro steroid competition assay involving the human vitamin D binding protein (DBP).[26] In this assay, each analogue was evaluated in terms of its ability to bind to human DBP in comparison to the natural hormone 1α,25-$(OH)_2$-D_3. Like the chick intestinal receptor assay results, the human DBP data are expressed as RCI values [the value for 1α,25-$(OH)_2$-D_3 is 100 by definition]. It should be noted that human DBP has the highest affinity for the natural metabolite 25-OH-D_3 (2, RCI = 66,800). The human DBP RCI values for analogues 4, 4′, 5, and 5′ were 3450 ± 960, 90 ± 67, 12.1 ± 2.1, and 2.2 ± 0.7, respectively. That 4 and 4′ bind more effectively than 5 and 5′ by ~280- and ~40-fold, respectively, parallels previous findings that the presence of a 1α-hydroxyl group in vitamin D is deleterious to an analog in effective binding to human

DBP. Binding to human DBP is normally reflective of how well an analogue is transported in serum as it migrates to various target sites in the aqueous, endocrine milieu. Although the 14-epi analogues **4** and **5** exhibit relatively low human DBP RCI values of 3450 and 12 as compared to the corresponding natural metabolites **2** (66 800) and **3** (100), respectively, the values are not exceptionally low. We conclude that transport of these analogues is not a major factor in their observed low in vivo calcemic activity.

In summary, **4**, **4'**, **5**, and **5'** are the first 14-epi analogues of natural vitamin D metabolites to be synthesized and biologically evaluated both in vivo and in vitro. The biological results indicate that the presence of the *cis*-CD ring junction is a deleterious structural feature for exerting calcemic activity. A comparison of the biological data for the vitamin forms (**4** and **5**) with the corresponding data from the previtamins (**4'** and **5'**) also reveals that the vitamin D structures are more important than the corresponding previtamins, at least for the biological assay systems examined. It remains for future studies to establish whether these analogues, particularly the previtamins **4'** and **5'**, might be active in other biological target systems recently found to accommodate 1α,25-$(OH)_2$-pre-D_3 (**3'**).[12b,27]

Experimental Section[28]

14-Epi-25-hydroxyvitamin D_3 (4). After treating a cooled (−78 °C) solution of **10a** (101 mg, 0.224 mmol) in dry THF (4 mL) with *n*-BuLi (0.144 mL, 0.23 mmol, 1.6 M in hexanes), the resulting deep red solution was stirred for 10 min, and then a solution of ketone **14** (51.8 mg, 0.147 mmol) in THF (0.8 mL) was added. The mixture was stirred for 5 h at −78 °C and then warmed to rt. The solvent was removed, and the residue redissolved in ether was washed with saturated $NaHCO_3$ (3 × 10 mL) and brine (3 × 10 mL). After concentration, the residue was purified by flash chromatography (100% hexanes) and then directly deprotected with TBAF (2.0 mL, 2.0 mmol, 1 M solution in THF). After stirring for 3 h, the solvent was removed, and the crude vitamin dissolved in EtOAc (5 mL) was washed with water (3 mL) and brine (3 mL) and dried (Na_2SO_4). Following filtration and concentration, the crude residue was purified by HPLC (50% EtOAc/hexanes, Rainin Dynamax 60 Å column) to afford after vacuum drying 66 mg (74%) of vitamin **4** as a thermally labile, colorless product. *^1H-NMR* (300 MHz, $CDCl_3$): δ 0.88 (3H, C_{21}-CH_3, d, $J \sim 6.4$ Hz), 0.92 (3H, C_{18}-CH_3, s), 1.22 (6H, $C_{26,27}$-CH_3, s), 3.91 (1H, H_3, m), 4.84 (1H, H_{19}, narrow m), 5.08 (1H, H_{19}, narrow m), 6.17 (2H, $H_{6,7}$-AB, s).

14-Epi-25-hydroxyprevitamin D_3 (4'). A solution of vitamin **4** (5 mg, 0.013 mmol) in benzene-d_6 (1 mL) was subjected to three freeze−thaw cycles under vacuum and then placed in a thermostated bath at 80.0 °C. After 2 h, the tube was cooled to room temperature and vitamin/previtamin distribution determined (5:95) by ^1H-NMR integration. The sample was then concentrated and purified by HPLC (50% EtOAc/hexanes, Rainin Dynamax 60 Å column) to afford, in order of elution, 14-epi-25-hydroxyvitamin D_3 (**4**, 0.3 mg) and 14-epi-25-dihydroxyprevitamin D_3 (**4'**, 4.5 mg). *^1H-NMR* (300 MHz, $CDCl_3$): δ 0.90 (3H, C_{18}-CH_3, s), 0.93 (3H, C_{21}-CH_3, d, $J \sim 6.4$ Hz), 1.21 (6H, $C_{26,27}$-CH_3, s), 2.45 (1H, broad d, $J \sim 16.2$ Hz), 3.8−3.9 (1H, H_3 m), 5.62 (1H, H_9, br s), 5.73 and 5.85 (2H, $H_{6,7}$-AB pattern, d, $J \sim 12.2$ Hz). *^{13}C-NMR* (75.5 MHz, $CDCl_3$): δ 19.6, 19.8, 21.1, 21.8, 22.9, 28.6, 29.1, 29.5, 29.7, 30.0, 31.4, 33.9, 34.2, 35.7, 37.7, 41.0, 44.4, 51.0, 52.3, 67.6, 71.1, 125.2, 126.3, 128.5, 129.3, 131.5, 139.0.

14-Epi-1α,25-dihydroxyvitamin D_3 (5). To a stirred solution of **10b** (67 mg, 0.11 mmol) in anhydrous THF (1.4 mL) at −78 °C under argon was added *n*-butyllithium (74 μL, 0.12 mmol, 1.55 M solution in hexanes) to give a deep orange solution. After adding CD ketone **14** (27.1 mg, 0.076 mmol) in dry THF (0.46 mL), the solution was stirred for 3 h at −78 °C and then warmed to rt. After concentration, the residue was dissolved in ether (3 mL) and washed with a saturated solution of $NaHCO_3$ (3 mL) and brine (3 mL). After drying ($MgSO_4$) and concentration of the ether solution, the crude residue was purified by flash chromatography to afford 48.2 mg (86% yield) of protected vitamin **15b**, which was treated with TBAF (0.79 mL, 0.79 mmol, 1 M solution in THF). After 3 h, the solvent was removed and the crude residue dissolved in EtOAc (5 mL). The solution was washed (water, 3 mL; and brine, 3 mL), dried (Na_2SO_4), filtered, and concentrated. Purification by HPLC (50% EtOAc/hexanes, Rainin Dynamax 60-Å column) afforded after vacuum drying 11 mg (81%) of vitamin **5**. *^1H-NMR* (300 MHz, $CDCl_3$): δ 0.87 (3H, C_{21}-CH_3, d, $J \sim 6.4$ Hz), 0.90 (3H, C_{18}-CH_3, s), 1.22 (6H, $C_{26,27}$-CH_3, s), 2.31 (1H, dd, $J \sim 13.2$ Hz, 7.2 Hz), 2.46 (1H, br d, $J \sim 14.3$ Hz), 2.60 (1H, dd, $J \sim 13.3$ Hz, 3.5 Hz), 4.23 (1H, H_3, m), 4.44 (1H, H_1, t, $J \sim 5.4$ Hz), 5.00 (1H, H_{19}, br s), 5.34 (1H, H_{19}, br s), 6.14 and 6.33 (2H, $H_{6,7}$-AB pattern, d, $J \sim 11.2$ Hz).

14-Epi-1α,25-dihydroxyprevitamin D_3 (5'). A solution of vitamin **5** (4.9 mg, 0.012 mmol) in benzene-d_6 (2 mL) was subjected to three freeze−thaw cycles under vacuum and then placed in a thermostated bath at 80.0 °C. After 4 h, the solution was cooled to room temperature and the vitamin/previtamin ratio determined by ^1H-NMR integration (~7:93). The solution was concentrated and purified by HPLC (100% EtOAc, Rainin Dynamax 60-Å column) to afford, in order of elution, epi-vitamin **5** (0.3 mg) and epi-previtamin **5'** (3.7 mg). *^1H-NMR* (300 MHz, $CDCl_3$): δ 0.91 (3H, C_{18}-CH_3, s), 0.94 (3H, C_{21}-CH_3, d, $J \sim 6.3$ Hz), 1.22 (6H, $C_{26,27}$-CH_3, s), 1.75 (3H, C_{19}-CH_3, br s), 2.55 (1H, br d, $J \sim 16.6$ Hz), 4.05 (1H, H_3, m), 4.18 (1H, H_1, br s), 5.65 (1H, H_9, m), 5.80 and 5.85 ($H_{6,7}$, AB pattern, d, $J \sim 12.5$ Hz).

De-*A,B*-25-hydroxycholestan-8-one (12). This compound was prepared in a manner similar to that recently communicated.[19] To a solution of ketone **11** (1.0 g, 3.8 mmol) in CCl_4 (15 mL), CH_3CN (15 mL), and pH 7 aqueous buffer solution (19 mL, 0.05 M KH_2PO_4 and 0.05 M in NaOH) was added $RuCl_3 \cdot H_2O$ (78.4 mg, 0.37 mmol) and $NaIO_4$ (2.83 g, 13.2 mmol). Upon vigorous stirring at 45 °C, the black solution turned yellow. The heterogeneous suspension returned to its original black color within 5 days. The organic phase was separated and the aqueous layer extracted with CH_2Cl_2 (4 × 20 mL). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered, and concentrated. Purification by flash chromatography (35% EtOAc/hexanes) afforded after vacuum drying 432 mg (40% yield) of 25-hydroxy-Grundmann's ketone as a colorless oil. *^1H-NMR* (300 MHz, $CDCl_3$): δ 0.62 (3H, C_{18}-CH_3, s), 0.95 (3H, C_{21}-CH_3, d, $J \sim 5.9$ Hz), 1.20 (6H, $C_{26,27}$-CH_3, s), 2.43 (1H, dd, $J \sim 11.6$, 7.6 Hz). *^{13}C-NMR* (75.5 MHz, $CDCl_3$): δ 12.4, 18.7, 19.0, 20.7, 24.0, 27.5, 29.1, 29.3, 35.4, 36.2, 38.9, 40.9, 44.2, 49.9, 56.6, 61.9, 70.9, 212.1.

De-*A,B*-14-epi-25-hydroxy-8-cholestanone (13). To a solution of 1.0 M NaOMe in methanol (421 μL) was added **12** (99.9 mg, 0.356 mmol) in MeOH (120 μL) at room temperature under an argon atmosphere. After stirring for 48 h, the ice-cooled mixture was quenched with acetic acid (49 μL, 0.86 mmol) and diluted with water. The crude mixture was extracted with hexanes, and the combined organic layers were washed with brine (3 × 10 mL), dried (Na_2SO_4), filtered, and concentrated to yield a mixture consisting of ~71% epi isomer **13** and ~29% starting material **12**. Purification by HPLC (25 × 1 cm Rainin, silica gel, 35% EtOAc/hexanes) afforded in order of elution 49 mg of the epi-ketone (49%) and 25 mg (25%) of starting material. *^1H-NMR* (300 MHz, $CDCl_3$): δ 0.89 (1H, C_{22}-CH_3, d, $J \sim 6.3$ Hz), 1.02 (3H, C_{18}-CH_3, s), 1.18 (6H, $C_{26,27}$-CH_3, s). *^{13}C-NMR* (75.5 MHz, $CDCl_3$): δ 19.0, 20.7, 21.1, 21.2, 23.0, 27.7, 29.1, 29.2, 34.3, 36.0, 36.3, 40.1, 44.2, 48.6, 50.4, 61.3, 70.9, 213.7.

De-*A,B*-14-epi-25-[(trimethylsilyl)oxy]-8-cholestanone (14). *N*-(Trimethylsilyl)imidazole (259.7 mg, 1.85 mmol) was added dropwise to a stirred solution of **13** (173.4 mg, 0.618 mmol) in dry THF (7 mL). The mixture was stirred for 4.5 h under argon and was then passed directly through a

short column of silica gel (15% EtOAc/hexanes). The eluent was concentrated and subjected to flash chromatography (15% EtOAc/hexanes) to afford after vacuum drying 194.2 mg (89%) of the TMS-protected alcohol **14** as a colorless oil. *¹H-NMR* (300 MHz, $CDCl_3$): δ 0.095 (9H, TMS methyl H's, s), 0.91 (3H, C_{21}, d, $J \sim 6.3$ Hz), 1.04 (3H, C_{18}, s), 1.19 (6H, $C_{26,27}$, s). *¹³C-NMR* (75.5 MHz, $CDCl_3$): δ 2.6, 19.0, 20.8, 21.2, 23.0, 27.7, 29.80, 29.83, 34.3, 36.0, 36.3, 40.1, 45.1, 48.6, 50.5, 61.3, 73.9, 213.4.

Kinetic Studies. For the kinetic studies, a solution of freshly purified vitamin was placed in benzene-d_6 such that the final concentration was approximately 2 mg/mL. This solution was then distributed into a series of NMR tubes and cooled to −78 °C. For a kinetic run, a sample was removed and placed in the precalibrated ¹H-NMR probe (QE-300 NMR spectrometer), which was preset to a specific temperature. After thermal equilibration of the sample (in benzene-d_6), the ¹H-NMR spectra were recorded at regular time intervals.

For the isomerization of 14-epi-25-hydroxyvitamin D_3 (**4**) to 14-epi-25-hydroxyprevitamin D_3 (**4'**), the rate of the reaction was monitored by following the disappearance of the δ 6.27 signal (H_7) of vitamin **4**. As a cross-check, the disappearance of the δ 6.55 signal (H_6) or the δ 4.98 signal ($2H_{19}$) was also periodically monitored to gauge the reliability of the electronic integration data. The studies were carried out in triplicate over the temperature range 59.8−89.7 °C. For the determination of the equilibrium constant at 80 °C, the ratio of previtamin to vitamin form was determined after heating the sample for extended periods (~8 half-lives starting from previtamin and ~131 half-lives starting from vitamin). At equilibrium there was present 5.41 ± 0.08% of vitamin at 80 °C. The isomerization of 14-epi-1α,25-dihydroxyvitamin D_3 (**5**) to its thermodynamically more stable previtamin form **5'** was carried out once at a single temperature (80 °C). At 80 °C, $t_{1/2}$ = 11.7 min for the conversion of the 14-epi-1α,25-dihydroxyvitamin D_3 to the corresponding previtamin form (this may be compared to $t_{1/2}$ = 13.7 min obtained for the isomerization of the substrate lacking the 1α-hydroxyl, namely 14-epi-25-hydroxyvitamin D_3). The results of the Arrhenius study are summarized in the text. The supplementary material contains data for the individual kinetic runs.

Biological Evaluation. Intestinal Calcium Absorption (ICA) and Bone Calcium Mobilization (BCM). ICA and BCM were determined in vivo in vitamin D deficient chicks as described previously.[23,24] Twelve hours before assay, the chicks, which had been placed on a zero-calcium diet 48 h before assay, were injected intramuscularly with the vitamin D metabolite or analogue in 0.1 mL of ethanol/1,2-propanediol (1:1, v/v) or with vehicle. At the time of assay, 4.0 mg of $^{40}Ca^{2+}$ + 5 μCi of $^{45}Ca^{2+}$ (New England Nuclear) were placed in the duodenum of the animals anesthetized with ether. After 30 min, the birds were decapitated and the blood collected. The radioactivity content of 0.2 mL of serum was measured in a liquid scintillation counter (Beckman LS8000) to determine the amount of $^{45}Ca^{2+}$ absorbed (which is a measure of ICA). BCM activity was estimated from the increase of total serum calcium as measured by atomic absorption spectrophotometry.

1α,25-$(OH)_2$-D_3 Chick Intestinal Receptor Steroid Competition Assay. A measure of competitive binding to the chick intestinal 1α,25-$(OH)_2$-D_3 receptor was performed by using the hydroxylapatite batch assay.[25] Increasing amounts of nonradioactive 1α,25-$(OH)_2$-D_3 or analogue were added to a standard amount of [³H]-1α,25-$(OH)_2$-D_3 and incubated with chick intestinal cytosol. The relative competitive index (RCI) for the analogues was determined by plotting the percent maximum 1α,25-$(OH)_2$-[³H]-D_3 bound × 100 on the ordinate versus [competitor]/[1α,25-$(OH)_2$-[³H]-D_3] on the abscissa. The slope of the line obtained for a particular analogue is divided by the slope of the line obtained for 1α,25-$(OH)_2$-D_3; multiplication of this value by 100 gives the RCI value. By definition, the RCI for 1α,25-$(OH)_2$-D_3 is 100.

25-OH-D_3 Human Vitamin D Binding Protein Steroid Competition Assay. A steroid competition assay for the human vitamin D binding protein (DBP) was carried out using human DBP (Gc-Globulin, Sigma, St. Louis, MO) as the binding protein. DBP (0.015 mg) in 0.8 μL of 0.1 M barbital buffer, pH 8.6, and [³H]-25-OH-D_3 (0.9 pmol, 20 Ci/mmol, Amersham, Arlington Heights, IL) and nonradioactive competitor (the test analogue or standard 25-OH-D_3 in an appropriate range of concentrations) in 0.1 mL of ethanol were incubated in duplicate in conical tubes on ice. After 2 h, 0.2 mL of a charcoal-dextran slurry (4 g of charcoal, 0.4 g of dextran in 200 mL of barbital buffer) was added. The incubation was continued for another 30 min on ice, and the tubes were centrifuged for 10 min at 900*g*. The tritium, representing [³H]-25-OH-D_3 bound to DBP, was determined in aliquots of the supernatant fluid by liquid scintillation measurement. At least triplicate assays were carried out on each analogue. The data was plotted as [competitor]/[³H]-25-OH-D_3] vs 1/[% maximum bound]. The relative competitive index or RCI was calculated as [slope of competitor]/[slope for 25-OH-D_3] × 100 as described previously.[26e,f] Such plots yield linear transformations characteristic for each analogue, the slopes of which are equal to the analogues competitive index value. Note that although each analogue was assayed in competition with [³H]-25-OH-D_3, the data are expressed as relative to the binding of 1α,25-$(OH)_2$-D_3, with its RCI set to 100. Thus, the RCI of 1α,25$(OH)_2$-D_3 = 100, and the RCI for 25-OH-D_3 = 66 800.

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Supplementary Material Available: Spectral, analytical, and kinetic data (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

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(28) Spectral and other analytical data as well as detailed description of the kinetic studies are given in the supplementary material. NMR spectral data in abbreviated form are presented in the Experimental Section as well. General experimental procedures are also presented in the supplementary material. The purity of all new compounds were judged by a combination of HPLC and ^{1}H- and ^{13}C-NMR analysis before mass spectral determination. The level of purity is indicated by the inclusion of copies of NMR spectra presented in the supplementary material.