Design, Synthesis, and Biological Evaluation of a 1α,25-Dihydroxy-19-norvitamin D_3 Analogue with a Frozen A-Ring Conformation

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To establish the conformation of vitamin D compounds responsible for biological activity, a 1α,25-dihydroxy-19-norvitamin D analogue **4** possessing a 1α-hydroxy group fixed in the axial orientation (β-chair form) was synthesized. The starting compounds were bicyclic lactones **6**, **7a**, and **7b**, derived from the quinic acid lactone, which were converted to the bicyclic ketone **13**. Julia coupling of this compound with sulfone **15** produced the 19-norvitamin D analogue **4**, possessing an additional ring connecting the 3β-oxygen and C-2, and the isomeric 3β-hydroxy compound **5**. *In vitro*, both analogues **4** and **5** exhibit reduced activity compared to the natural hormone **1**, but the binding, differentiation, and transcriptional activities of analogue **5** are markedly higher than that of **4** constrained in the α-chair conformation. Surprisingly, *in vivo* tests in mice showed that the analogue **4** significantly increases serum calcium at dose levels similar to 1α,25-$(OH)_2D_3$. These seemingly discordant results are discussed.

Introduction

It has been established that 1α,25-dihydroxyvitamin D_3[a] (1α,-25-$(OH)_2D_3$, calcitriol, **1**; Figure 1), the functional metabolite of vitamin D, exerts its control over a multitude of biological processes related to calcium and phosphorus homeostasis, cell proliferation and differentiation, and immune regulation.[1,2] Numerous studies have indicated that these biological functions of the natural hormone 1α,25-$(OH)_2D_3$ are mediated through the vitamin D receptor (VDR),[3] a nuclear protein which belongs to the nuclear receptor superfamily.[4] A specific interaction between 1α,25-$(OH)_2D_3$ and the VDR promotes some conformational changes in the protein and allows its subsequent binding to the retinoid X receptor (RXR) and several coactivators. The formed complex binds to a vitamin D response element (VDRE) inducing transcription.[5] It is well-known that the presence of a 1α-hydroxyl group in the vitamin D molecule is crucial for the receptor binding.[6,7] Accumulated crystallographic data indicate that vitamin D compounds are bound to the VDR with the ring A existing in a β-chair conformation (Figure 2a) and possessing an equatorially oriented 1α-hydroxyl. However, in all vitamins which formed these crystalline complexes with the human, rat, or zebra fish VDR, i.e., the natural hormone 1α,25-$OH)_2D_3$[8−10] and its analogues with the modified side chains[10−14] or 2α-substituents[15] as well as 19-nor-1α-hydroxylated D vitamins with 2-methyl[9] or 2-methylene[9,16] substituents, equilibration between the ligand's A-ring chair forms was possible. As a consequence of this fact, the actual A-ring conformation of the complexed ligand, dissolved in the physiological milieu, could be still considered as uncertain. Therefore, a vitamin D analogue existing only in the opposite α-chair A-ring conformation and possessing a 1α-hydroxyl group "frozen" in the axial orientation seemed to be an interesting synthetic target.

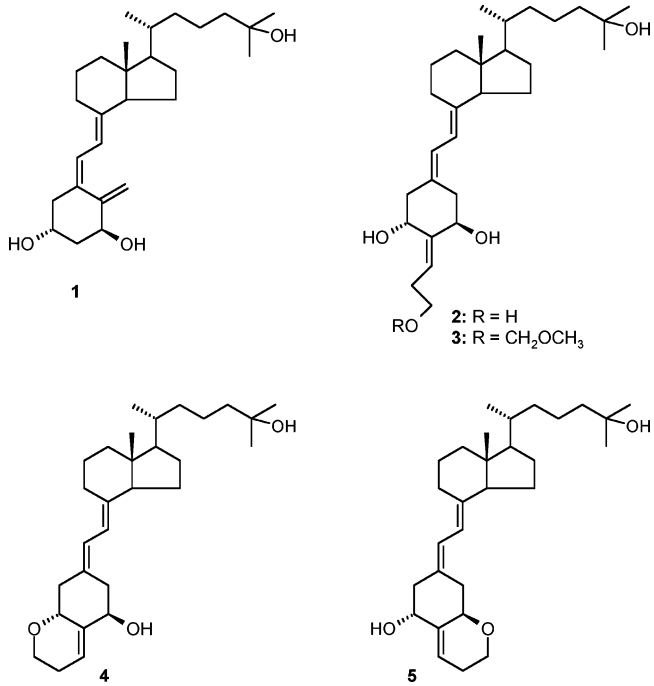

Figure 1. Chemical structure of 1α,25-dihydroxyvitamin D_3 (calcitriol, **1**) and its analogues.

We have recently described the preparation of 2-(3′-hydroxypropylidene)-19-nor-1α,25-$(OH)_2D_3$ (**2**) and its alkoxy derivative **3**.[17,18] Since both of these analogues were characterized by high binding ability to the nuclear vitamin D receptor (VDR), it encouraged us to attempt the synthesis of a closely related 19-norvitamin D compound **4** in which the propylidene substituent at C-2 is connected to a 3β-oxygen atom.[19] The structural features of this molecule (presence of an additional ring and a "flattening bond" system)[20] should restrain the A-ring from interconversion to the alternative chair conformation and "freeze" it in the α-form (Figure 2b).

The strategy of our synthesis was based on the Julia coupling[21,22] of the bicylic ketone of structure **13** (Scheme 1) with the protected sulfone **15** serving as the C,D-ring fragment.

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[a] Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; hVDR, human vitamin D receptor; LBD, ligand binding domain; MOM, methoxymethyl; SEM, 2-(trimethylsilyl)ethoxymethyl.

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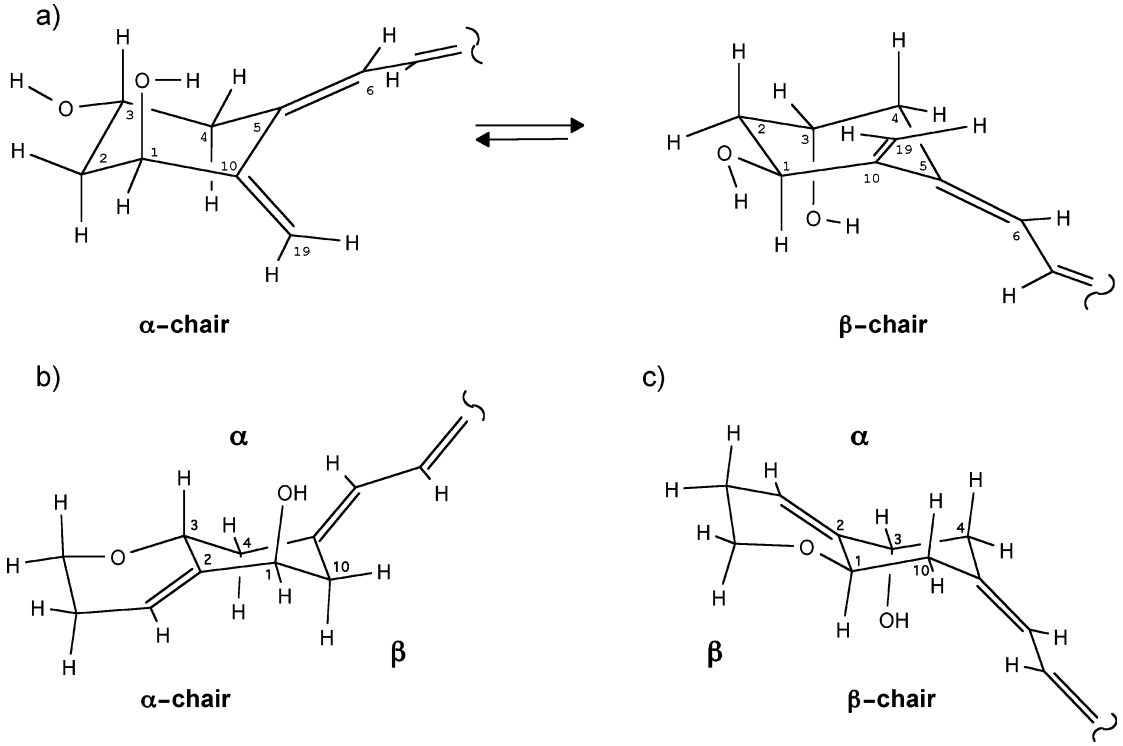


Figure 2. (a) A-Ring conformational equilibrium of 1α-hydroxyvitamin D analogues. The A-ring conformations of the synthesized vitamin D analogues **4** (b) and **5** (c).

We have recently described a conversion of the commercially available (1*R*,3*R*,4*S*,5*R*)-(−)-quinic acid into the bicyclic lactones substituted with alkoxyalkylidene groups (**6** and **7a**,**b**).[17] We anticipated that these compounds, especially the *Z*-isomer **6**, might be useful for the construction of the desired A-ring unit **13**. Sulfone **15**, in turn, could be easily prepared[22] from the protected 25-hydroxy Grundmann ketone.

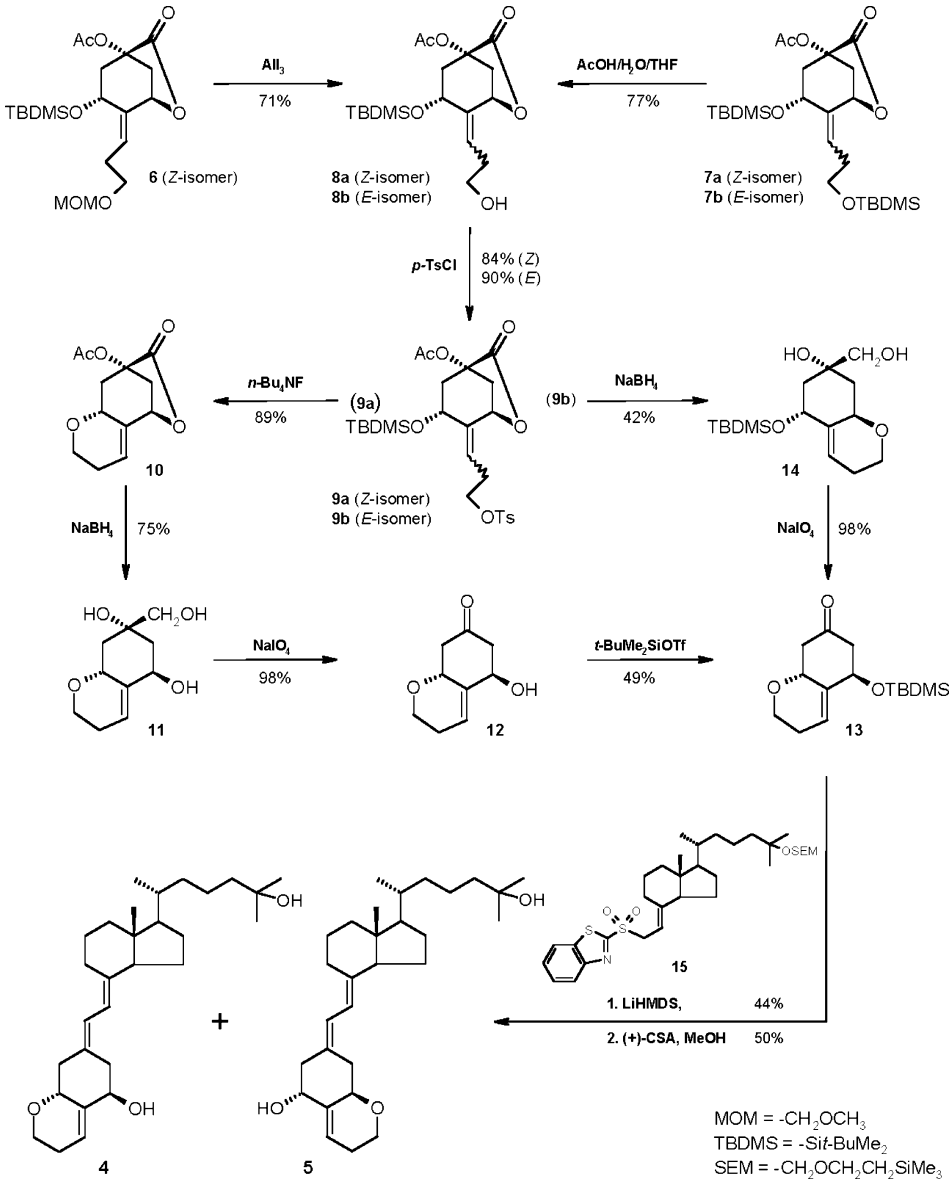
Results and Discussion

Chemistry. As a first starting compound for the synthesis of the ketone **13**, we chose bicyclic lactone **6** (Scheme 1) prepared from the quinic acid as described previously.[17] A wide array of different reagents has been tried for deprotection of the terminal primary hydroxy group in **6**. Unsuccessful attempts to remove the MOM group included HCl in 2-propanol, AG 50W-X4 in methanol, CF_3COOH in methylene chloride, $LiBF_4$ in acetonitrile, Me_3SiBr in methylene chloride, and Ph_3CBF_4 in methylene chloride, whereas low yields of deprotected derivative were achieved with BuSH and $MgBr_2$ in ethyl ether and B-chlorocatecholborane in methylene chloride. Treatment of **6** with aluminum iodide in acetonitrile provided the best yield (71%) of the desired 3′-hydroxypropylidene compound **8a** that was subsequently tosylated under standard conditions. Consistent with our expectations,[23,24] reaction of the tosylate **9a** with tetrabutylammonium fluoride gave an excellent yield (89%) of the cyclized product **10**. The ^{1}H NMR spectrum of **10** supported its tricyclic structure. The signals of the olefinic proton at C-3 and a methine proton at C-7 appeared as a narrow ($w/2 = 11$ Hz) and a broad ($w/2 = 22$ Hz) multiplet, respectively. Spin decoupling experiments confirmed that broadening of the latter signal was due to additional homoallylic couplings with the protons at C-4; such effects are often observed in cyclic systems. Detailed analysis of proton−proton coupling data allowed us to establish that an additional 5,6-dihydro-2*H*-pyran ring exists in the half-chair conformation. Molecular modeling calculations proved that a form of **10**, which possess an oxygen atom situated "below" (α) the plane of C(4)−C(3)═C(2)−C(7) fragment (Figure 3a), is energetically preferred (steric energy lower by 2.44 kcal/mol) to an alternative conformation in which five-atom moiety C(4)−C(3)═C(2)−C(7)−O is almost planar (Figure 3b). Also, the comparison of the respective values of dihydropyran proton couplings observed in the ^{1}H NMR spectrum of **10** with those predicted by the molecular modeling program fully supported the half-chair form (Figure 3a,b). Sodium borohydride reduction of **10** furnished a bicyclic triol **11**. Periodate cleavage of the vicinal diol and subsequent silylation of the formed hydroxy ketone **12** provided the desired A-ring fragment **13**. Interestingly, analysis of ^{1}H NMR data seem to indicate that half-chair conformation of the dihydropyran moiety is also favored in all compounds subsequently synthesized from **10**. Magnitudes of the vicinal couplings of 5-H confirmed the axial orientation of the oxygen substituent at C-5 in both hexahydrochromenone derivatives **12** (Figure 3c) and **13** (Figure 3d).

We also explored an alternative pathway to **13** starting from the mixture of isomeric compounds **7a**,**b** prepared by us previously.[17] Selective deprotection of the primary hydroxyl was achieved without problems by acetic acid-catalyzed hydrolysis, and the formed alcohols **8a**,**b** were separated by column chromatography. The main *E*-isomer **8b** was tosylated and the tosylate **9b** subjected to reduction with sodium borohydride. Spectral data of the obtained product **14** revealed that reduction of the lactone moiety was followed by nucleophilic substitution of the tosyl group resulting in the closure of the dihydropyran ring. Periodate oxidation of the diol **14** furnished the ketone **13**, identical with the previously obtained. Thus, it was proved that both isomeric hydroxypropylidene lactones **8a**,**b** can be efficiently converted to the bicyclic ketone **13**. This hexahydrochromenone derivative was in the next step subjected to modified Julia olefination.

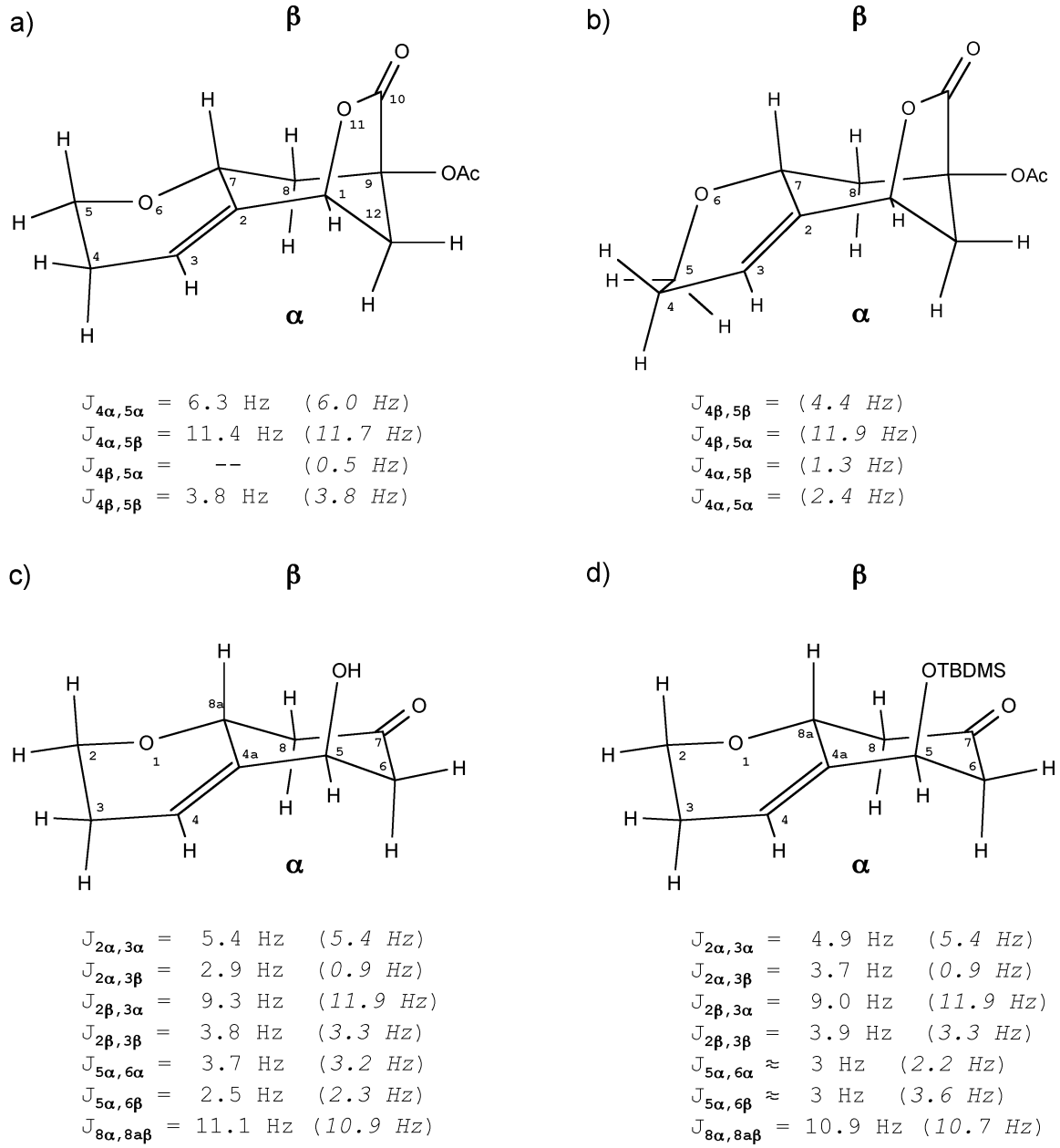
Through preparation of the C,D-ring fragment, sulfone **15** was achieved by the synthetic path depicted in the Scheme 2.

Scheme 1



Thus, the tertiary hydroxyl in the starting 25-hydroxy Grundmann ketone **16**[25] was protected as an SEM ether, and the product was transformed into an α,*β*-unsaturated aldehyde **17** by treatment with the deprotonated *N*-*tert*-butyl-2-(trimethylsilyl)acetaldimine[26] followed by oxalic acid hydrolysis. It was established that only the product with *E*-stereochemistry was formed in such reaction sequence. The aldehyde **17** was reduced with DIBALH, and the resulting allylic alcohol **18** was subjected to Mitsunobu reaction with 2-mercaptobenzothiazole followed by oxidation to form the desired C,D-ring fragment **15**. Coupling of the ketone **13** with an anion, generated from the thiazoline sulfone **15** and lithium bis(trimethylsilyl)amide, followed by removal of the silyl protecting groups gave the expected mixture of two 19-norvitamin D analogues **4** and **5** which were purified and separated by straight- and reversed-phase HPLC. The structures of both products were unequivocally established by analysis of their ^{1}H NMR, UV, and mass spectra. Spin decoupling and ^{1}H NOE difference spectroscopy experiments involving olefinic protons at C-6 and C-7 were helpful in establishing the A-ring conformations of the synthesized 19-norvitamins. Comparison of their proton NMR spectra with those of the previously synthesized 2-ethylidene analogues of 1α,25-dihydroxy-19-norvitamin D_3,[27] characterized by a significant bias toward one chair form of the ring A, proved especially useful. Chemical shifts of the corresponding olefinic and A-ring protons as well as their multiplet patterns show close similarity in the respective vitamin D pairs: (a) analogue **4** and (*Z*)-2-ethylidene-19-nor-1α,25-$(OH)_2D_3$ (Figure 4a), (b) analogue **5** and (*E*)-2-ethylidene-19-nor-1α,25-$(OH)_2D_3$ (Figure 4b). It has, therefore, been confirmed that the secondary A-ring hydroxyl in vitamin D analogues **4** (Figure 2b) and **5** (Figure 2c) has an axial orientation. Ring A in these compounds, because of the presence of an exocyclic double bond being a part of an additional six-membered ring, is prevented from interconversion to the alternative chair conformer. Thus, the compound **4** represents a unique analogue of the vitamin D hormone, with a "frozen" A-ring conformation and can be used for evaluation of the role of 1α-OH orientation in binding with VDR and subsequent biological activity.

Biological Evaluation. Among the synthesized vitamin D compounds **4** and **5**, possessing an additional dihydropyran ring, only the latter has significant binding affinity to the vitamin D receptor (Table 1) albeit decreased more than 500 times compared to 1α,25-$(OH)_2D_3$ (**1**). Studies on the ability of the



$J_{4\alpha,5\alpha}$ = 6.3 Hz (*6.0 Hz*)
$J_{4\alpha,5\beta}$ = 11.4 Hz (*11.7 Hz*)
$J_{4\beta,5\alpha}$ = -- (*0.5 Hz*)
$J_{4\beta,5\beta}$ = 3.8 Hz (*3.8 Hz*)

$J_{4\beta,5\beta}$ = (*4.4 Hz*)
$J_{4\beta,5\alpha}$ = (*11.9 Hz*)
$J_{4\alpha,5\beta}$ = (*1.3 Hz*)
$J_{4\alpha,5\alpha}$ = (*2.4 Hz*)

$J_{2\alpha,3\alpha}$ = 5.4 Hz (*5.4 Hz*)
$J_{2\alpha,3\beta}$ = 2.9 Hz (*0.9 Hz*)
$J_{2\beta,3\alpha}$ = 9.3 Hz (*11.9 Hz*)
$J_{2\beta,3\beta}$ = 3.8 Hz (*3.3 Hz*)
$J_{5\alpha,6\alpha}$ = 3.7 Hz (*3.2 Hz*)
$J_{5\alpha,6\beta}$ = 2.5 Hz (*2.3 Hz*)
$J_{8\alpha,8a\beta}$ = 11.1 Hz (*10.9 Hz*)

$J_{2\alpha,3\alpha}$ = 4.9 Hz (*5.4 Hz*)
$J_{2\alpha,3\beta}$ = 3.7 Hz (*0.9 Hz*)
$J_{2\beta,3\alpha}$ = 9.0 Hz (*11.9 Hz*)
$J_{2\beta,3\beta}$ = 3.9 Hz (*3.3 Hz*)
$J_{5\alpha,6\alpha}$ ≈ 3 Hz (*2.2 Hz*)
$J_{5\alpha,6\beta}$ ≈ 3 Hz (*3.6 Hz*)
$J_{8\alpha,8a\beta}$ = 10.9 Hz (*10.7 Hz*)

Figure 3. Preferred, energy-minimized conformations of the synthesized compounds **10** (a), **12** (c), and **13** (d); a higher energy conformer of **10** (b) is also shown. The most informative $^1H-^1H$ coupling constants are given. Values of the calculated couplings are given in parentheses.

vitamins **4** and **5** to induce differentiation of human promyelocyte HL-60 cells into monocytes show that their potency is decreased by 3 and 2 orders of magnitude, respectively, in comparison with the natural hormone **1**. Analogue **5** also has weak transcriptional activity, indicated in the 24-hydroxylase (CYP-24) promoter driving luciferase reporter gene system, whereas **4** has been found completely inactive in this regard.

Taking into account the above-described *in vitro* test results, very low calcemic activity or even complete lack of *in vivo* activity might be expected for the synthesized analogues **4** and **5**. However, studies conducted with these compounds *in vivo* in vitamin D-deficient mice in doses exceeding 30 times that of the natural hormone, showed that these compounds have a similar calcemic response after 24 h, as **1** and isomer **4** was markedly more active 48 h after the dose was administered (Figure 5). A second study conducted in vitamin D-sufficient CD-1 mice again showed the remarkable *in vivo* activity of analogue **4** to raise blood calcium levels (Figure 6). However, no significant increase in serum calcium was detected with analogue **5**, which is consistent with the vitamin D-deficient mouse study in that the apparent increase in serum calcium after administration of analogue **5** did not turn out to be statistically significant given the variation and the few animals per group ($n = 3$). Interestingly, the analogue that had the least amount of activity *in vitro* (analogue **4** in the α-chair form) has the most *in vivo* activity, and furthermore its potency *in vivo* is similar to that of the native hormone. The last experiment conducted with analogue **4** is shown in Figure 7. This study was again performed in vitamin D-sufficient CD-1 mice. Two different routes of administration (intraperitoneal injection vs oral gavage) were studied. Both routes of administration are effective at causing an increase in serum calcium; however, intraperitoneal injection is more effective than oral gavage. This observation is similar to that of the native hormone. It should also be noted that in the vitamin D-deficient animals, there is a significant delay compared to 1α,25-$(OH)_2D_3$ for an observed increase in serum calcium to occur when the animals are given analogue **4**. It can be suggested that *in vivo* both analogues **4**

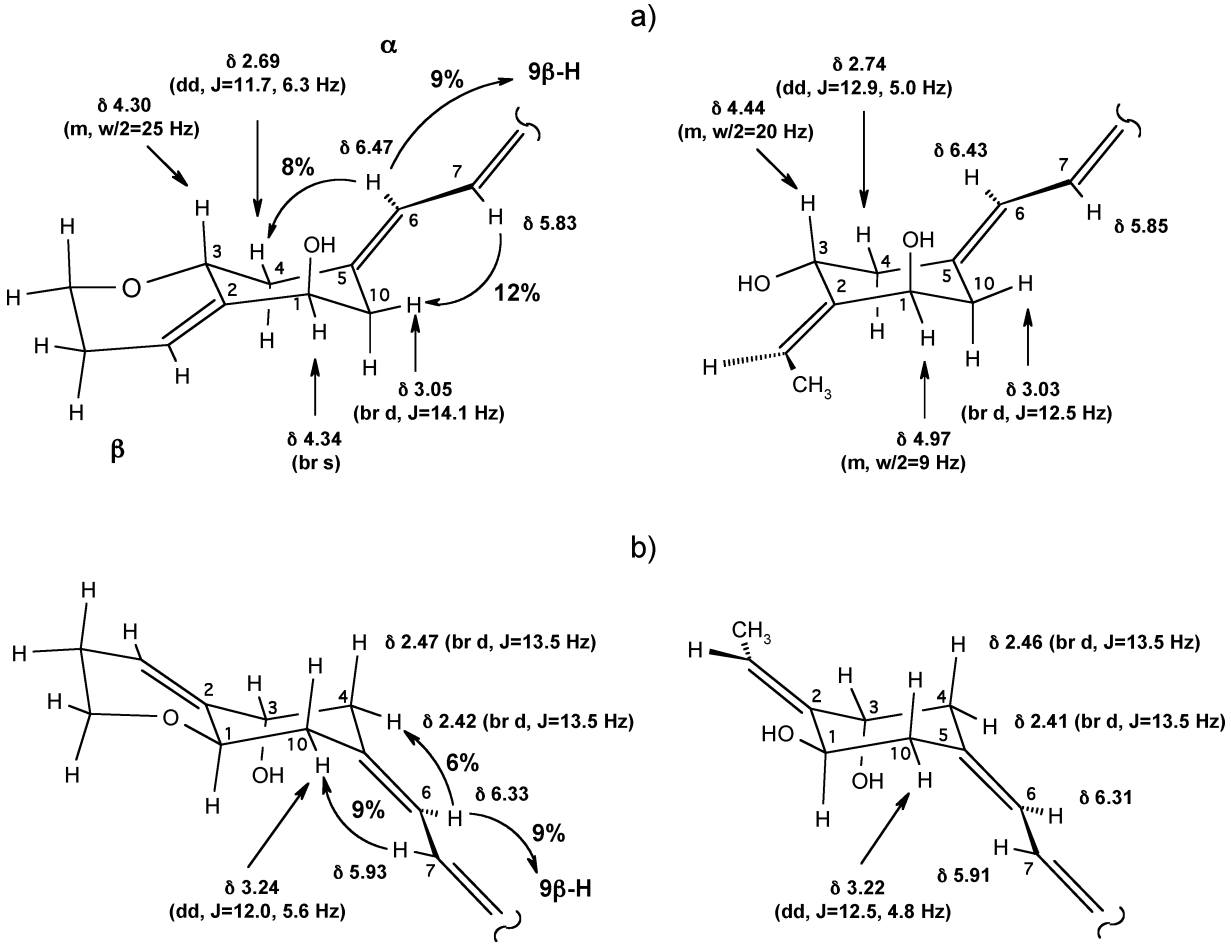
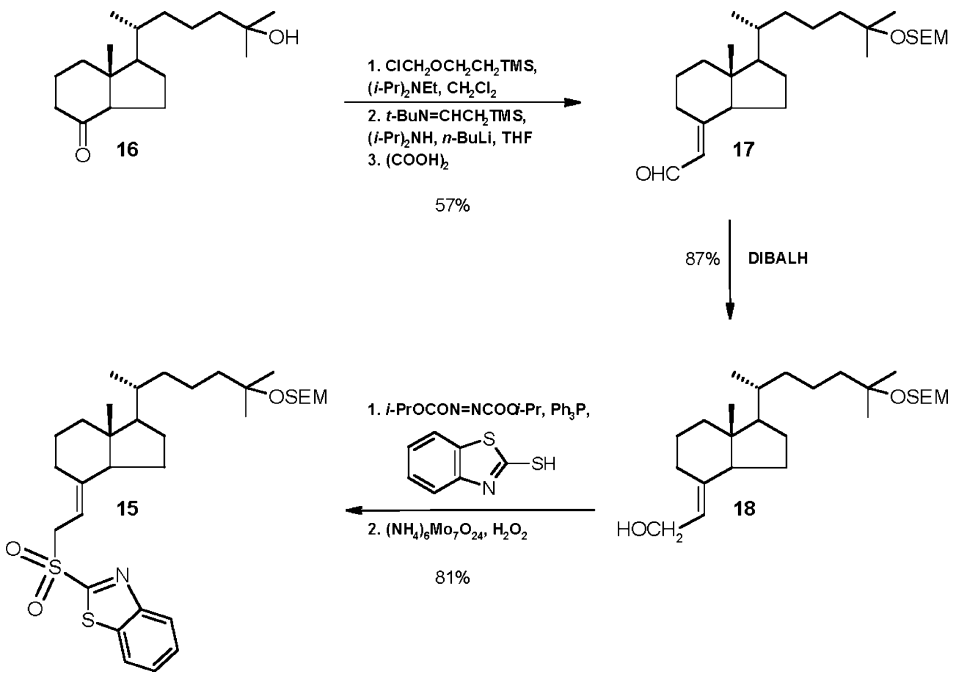


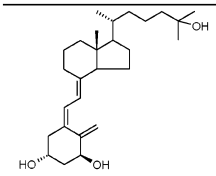
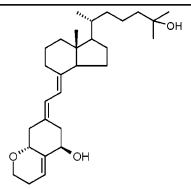
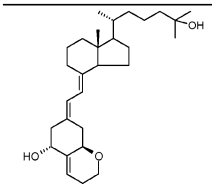
Figure 4. Comparison of preferred, energy-minimized A-ring conformations of (a) the synthesized analogue **4** and (*Z*)-2-ethylidene-19-nor-1α,-25-$(OH)_2D_3$, (b) the synthesized analogue **5** and (*E*)-2-ethylidene-19-nor-1α,25-$(OH)_2D_3$. The most informative proton NMR data (chemical shifts and multiplet patterns) and NOEs are given.

Scheme 2



and **5** might undergo some metabolic change (cleavage of dihydropyran rings at the ether bond?)[28] resulting in one compound with very little biological activity and the other with pronounced *in vivo* activity. If ring cleavage is the metabolic conversion taking place, it is understandable why analogue **4** is much more active than analogue **5** *in vivo*. Unlike analogue **4**, cleavage of the dihydropyran ring in analogue **5** would not produce a 1α-hydroxyl group which is required for VDR

Table 1. VDR Binding Properties,[a] HL-60 Differentiating Activities,[b] and Transcriptional Activities[c] of the Vitamin D Analogues **4** and **5**

compound	compd no.	VDR binding EC_{50}	VDR binding ratio	HL-60 differentiation EC_{50}	HL-60 differentiation ratio	24-OHase transcription EC_{50}	24-OHase transcription ratio
	**1**	5 ± 10^{-11} M	1	2 ± 10^{-9} M	1	2 ± 10^{-10} M	1
	**4**	$> 10^{-5}$ M	$> 10^{6}$	ca. 10^{-6} M	$> 10^{3}$	no activity	
	**5**	3 ± 10^{-8} M	560	ca. 10^{-7} M	$> 10^{2}$	1 ± 10^{-7} M	500

[a] Competitive binding of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized vitamin D analogues to the full-length recombinant rat vitamin D receptor. The experiments were carried out in duplicate on two different occasions. The K_i values are derived from dose−response curves and represent the inhibition constant when radiolabeled 1α,25-$(OH)_2D_3$ is present at 1 nM and a K_d of 0.2 nM is used. The binding ratio is the average ratio of the analogue K_i to the K_i for 1α,25-$(OH)_2D_3$. [b] Induction of differentiation of HL-60 promyelocytes to monocytes by 1α,25-$(OH)_2D_3$ (**1**) and the synthesized vitamin D analogues. Differentiation state was determined by measuring the percentage of cells reducing nitro blue tetrazolium (NBT). The experiment was repeated in duplicate two times. The ED_{50} values are derived from dose−response curves and represent the analogue concentration capable of inducing 50% maturation. The differentiation activity ratio is the average ratio of the analogue ED_{50} to the ED_{50} for 1α,25-$(OH)_2D_3$. [c] Transcriptional assay in rat osteosarcoma cells stably transfected with a 24-hydroxylase gene reporter plasmid. The ED_{50} values are derived from dose−response curves and represent the analogue concentration capable of increasing the luciferase activity 50%. The lucerifase activity ratio is the average ratio of the analogue ED_{50} to the ED_{50} for 1α,25-$(OH)_2D_3$.

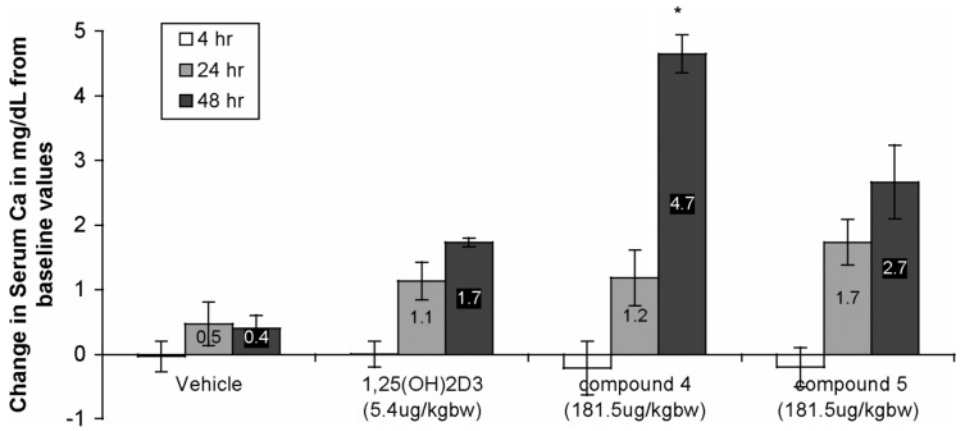


Figure 5. Serum calcium change in response to a single, intraperitoneal injection in D-deficient CD-1 mice. Statistical significance ($p < 0.05$) compared to the vehicle group is indicated by an asterisk.

binding.[6,7] Further studies will be required to provide an understanding for the *in vivo* biological activity.

Discussion

Conformational equilibrium of the cyclohexane ring A of vitamin D compounds and its influence on biological activity has been studied for more than three decades.[29,30] In 1974 Okamura hypothesized that the β-chair form, which possesses an equatorial 1α-hydroxyl, is responsible for the biological activities of vitamin D analogues.[31] Our early studies on 1α,-25-dihydroxy-10,19-dihydrovitamin D_3 seemed to contradict this suggestion.[32] Then, the results of biological testing and conformational analysis of 2-methyl-substituted analogues of the hormone **1**, synthesized by Japanese scientists,[33] and their 19-nor counterparts obtained in our laboratory[34] prompted us to suggest that an axial orientation of 1α-OH might be of crucial importance for exertion of calcemic activity. It was found that 2α-alkylated vitamins, characterized by strong bias (above 90%) toward conformers with the axial hydroxyl at C-1, are much more biologically potent then the respective 2β-isomers existing in solution primarily in the opposite β-chair form.[34] Afterward, however, the Moras group reported the crystal structure of the hVDR ligand binding domain (LBD) complexed with hormone **1**[8] and several other ligands characterized by an unnatural configuration at C-20.[11] All these results clearly indicated that the receptor binds (at least in the crystalline state) vitamin D compounds having their A-rings in the β-chair conformation. Even more convincing was the recent report from our laboratory,

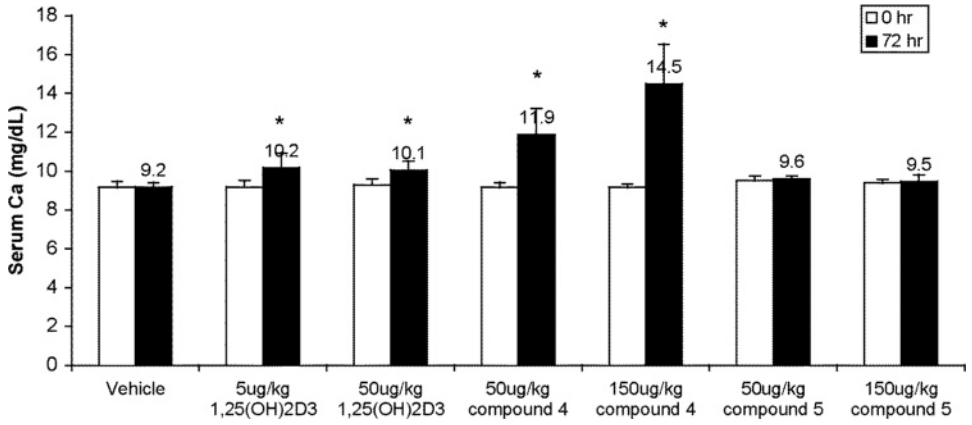


Figure 6. Serum calcium change in response to a single, intraperitoneal injection in D-sufficient CD-1 mice. Statistical significance ($p < 0.05$) compared to the vehicle group is indicated by an asterisk.

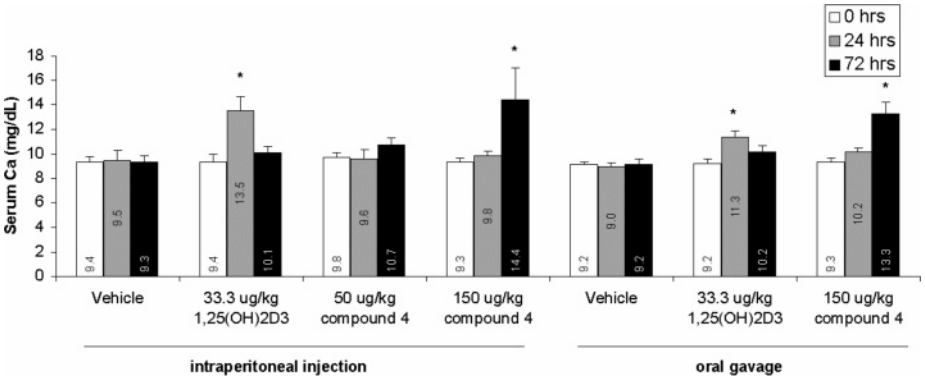


Figure 7. Serum calcium change in response to different routes of administration in D-sufficient CD-1 mice. Statistical significance ($p < 0.05$) compared to the vehicle group is indicated by an asterisk.

in which Vanhooke described the crystal structure of the rat VDR LBD complexed with a 1α,25-dihydroxy-2α-methyl-19-norvitamin D_3,[9] possessing highly elevated biopotency in both intestine and bone.[34] The study shows that this vitamin D compound also adopts the β-chair A-ring conformation in its crystalline complex with VDR. However, in all these cases interconversion between the ligand's A-ring chair conformers was not prohibited, and therefore some doubts could arise which form of the ligand−VDR complex actually exists in the real physiological environment. Thus, it was tempting to synthesize and biologically evaluate a vitamin D compound possessing an axially oriented hydroxyl group at C-1 and unable to change its A-ring conformation. The 1α,25-dihydroxyvitamin D_3 analogue **4**, described in the present work, fulfills these requirements. It was established that such vitamin D does not bind to the VDR and lacks activity in cellular differentiation and in inducing transcription of a vitamin D-responsive gene. Notably, its isomer **5** possessing free 3β- and 25-hydroxyl groups, but characterized by a "frozen" A-ring β-chair conformation, was found to be ca. 560 times less potent in binding to the receptor than the hormone **1**. Such binding ability could be expected for a 25-OH-D_3 derivative in which the 1α-oxygen function cannot act as a hydrogen donor to create the hydrogen bonds with the amino acids from the LBD.[6] Considering the structure of the vitamins **4** and **5**, possessing a *trans*-conformation of the intercyclic 5,7-diene fragment and the 25-hydroxycholestane side chain, it would be expected that only genomic VDR ligand binding pocket[35] can accommodate both these compounds.

The biological results obtained *in vitro* on the synthesized analogues **4** and **5** clearly confirm that the A-ring β-chair conformation and, consequently, equatorial orientation of the 1α-OH are necessary for the vitamin D compound to ensure its binding with VDR and exertion of biological activity. Biological evaluation of the tested vitamins *in vivo* does not generate results consistent with those obtained *in vitro*, most likely due to metabolic transformation of both compounds occurring in the animal.

Conclusions

The conformations of the A-rings of vitamins **4** and **5**, as well as the structures of intermediate compounds used for the construction of their A-ring parts, were established by NMR spectroscopic methods. Analysis of the observed vicinal proton coupling constants proved that in the synthesized vitamin D analogues **4** and **5**, possessing an additional dihydropyran ring, their A rings could only exist in a single conformation, α- and β-chair, respectively. Biological *in vitro* testing of the analogues **4** and **5** allowed us to conclude that the presence of an equatorially oriented free hydroxyl at C-1 is necessary for binding to the vitamin D receptor. Thus, only a vitamin D analogue that can assume a β-chair A-ring conformation can be accepted by the VDR, further inducing conformational changes crucial for the ligand−receptor activation.

Experimental Section

Chemistry. Melting points (uncorrected) were determined on a Thomas-Hoover capillary melting-point apparatus. Ultraviolet (UV) absorption spectra were recorded with a Perkin-Elmer Lambda 3B UV−vis spectrophotometer in ethanol. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 400 and 500 MHz with a Bruker Instruments DMX-400 and DMX-500 Avance console spectrometers in deteriochloroform. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 125 MHz with a Bruker Instruments DMX-500 Avance console in deuteriochloroform. Chemical

shifts (δ) are reported downfield from internal Me_4Si (δ 0.00). Electron impact (EI) mass spectra were obtained with a Micromass AutoSpec (Beverly, MA) instrument. High-performance liquid chromatography (HPLC) was performed on a Waters Associates liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model U6K Universal injector, and a Model 486 tunable absorbance detector. THF was freshly distilled before use from sodium benzophenone ketyl under argon.

The starting compounds **6** and **7a**,**b** were obtained from commercial (−)-quinic acid according to the published procedures.[17]

(4*Z*)-(1*R*,3*R*,5*R*)-1-Acetoxy-3-[(*tert*-butyldimethylsilyl)oxy]-4-[3′-hydroxypropylidene]-6-oxabicyclo[3.2.1]octan-7-one (8a). To a solution of **6** (26 mg, 63 μmol) in anhydrous CH_3CN (0.6 mL) was added AlI_3 (170 mg, 0.42 mmol) at 0 °C under argon. The mixture was stirred at 0 °C for 50 min, poured into aq $Na_2S_2O_3$, and extracted with ethyl acetate. The extract was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by flash chromatography. Elution with hexane/ethyl acetate (6:4) afforded pure alcohol **8a** (16.5 mg, 71%) as a colorless oil.

(4*E*)-(1*R*,3*R*,5*R*)-1-Acetoxy-3-[(*tert*-butyldimethylsilyl)oxy]-4-[3′-hydroxypropylidene]-6-oxabicyclo[3.2.1]octan-7-one (8b). To a solution of a mixture of isomeric compounds **7a** and **7b** (2:3 ratio; 700 mg, 1.49 mmol) in THF (6 mL) were added acetic acid (18 mL) and water (6 mL). The solution was stirred at room temperature for 5 h, cooled to 0 °C, poured into saturated $NaHCO_3$, and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and evaporated. The oily residue was purified by column chromatography on silica. Elution with hexane/ethyl acetate (75:25) afforded a less polar *Z*-isomer **8a** (84 mg), a mixture of **8a** and **8b** (147 mg), and a pure more polar *E*-isomer **8b** (180 mg); overall yield of both products 77%.

(4*Z*)-(1*R*,3*R*,5*R*)-1-Acetoxy-3-[(*tert*-butyldimethylsilyl)oxy]-6-oxa-4-[3′-(*p*-toluenesulfonyloxy)propylidene]bicyclo[3.2.1]octan-7-one (9a). To a solution of hydroxy compound **8a** (16 mg, 43 μmol) in anhydrous pyridine (140 μL) were added at 0 °C *p*-toluenesulfonyl chloride (24 mg, 127 μmol) and a catalytic quantity of 4-(dimethylamino)pyridine. The mixture was stirred at 0 °C for 1 h and at 6 °C for 18 h. It was then poured into ice/saturated $NaHCO_3$, shaken for 15 min, and extracted with ethyl acetate and benzene. The combined extracts were washed with saturated $NaHCO_3$, water, saturated $CuSO_4$, and water again, dried (Na_2SO_4), and evaporated. The residue (ca. 16 mg) was dissolved in benzene/hexane, applied on a silica Sep-Pak cartridge, and washed with hexane/ethyl acetate (95:5, 10 mL) to remove polar impurities. Elution with hexane/ethyl acetate (85:15, 20 mL) provided a pure oily tosylate **9a** (19 mg, 84%).

(4*E*)-(1*R*,3*R*,5*R*)-1-Acetoxy-3-[(*tert*-butyldimethylsilyl)oxy]-6-oxa-4-[3′-(*p*-toluenesulfonyloxy)propylidene]bicyclo[3.2.1]octan-7-one (9b). Tosylate **9b** was obtained from hydroxy compound **8b** by tosylation performed analogously to the process described above for **8a** except the product was purified by column chromatography. Apolar impurities were removed with hexane/ethyl acetate (95:5). Further elution with hexane/ethyl acetate (75:25) provided a pure oily tosylate **9b** in 90% yield.

(1*R*,7*R*,9*S*)-(9-Acetoxy-6,11-dioxa-tricyclo[7.2.1.0*2,7*]dodec-2-en-10-one (10). Solution of tosylate **9a** (18.8 mg, 36 μmol) in dry THF (8 mL) was treated with tetrabutylammonium fluoride (1.0 M in THF, 180 μL, 180 μmol). The mixture was stirred under argon at room temperature for 18 h, poured into brine, and extracted with ethyl acetate and benzene. Organic extracts were washed with brine, dried (Na_2SO_4), and evaporated. The oily residue was dissolved in hexane, applied on a silica Sep-Pak cartridge, and washed with hexane/ethyl acetate (85:15, 20 mL) to give a pure tricyclic product **10** (7.6 mg, 89%) as an oil.

(5*R*,7*R*,8a*R*)-7-Hydroxymethyl-3,5,6,7,8,8a-hexahydro-2*H*-chromene-5,7-diol (11). To a solution of tricyclic compound **10** (9.5 mg, 40 μmol) in anhydrous ethanol (1.0 mL) at 0 °C was added $NaBH_4$ (15 mg, 0.4 mmol). The resultant mixture was then stirred at room temperature for 18 h, a small volume of brine/saturated NH_4Cl was added, and the solvents were removed in vacuum. The residue was washed several times with warm ethanol. The ethanol extracts were combined and evaporated to dryness with benzene. The solid residue was then washed a few times with warm chloroform. The combined chloroform extracts were concentrated to a small volume and applied on a silica Sep-Pak cartridge. Elution with hexane/ethyl acetate (1:9, 20 mL) yielded a pure semisolid triol **11** (6 mg, 75%).

(5*R*,8a*R*)-5-Hydroxy-2,3,5,6,8,8a-hexahydrochromen-7-one (12). Sodium periodate−saturated water (50 μL) was added to a solution of the triol **11** (5 mg, 2.6 μmol) in methanol (200 μL) at 0 °C. The mixture was stirred at 0 °C for 1 h, thioanisole (3 μL) was added, and stirring was continued for 10 min. The mixture was diluted with benzene/ethyl acetate (1:1, 1 mL) and filtered through a silica Sep-Pak. The Sep-Pak was washed with an additional 5 mL of the same solvent system, the combined solutions were evaporated, and the residue was redissolved in hexane/ethyl acetate (7:3) and applied on a silica gel Sep-Pak. Elution with the same solvent system (10 mL) provided aromatic compounds whereas a pure hydroxy ketone **12** (4.1 mg, 98%) was eluted with hexane/ethyl acetate (1:1, 10 mL) as a colorless oil.

(5*R*,8a*R*)-5-[*tert*-Butyldimethylsilyl)oxy]-2,3,5,6,8,8a-hexahydrochromen-7 -one (13). (a) To a solution of hydroxy ketone **12** (4 mg, 24 μmol) in anhydrous methylene chloride (90 μL) were added 2,6-lutidine (7 μL, 60 mmol) and *tert*-butyldimethylsilyl trifluoromethanesulfonate (12 μL, 51 mmol) at −50 °C. The reaction mixture was stirred at −50 °C for 50 min, diluted with cold methylene chloride, and poured into water. The organic phase was washed with saturated $CuSO_4$ and water, dried (Na_2SO_4), and evaporated. The residue was redissolved in hexane and applied on a silica gel Sep-Pak. Elution with hexane/ethyl acetate (95:5, 10 mL) provided a less polar compound (2.1 mg), the TBDMS derivative of the enol ether derived from **13**. The desired protected hydroxy ketone **13** (3.3 mg, 49%) was eluted with hexane/ethyl acetate (9:1, 10 mL) as a colorless oil. Further elution with the same solvent system afforded 2,3,8,8a-tetrahydrochromen-7-one (0.6 mg).

(b) To a solution of compound **9b** (20 mg, 38 μmol) in anhydrous ethanol (0.5 mL) at 0 °C was added $NaBH_4$ (14 mg, 0.38 mmol), and the mixture was stirred at 5 °C for 18 h and then for 20 h at room temperature. The saturated NH_4Cl was added, and the mixture was poured into brine and extracted several times with ether and methylene chloride. The extracts were washed with brine, combined, dried ($MgSO_4$), and evaporated. The oily residue was purified by silica Sep-Pak cartridge. Elution with hexane/ethyl acetate (7:3) gave pure product **14** as semicrystals (115 mg, 79%).

Sodium periodate cleavage of the diol **14** was performed analogously to the process described above for **11**. The semicrystalline ketone **13** (97% yield) was eluted from a Sep-Pak cartridge with hexane/ethyl acetate (9:1).

[(1*R*,3a*S*,7a*R*)-7a-Methyl-1-[(*R*)-6-[(2-trimethylsilyl)-ethoxymethoxy]-6-methylheptan-2-yl]-octahydroinden-(4*E*)-ylidene]acetaldehyde (17). To a solution of 25-hydroxy Grundmann ketone **16**[25a] (186 mg, 0.663mmol) in anhydrous methylene chloride (0.3 mL) and *N*,*N*-diisopropylethylamine (448 mg, 3.46 mmol) was added 2-(trimethylsilyl)ethoxymethyl chloride (166 mg, 0.995 mmol). The mixture was strirred at room temperature for 1.5 h and then poured into 2% aq HCl solution and extracted with methylene chloride. The extracts were washed with diluted $NaHCO_3$, dried ($MgSO_4$), and evaporated. An oily residue was purified by column chromatography. Elution with hexane/ethyl acetate (95:5) gave pure protected Grundmann ketone, (1*R*,3a*R*,7a*R*)-7a-methyl-1-[(*R*)-6-[(2-trimethylsilyl)ethoxymethoxy]-6-methylheptan-2-yl]-octahydroinden-4-one (220 mg, 81%) as a colorless oil.

To a solution of diisopropylamine (70 μL, 0.507 mmol) in anhydrous THF (0.58 mL) was added *n*-BuLi (2.0 M in cyclohexane, 254 μL, 0.507 mmol) under argon at −78 °C. After being stirred for 5 min, the mixture was warmed to 0 °C and a solution of *N*-*tert*-butyl-2-(trimethylsilyl)acetaldimine[26c] (504 μmol, 86 mg) in anhydrous THF (0.5 mL) was added. The mixture was stirred for 20 min and cooled to −78 °C, and a solution of protected Grundmann ketone (86 mg, 0.205 mmol) in anhydrous THF (1.1 mL) was added. The resulting mixture was allowed to warm to

−20 °C during 30 min. After the mixture was stirred for additional 1.5 h at this temperature, 5% aq solution of oxalic acid solution (1.4 mL) was added and the mixture was stirred for 1.5 h at room temperature. Then it was poured into water and extracted with ether. The organic phase was washed with diluted $NaHCO_3$ and water, dried ($MgSO_4$), and evaporated. An oily residue was purified by column chromatography. Elution with hexane/ethyl acetate (95:5) gave pure oily aldehyde **17** (64 mg, 70%).

2-[(1*R*,3a*S*,7a*R*)-7a-Methyl-1-[(*R*)-6-[(2-trimethylsilyl)-ethoxymethoxy]-6-methylheptan-2-yl]octahydroinden-(4*E*)-ylidene]ethanol (18). Diisobutylaluminum hydride (1.0 M in toulene, 357 μL, 0.357 mmol) was added to a solution of the aldehyde **17** (57 mg, 0.131 mmol) in anhydrous toluene (1 mL) at −78 °C under argon. The mixture was stirred for 1 h at −78 °C, quenched by addition of 2 N potassium sodium tartrate, poured into the water, and extracted with ethyl acetate. The organic extract was washed with brine, dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography. Pure allylic alcohol **18** (50 mg, 87%) was eluted with hexane/ethyl acetate (9:1).

(1*R*,3a*S*,7a*R*)-4-[2-(Benzothiazole-2-sulfonyl)-(4*E*)-ethylidene]-7a-methyl-1-[(*R*)-6-[(2-trimethylsilyl)ethoxymethoxy]-6-methylheptan-2-yl]-octahydroindene (15). To a stirred solution of 2-mercaptobenzotriazole (52.9 mg, 317 μmol) and triphenylphosphine (82.4 mg, 317 μmol) in anhydrous methylene chloride (750 μL) were added a solution of the allylic alcohol **18** (91.5 mg, 210 μmoL) in anhydrous methylene chloride (750 μL) and DIAD (61 μL, 210 μmol) at 0 °C. After being stirred for 1 h at this temperature, the solvents were evaporated in vacuo. The residue was redissolved in ethanol and cooled to 0 °C, and 30% H_2O_2 (130 μL) followed by $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (51.6 mg, 41.9 μmol) was added. After being stirred for 3 h at room temperature, the mixture was poured into cold saturated Na_2SO_3 and extracted with ethyl acetate. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was redissolved in a small volume of benzene/hexane (1:1) and applied on a silica column. Pure oily allylic sulfone **15** (105 mg, 81%) was eluted with hexane/ethyl acetate (98:2).

1α,25-Dihydroxy- and 25-Hydroxy-19-norvitamin D_3 Analogues (4 and 5). To a solution of sulfone **15** (38.0 mg, 61 μmol) in dry THF (200 μL) was added LiHMDS (1 M in THF, 60 μL, 60 μmol) at −78 °C under argon. The solution turned deep red. The mixture was stirred at −78 °C for 20 min, and a solution of the ketone **13** (7.5 mg, 26.6 μmol) in THF (100 + 80 μL) was added. The stirring was continued at −78 °C for 1.5 h, and the reaction mixture was allowed to warm to −10 °C during ca. 1.5 h. Then it was poured into saturated NH_4Cl and extracted with ether. The extract was washed with brine, dried (Na_2SO_4), and evaporated. The yellow oily residue was purified by column chromatography. Elution with hexane/ethyl acetate (97:3) afforded the silylated 19-norvitamins (7 mg, 44%). Further elution with hexane/ethyl acetate (95:5) gave unreacted sulfone **15** (27 mg) whereas unchanged ketone **13** (1 mg) was eluted with hexane/ethyl acetate (94:6).

The obtained mixture of protected 19-norvitamins (3.5 mg, 5.1 μmol) was dissolved in anhydrous methanol (0.6 mL) and treated with (+)-10-camphorosulfonic acid (10 mg, 43 μmol). The solution was stirred under argon at room temperature for 19 h, poured into brine, and extracted with ethyl acetate. The extract was washed with diluted $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated. The residue was purified by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using a hexane/2-propanol (9:1) solvent system. Isomeric 19-norvitamins **4** (0.25 mg, 11%) and **5** (0.87 mg, 39%) were collected at R_V 31 mL and R_V 35 mL, respectively. Final purification and separation of both isomers was achieved by reversed-phase HPLC (9.4 mm × 25 cm Zorbax-ODS column, 4 mL/min) using methanol/water (97:3) solvent system. 1α,25-Dihydroxy-19-norvitamin D analogue **4** was collected at R_V 31.5 mL and its isomer **5** at R_V 18 mL.

Biological Studies. 1. *In Vitro* Studies. VDR binding, HL-60 differentiation and 24-hydroxylase transcription assays were performed as previously described.[18]

2. *In Vivo* Studies. First Study. Female, CD-1 mice (6 weeks old) were purchased from Harlan Sprague−Dawley Co. (Indianapolis, IN). Upon arrival, the animals were placed in a room with filtered lighting and fed a purified, vitamin D-deficient diet[36] for 17 weeks. The mice were then assigned to four groups (n = 3/group) and given a single intraperitoneal injection of vehicle (95:5 propylene glycol/ethanol), 1α,25-$(OH)_2D_3$, analogue **4**, or analogue **5**. Blood was collected prior to dose administration and multiple time points thereafter. Serum calcium was measured by diluting with 0.1% lanthanum chloride and reading the absorbance using an atomic absorption spectrometer. The change in serum calcium from predose values (baseline) is reported.

Second Study. Female CD-1 mice (6−7 week old) were purchased from Harlan (Indianapolis, IN). The animals were group housed and fed a purified diet containing 0.47% calcium.[36] After a 5−7 day acclimation period, the animals were assigned to treatment groups (n = 5−6/group) and given a single dose of the designated analogues by intraperitoneal injection. Blood was collected for serum calcium concentration analyses immediately prior to dose administration and 72 h following dose delivery. Serum calcium was analyzed as described above.

Third Study. Female, CD-1 mice (6−7 week old) were purchased from Harlan (Indianapolis, IN). The animals were group housed and fed a purified diet containing 0.47% calcium.[36] After a 5−7 day acclimation period, the animals were assigned to treatment groups (n = 5/group) and given a single dose of the designated analogues by intraperitoneal injection or oral gavage. Blood was collected for serum calcium concentration analyses at various timepoints following dose delivery. Serum calcium was analyzed as described above.

Statistical Analysis. In vivo data were analyzed by one-way ANOVA followed by pairwise comparisons when significant overall differences were detected. Post-hoc analyses included Tukey's, Scheffe's, and Fisher's LSD tests. Only differences ($p < 0.05$) that were present in two out of the three post-hoc tests were considered significant.

Molecular Modeling. Molecular mechanism studies were used to establish the energy-minimized structures of the most important synthetic intermediates. The calculation of optimized geometries and steric energies was carried out using the algorithm from the MM^+ HyperChem (release 7.0) software package (Autodesk, Inc.). MM^+ is an all-atom force field based on the MM2 functional form. The couplings observed in the ^1H NMR spectra of the synthesized compounds were compared to those calculated using PC MODEL (release 9.0) molecular modeling software (Serena Software); molecular modeling was performed in the MMX mode. The force field MMX is an enhanced version of MM2, with the pi-VESCF routines taken from MMP1.

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Supporting Information Available: Purity criteria and spectral data of the synthesized compounds; figures with either the competitive binding curves or dose−response curves derived from the binding and cellular differentiation and transcriptional assays of the vitamin D analogues **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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