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Synthesis and Biological Activity of 25-Hydroxy-2-methylene-vitamin D_3 Analogues Monohydroxylated in the A-ring

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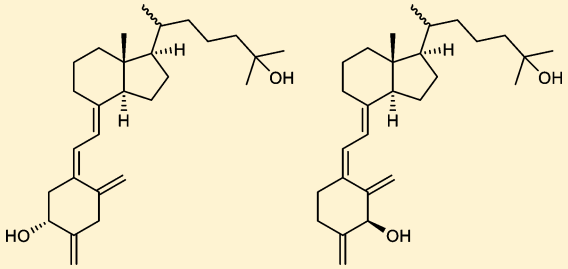
Supporting Information

ABSTRACT: The 20*R*- and 20*S*-isomers of 25-hydroxy-2-methylene-vitamin D_3 and 3-desoxy-1α,25-dihydroxy-2-methylene-vitamin D_3 have been synthesized. Two alternative synthetic routes were devised for preparation of the required A-ring synthons, starting from the chiral compound derived from the (−)-quinic acid and, alternatively, from the commercially available achiral precursor, monoprotected 1,4-cyclohexanedione. The A-ring dienynes were coupled by the Sonogashira process with the respective C,D-ring fragments, the enol triflates derived from the protected (20*R*)- or (20*S*)-25-hydroxy Grundmann ketones. All four compounds possessed significant *in vivo* activity on bone calcium mobilization and intestinal calcium transport. The presence of a 2-methylene group increased intestinal calcium transport activity of all four analogues above that of the native hormone, 1α,25-dihydroxyvitamin D_3. In contrast, bone calcium mobilization was equal to that produced by 1α,25-dihydroxyvitamin D_3 in compounds having a (20*S*)-configuration or diminished to one-tenth that of 1α,25-dihydroxyvitamin D_3 in compounds with a (20*R*)-configuration.



INTRODUCTION

25-Hydroxyvitamin D_3 (25-OH-D_3, **1**, Figure 1) is the major metabolite of vitamin D_3 circulating in the blood, and undergoing hydroxylation at C-1 in the kidneys to biologically active form, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$, calcitriol, **2**].[1] This natural hormone is not only responsible for regulation of mineral homeostasis, but it also has been recognized for numerous antiproliferative, pro-differentiative, and immunomodulatory activities.[2] Calcitriol exerts its major actions through the classical genomic pathway, which involves binding to the vitamin D receptor (VDR)−retinoid X receptor (RXR) complex and subsequent modulation of gene expression;[3,4] rapid actions of 1α,25-$(OH)_2D_3$ have been also established.[5] The unusually broad range of these activities explains increasing interest in the preparation of its analogues characterized by more selective biological profiles and considered as promising therapeutic agents.[6] As a consequence of the efforts of many research groups, directed to the syntheses of calcitriol analogues with interesting structural modifications, some of the discovered compounds have been already clinically used in the treatment of bone diseases, secondary hyperparathyroidism, and psoriasis.[1,6−8]

One of the most interesting structural alterations of the calcitriol molecule, discovered in our laboratory,[9] was the "shift" of its 10-exomethylene substituent from C-10 to C-2. Such modification, combined with an inversion of the natural configuration at C-20, resulted in the analogue, named 2MD (**3**), that strongly acts on bone and leads to dramatic increase in bone density in ovariectomized rats.[10] Taking into account the structure of 2MD, characterized by the presence of allylic 1α- and 3β-hydroxyl groups, it was of interest to investigate whether 2-methylene-19-norvitamins can be enzymatically 1α-hydroxylated in kidney by cytochrome P450 (CYP27B1), analogously to 25-hydroxyvitamin D_3. Therefore, we synthesized such compounds (analogues **4** and **5**) and examined their activity.[11,12] It has been established that 1-desoxy-2MD (**5**) binds to the VDR 1 log less strongly than the natural hormone and 2MD. Further, in comparison with 2MD, the examined compound is significantly less active *in vivo*, which might indicate its reduced tendency to undergo enzymatic 1α-hydroxylation. Also, other A-ring monohydroxylated 19-norvitamin D compounds were obtained in our laboratory, namely, 3-desoxy-2MD (**7**) and its (20*R*)-epimer **6**, and it turned out that they are characterized by significant calcemic potency.[13] High biological activity of compounds discussed above, as well as an intriguing problem of enzymatic oxidation at C-1, encouraged us to perform synthesis of the related series of vitamin D analogues possessing the "natural" 10-exo-methylene group, which can play a crucial role in the 1-hydroxylation process. Thus, to elucidate further the effects of an A-ring possessing two exocyclic methylene groups at C-2 and C-10 on biological *in vitro* and *in vivo* activities, we designed 25-hydroxy-2-methylene-vitamin D_3 analogues **8**−**11** (Figure 2). As a convenient method for their synthesis, we chose the Sonogashira coupling[14] of the A-ring dienynes (**12**−**14**) and CD-ring vinyl triflates (**17** and **18**), derived from the

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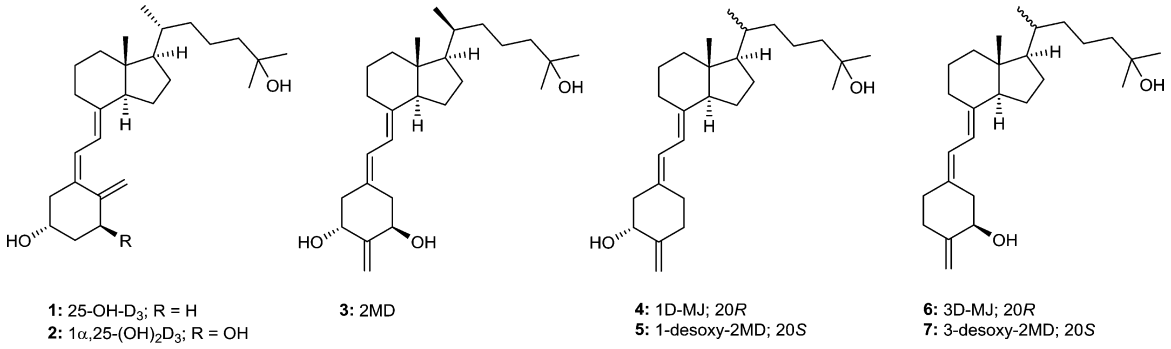


Figure 1. Chemical structure of 25-hydroxyvitamin D_3 (**1**), 1α,25-dihydroxyvitamin D_3 (calcitriol, **2**), 2MD (**3**), and its desoxy analogues **4**–**7**.

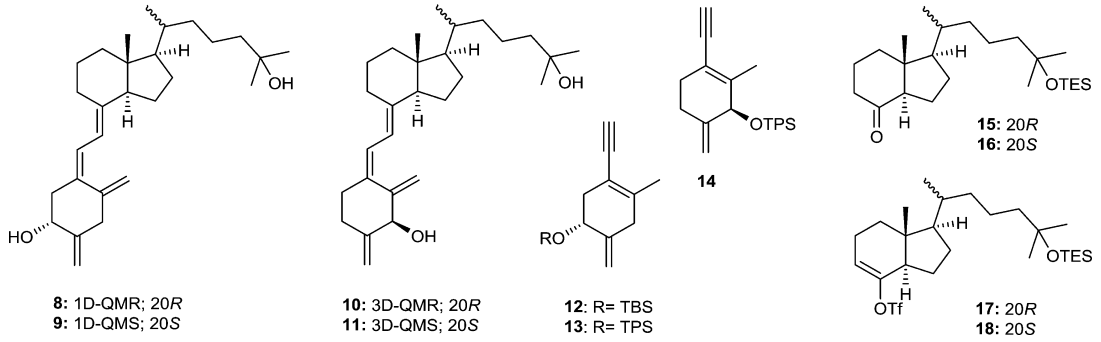
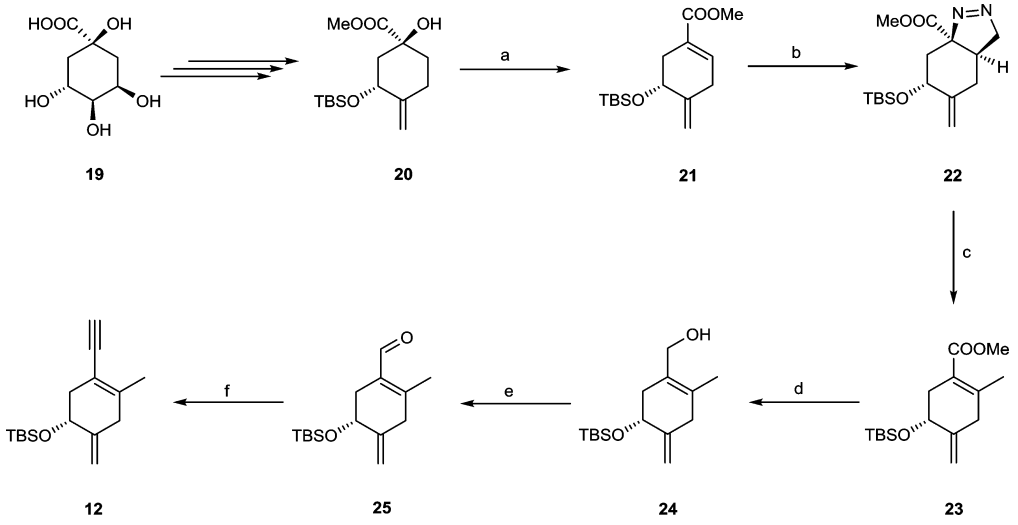


Figure 2. Chemical structures of the obtained 25-hydroxy-2-methylene-vitamin D_3 analogues **8**–**11** and the building blocks for their synthesis.

Scheme 1[a]



[a](a) Martin sulfurane, CCl_4, 91%; (b) CH_2N_2, Et_2O, 99%; (c) DMSO, 125 °C, 98%; (d) DIBALH, CH_2Cl_2, toluene, 96%; (e) PDC, CH_2Cl_2, 44%; (f) *n*-BuLi, $TMSCHN_2$, 52%.

known Grundmann ketones (**15** and **16**). Part of this work has been previously communicated.[15]

RESULTS AND DISCUSSION

Chemistry. First, we focused our efforts on the preparation of the A-ring fragment **12**. The synthesis started from the hydroxy ester **20** (Scheme 1), obtained from the commercially available (−)-(1*S*,3*R*,4*S*,5*R*)-quinic acid (**19**) by the method elaborated by Sibilska et al.[12] The synthetic strategy was based on the work of Desmaele and Tanier.[16] Thus, the tertiary alcohol was dehydrated using Martin sulfurane reagent, and the formed α,β-unsaturated ester **21** was subjected to reaction with diazomethane. Such 1,3-dipolar cycloaddition proved to be a regio- and stereospecific process and provided the single bicyclic product in very high yield. The structure of the adduct **22**, resulting from the attack of the diazomethane from the less hindered side of the molecule, was unequivocally established by consideration of literature data concerning similar processes,[17,18] molecular modeling, and inspection of the ^{1}H NMR spectrum of the product. The most informative were proton resonances derived from the N=N−CH_2 fragment of the formed dihydropyrazole ring: multiplicity of these signals (doublets of doublets) proves that this methylene group is attached to a hydrogen-bearing carbon, whereas the magnitudes

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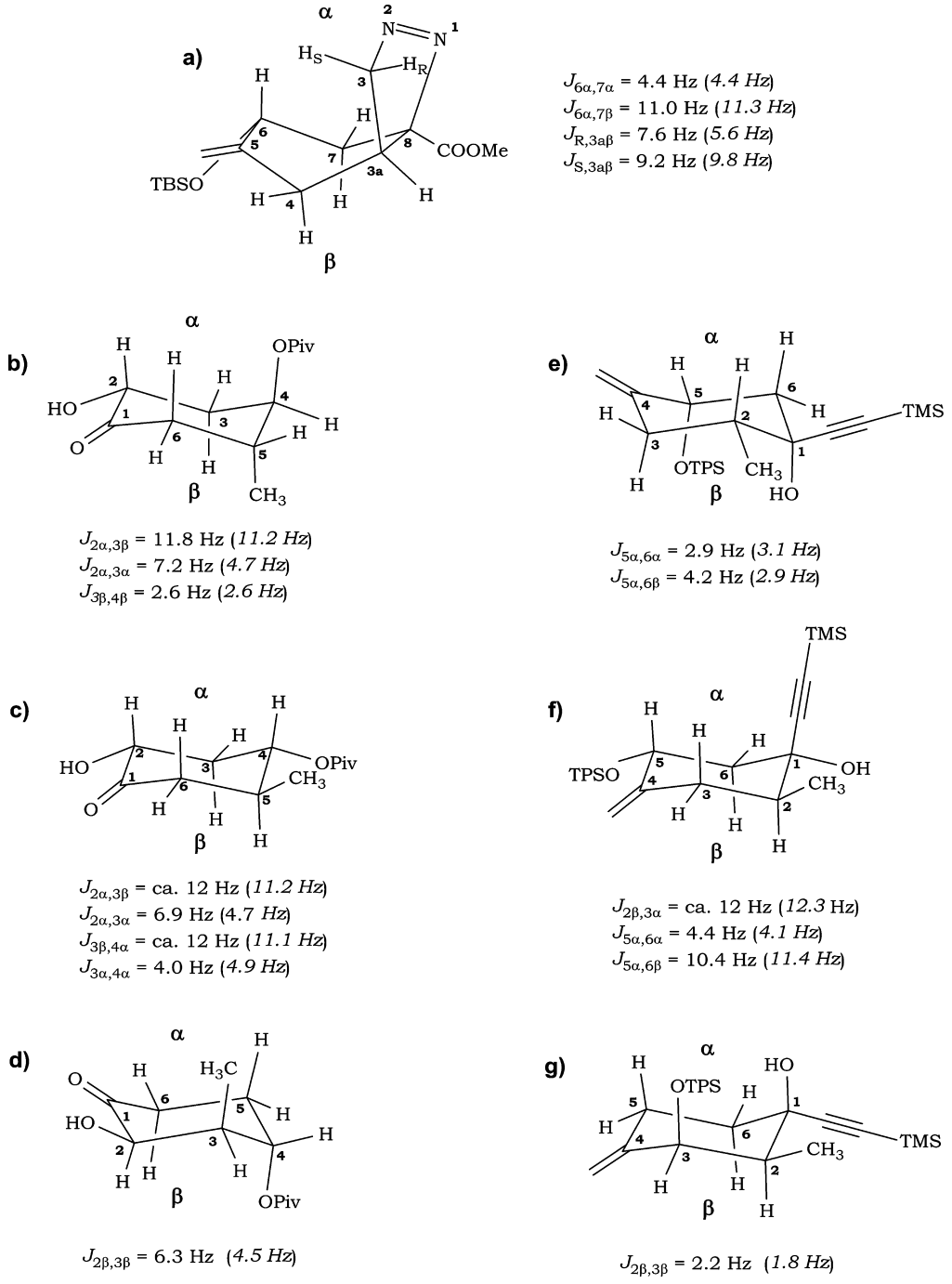

Figure 3. Preferred energy-minimized (HyperChem) conformations of bicyclic diazomethane adduct **22** (a), three hydroxy ketones **29** (b), **30** (c), and **31** (d), and three propargylic alcohols **36** (e), **42** (f), and **48** (g). The most informative ^{1}H−^{1}H constants are listed; values of calculated couplings (PC MODEL) are enclosed in parentheses.

of the respective vicinal couplings confirm the ascribed structure (Figure 3a).

The subsequent thermolysis of the bicyclic compound **22** led to efficient extrusion of nitrogen and formation of unsaturated ester **23**. Its reduction with DIBALH furnished the allylic alcohol **24**, which was oxidized with PDC to the *α,β*-unsaturated aldehyde **25**. Treatment of this product with (trimethylsilyl)diazomethane provided the desired A-ring building block **12**.

Another synthetic path to the A-ring precursors of 2-methylene-vitamin D analogues was also elaborated, involving total synthesis of both fragments **13** and **14**, differing in the position of the silyl-protected hydroxyl group. A racemic mixture of *trans*-3-methyl-4-pivaloyloxy-cyclohexanones **27** and **28** (Scheme 2) was easily obtained in four steps from commercially available 1,4-cyclohexanedione monoethylene acetal **26** in 55% overall yield.[19] These compounds were subjected to enantioselective *α*-aminoxylation using the procedure described by Hayashi et al.[20] Thus, enantiomers **27** and **28** were subjected to the reaction with nitrosobenzene carried out in the presence of a catalytic amount of L-proline, and three main products, **29**, **30**, and **31**, were isolated by column chromatography in 23%, 19%, and 22% yield, respectively. The synthetically advantageous outcome of this

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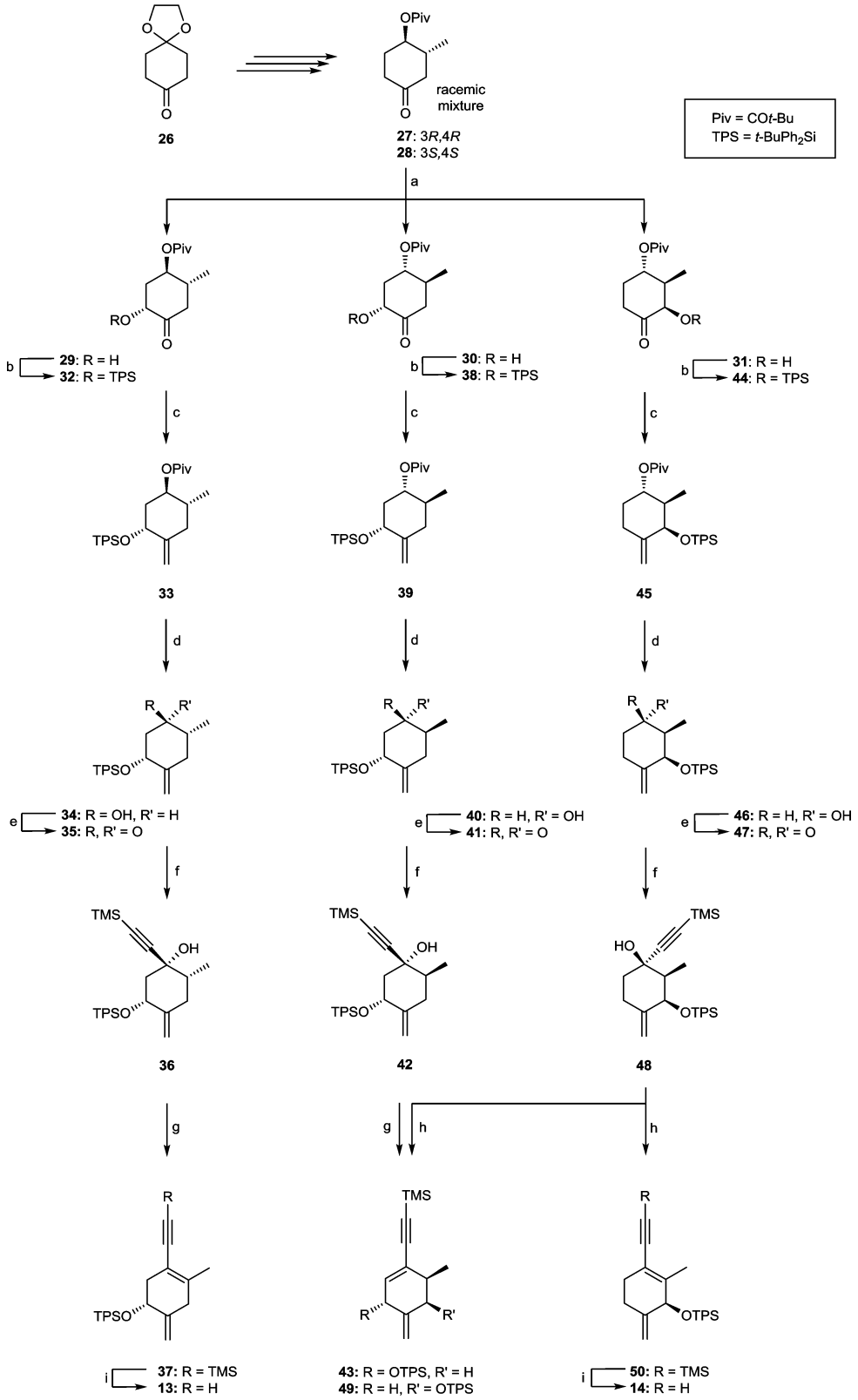
Scheme 2[a]



[a](a) PhNO, L-Pro, $CHCl_3$, **29**/**30**/**31** = 1.14:1:1.17, 64%; (b) TBDPSCl, $AgNO_3$, DMF; **32** 97%, **38** 95%, **44** 73%; (c) $Ph_3P^+CH_3Br^-$, *n*-BuLi, THF, **33** 97%, **39** 96%, **45** 79%; (d) DIBALH, CH_2Cl_2, toluene; **34** 96%, **40** 96%, **46** 97%; (e) Dess–Martin periodinane, CH_2Cl_2; **35** 95%, **41** 96%, **47** 95%; (f) TMSC≡CH, *n*-BuLi, THF; **36** 96%, **42** 94%, **48** 99%; (g) MsCl, TEA, CH_2Cl_2; **37** 38%, **43** 97%; (h) Martin sulfurane, CCl_4; **43** 98%, **49**/**50** = 2:1, 81%; (i) K_2CO_3, THF, MeOH; **13** 96%, **14** 60%.

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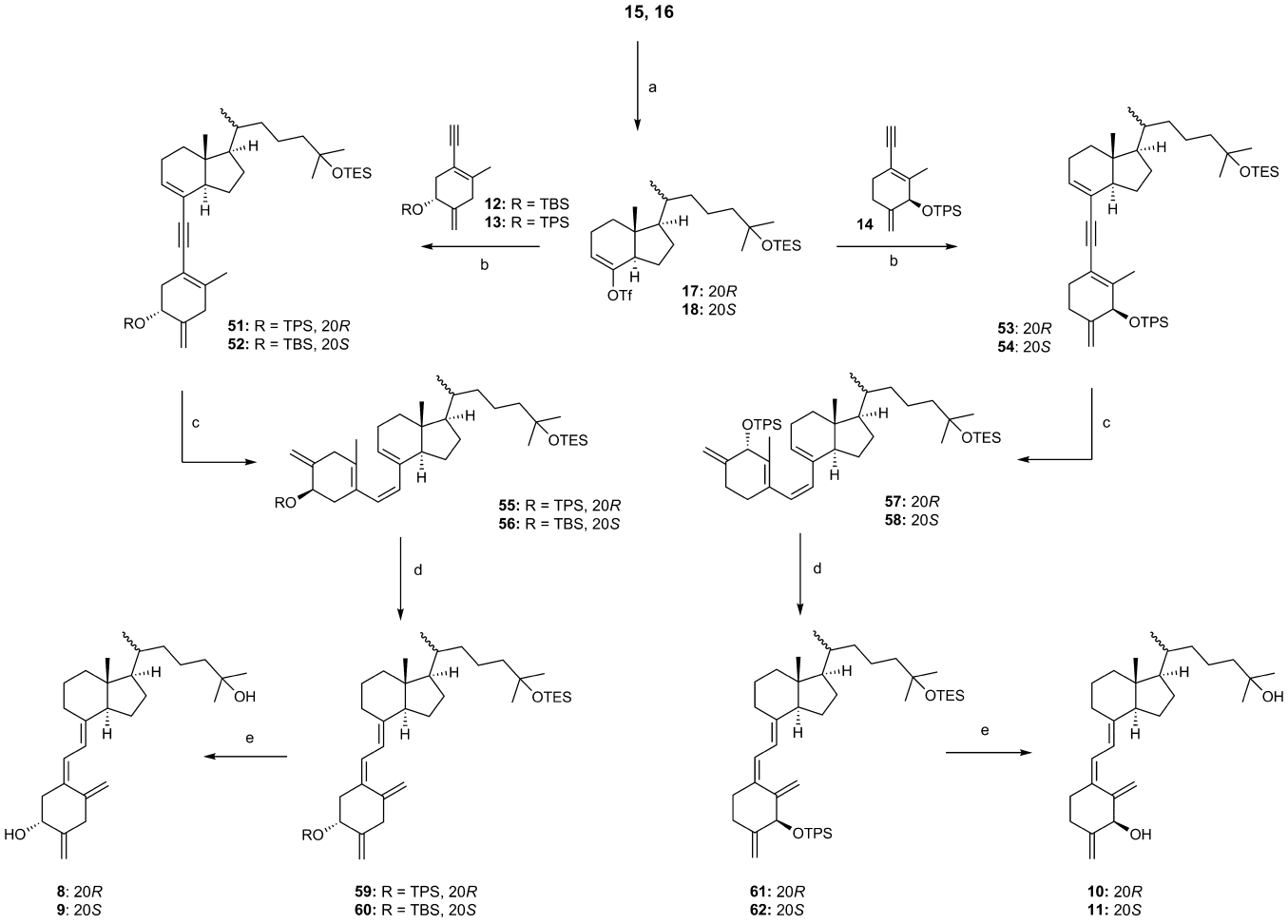
Scheme 3[a]



[a](a) LDA, $PhNTf_2$, THF; **18** 82%; (b) $(PPh_3)_2Pd(OAc)_2$, CuI, Et_2NH, DMF; **51** 87%, **52** 93%, **53** 74%, **54** 58%; (c) H_2, Lindlar cat., quinoline, hexane; **55** 25%, **56** 80%, **57** 80%, **58** 80%; (d) 65 °C, hexane; **59** 76%, **60** 87%, **61** 96%, **62** 64%; (e) TBAF, THF; **8** 29%, **9** 23%, **10** 33%, **11** 56%.

process, directly providing the required α-hydroxylated products, was rather unexpected. Absence of the usual α-aminoxylated products is obviously a consequence of the different reaction conditions. The procedures described in the literature[20] used an excess of carbonyl compound to nitrosobenzene (from 1.2 to as much as 10 equiv) whereas we employed 1.75 equiv of nitrosobenzene and higher catalyst loading. The structural assignments of the obtained hydroxy ketones was based on molecular modeling and careful analysis of their ^{1}H NMR spectra as well as assumption that an introduced secondary hydroxyl has the *R*-configuration.[20] Thus, for example, multiplicity (doublet of dublets) of the signal derived from the proton attached to the carbinol C-2 in hydroxy ketones **29** and **30** indicates that the newly introduced hydroxyl and the methyl substituent are located in these products on the opposite site of the cyclohexanone ring, whereas a single splitting of 2-H signal in the spectrum of isomeric compound **31** confirms that hydroxyl is located between carbonyl and methyl group. Configurational assignment of methyl and pivaloyloxy substituents is facilitated by the fact that they remain in the *trans*-relationship. Molecular mechanics calculations have shown that, for all three products, their conformations possessing equatorially oriented hydroxyl (Figure 3b–d) are strongly preferred (steric energies lower by at least 1.3 kcal/mol). These results were supported by comparison of selected vicinal coupling constants found in the ^{1}H NMR spectra of the hydroxy ketones with the data reported by Anet for cyclohexanol analogues ($J_{ax,ax}$ = 11.1 Hz, $J_{eq,eq}$ = 2.7 Hz).[21]

Three hydroxy ketones **29**–**31** were then separately transformed to the respective cyclohexanones **35**, **41**, and **47**, employing the following four-step reaction sequence: silylation with *tert*-butyldiphenyl silyl chloride, Wittig methylenation with an ylide generated from methyltriphenylphosphonium bromide, reductive removal of the pivalate through treatment with DIBALH, and finally oxidation of cyclohexanol derivatives with Dess–Martin periodinane. The obtained cyclohexanones were then subjected to reaction with lithium (trimethylsilyl)acetylide, which provided in each case the single product. The structures of the formed propargylic alcohols **36** and **48** were proposed by assuming an anion addition at the less hindered face of carbonyl and in the case of **42** by an attack of nucleophile from the side opposite to the bulky OTPS group. Although the analysis of the ^{1}H NMR spectra (Figure 3e–g) of products provided some support for these structural assignments, the important proof for the ascribed configurations of the tertiary hydroxyls was obtained from the results of the subsequent dehydration processes. Thus, a facile although not very efficient conversion of tertiary alcohol **36** to the desired dienyne product **37** was achieved by its treatment with methanesulfonyl chloride-

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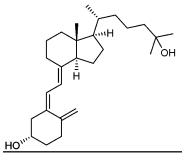
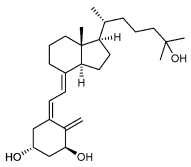
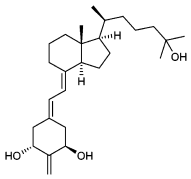
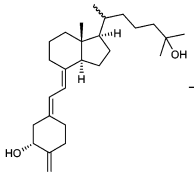
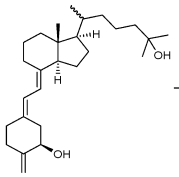
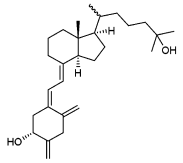
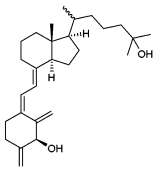
Table 1. Relative VDR Binding Activities,[a] HL-60 Differentiating Activities,[b] and Transcriptional Activities[c] of the 25-OH-D_3 (1), Vitamin D Hormone (2), 2MD (3), and the Vitamin D Analogues 4−11

Compd. Structure	Comp. No.	VDR Binding[a]	HL-60[b] differentiation	24OHase[c] transcription
		K_i Ratio	ED_{50} Ratio	ED_{50} Ratio
	**1**	0.01	0.001	0.001
	**2**	1	1	1
	**3**	1	25	29
	**4**	0.02	0.01	0.001
	5	0.1	0.07	0.07
	**6**	0.2	2.2	0.7
	7	0.5	2.5	2.0
	**8**	0.05	0.005	0.0005
	9	0.33	0.22	0.2
	**10**	0.25	2.0	1.0
	11	0.33	4.0	1.0

[a]Competitive binding of 1α,25-$(OH)_2D_3$ (**2**) 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 the dose−response curves (refs 11−13; Figures 12 and 13 in Supporting Information) 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 numbers shown in the table are expressed as the average ratio of the 1α,25-$(OH)_2D_3$ K_i to the K_i for the analogue. [b]Induction of differentiation of HL-60 promyelocytes to monocytes by 1α,25-$(OH)_2D_3$ (**2**) 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 the dose−response curves (refs 11−13; Figures 14 and 15 in Supporting Information) and represent the analogue concentration capable of inducing 50% maturation. The numbers shown in the table are expressed as the average ratio of the 1α,25-$(OH)_2D_3$ ED_{50} to the ED_{50} for the analogue. [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 (refs 11−13; Figures 16 and 17 in Supporting Information) and represent the analogue concentration capable of increasing the luciferase activity by 50%. The numbers shown in the table are expressed as the average ratio of the 1α,25-$(OH)_2D_3$ ED_{50} to the ED_{50} for the analogue.

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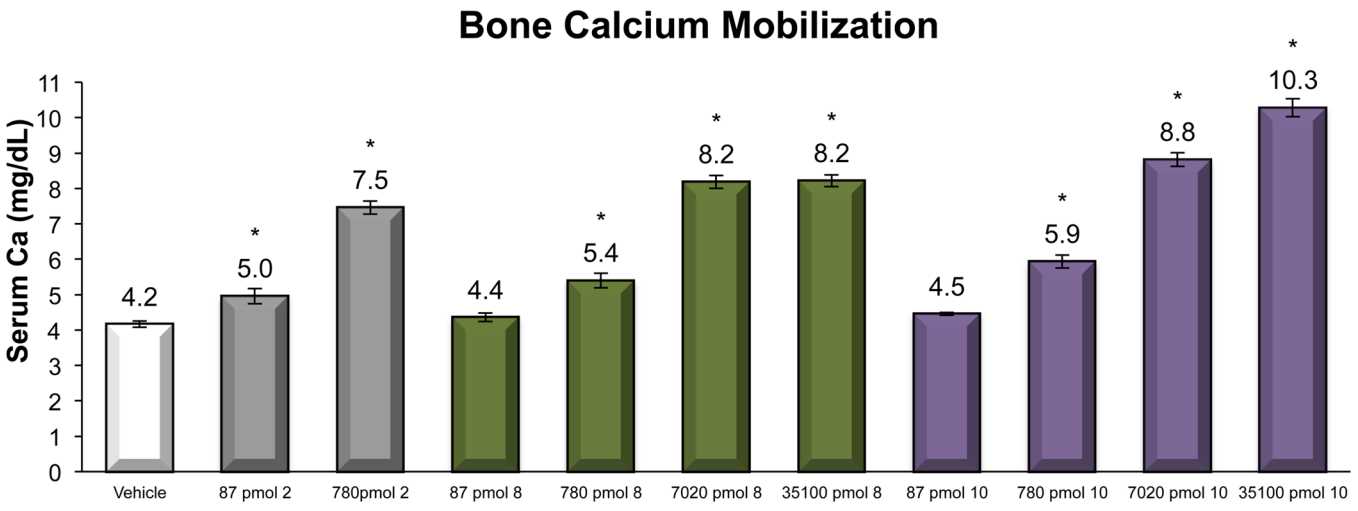

Figure 4. Bone calcium mobilization of 1α,25-$(OH)_2D_3$ (**2**) and the synthesized 25-hydroxy-2-methylene-vitamin D_3 analogues **8** and **10**. Asterisks indicate $p < 0.05$.

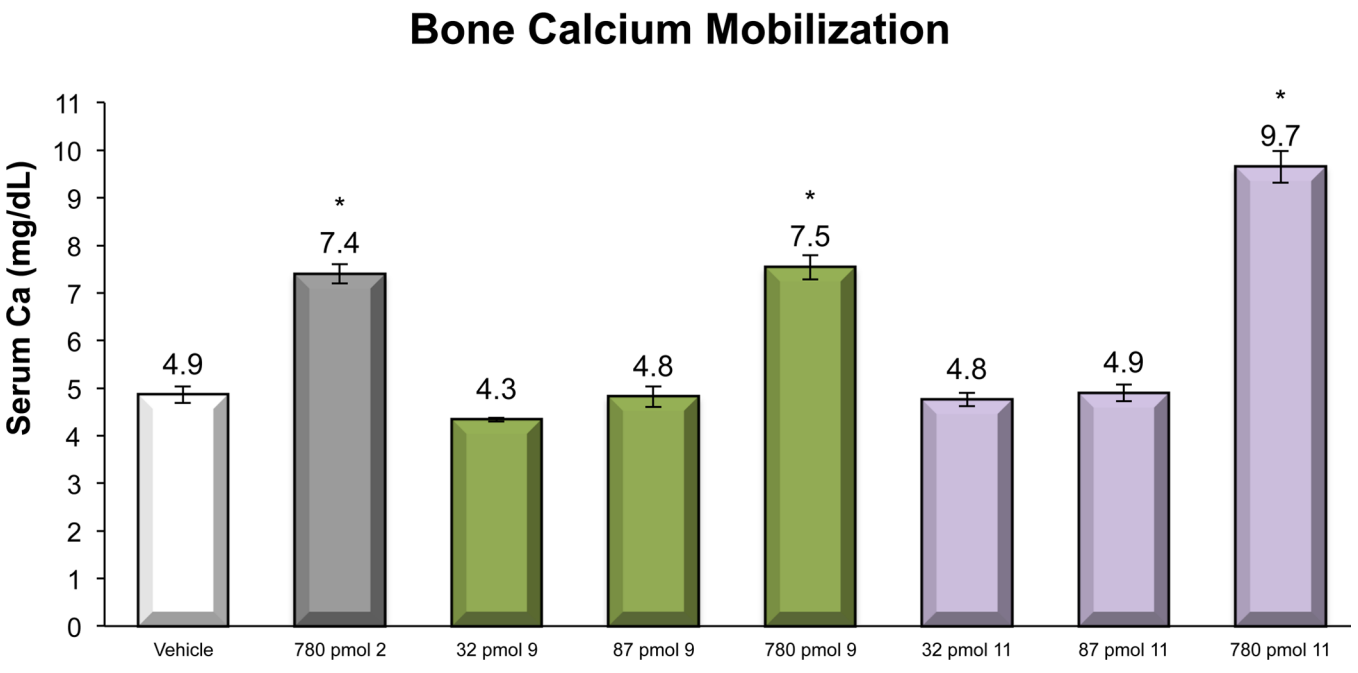

Figure 5. Bone calcium mobilization of 1α,25-$(OH)_2D_3$ (**2**) and the synthesized 25-hydroxy-2-methylene-vitamin D_3 analogues **9** and **11**. Asterisks indicate $p < 0.05$.

triethylamine. In contrast, the same procedure applied to the epimeric propargylic alcohol **42** resulted in its smooth and quantitative conversion into the *anti*-Zaitsev elimination product **43**. An almost identical result of the process was observed with Martin sulfurane as a dehydrating agent, whereas very poor yield of **43** was obtained with Burgess reagent (*syn*-elimination). Also in the case of alcohol **48**, its dehydration with Martin sulfurane reagent proved to be an efficient process; however, the prevailing product was the undesired dienyne **49** with the newly formed trisubstituted double bond, whereas the Zaitsev product **50** represented the minor component of the reaction mixture. Attempted dehydration of the alcohol **48**, performed according to the procedure used for **36**, yielded a complex mixture of products with the dienyne **49** as the main component. Removal of the trimethylsilyl group in the dienyne **37** provided in quantitative yield the A-ring building block **13** suitable for synthesis of the target 25-hydroxy-2-methylene-vitamin D_3 compounds **8** and **9**. Desilylation of the mixture of **49** and **50** allowed us to separate the isomeric mixture of dienynes; the obtained compound **14** also constituted an useful building fragment that could be used for a construction of 3-desoxy-1α,25-dihydroxy-2-methylene-vitamin D_3 (**10**) and its (20*S*)-epimer **11**.

The enol triflate **18**, representing a C,D-ring/side chain fragment, was obtained from the protected (20*S*)-25-hydroxy Grundmann ketone **16**,[9] employing the method used by De Clercq for the preparation of the epimeric enol triflate **17** from the hydrindanone **15**.[22] Thus, treatment of the kinetic enolate, generated from the ketone **16** by addition of the LDA at −78 °C, with *N*-phenyltriflimide afforded **18** in a good yield (Scheme 3). Then, the enol triflates **17** and **18** were subjected to Sonogashira coupling[13] with the dienynes **13** and **12**, respectively. The coupling reactions were carried out in the presence of bis(triphenylphosphine)palladium(II) acetate–

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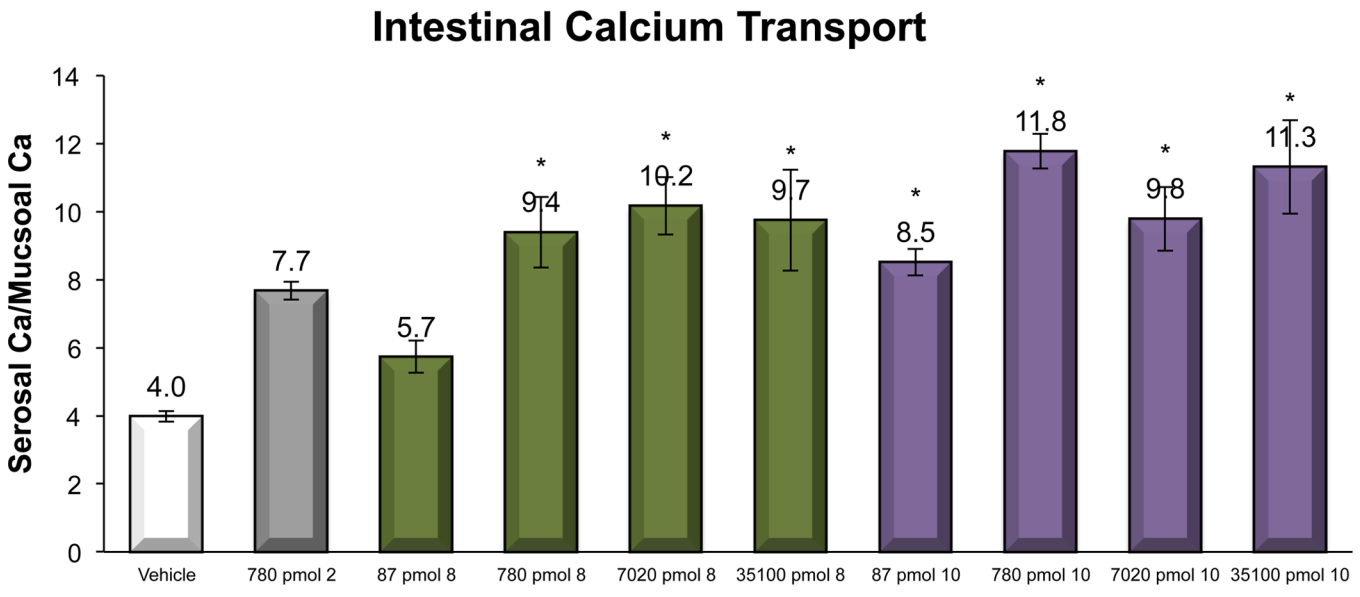

Figure 6. Intestinal calcium transport of 1α,25-$(OH)_2D_3$ (**2**) and the synthesized 25-hydroxy-2-methylene-vitamin D_3 analogues **8** and **10**. Asterisks indicate $p < 0.05$.

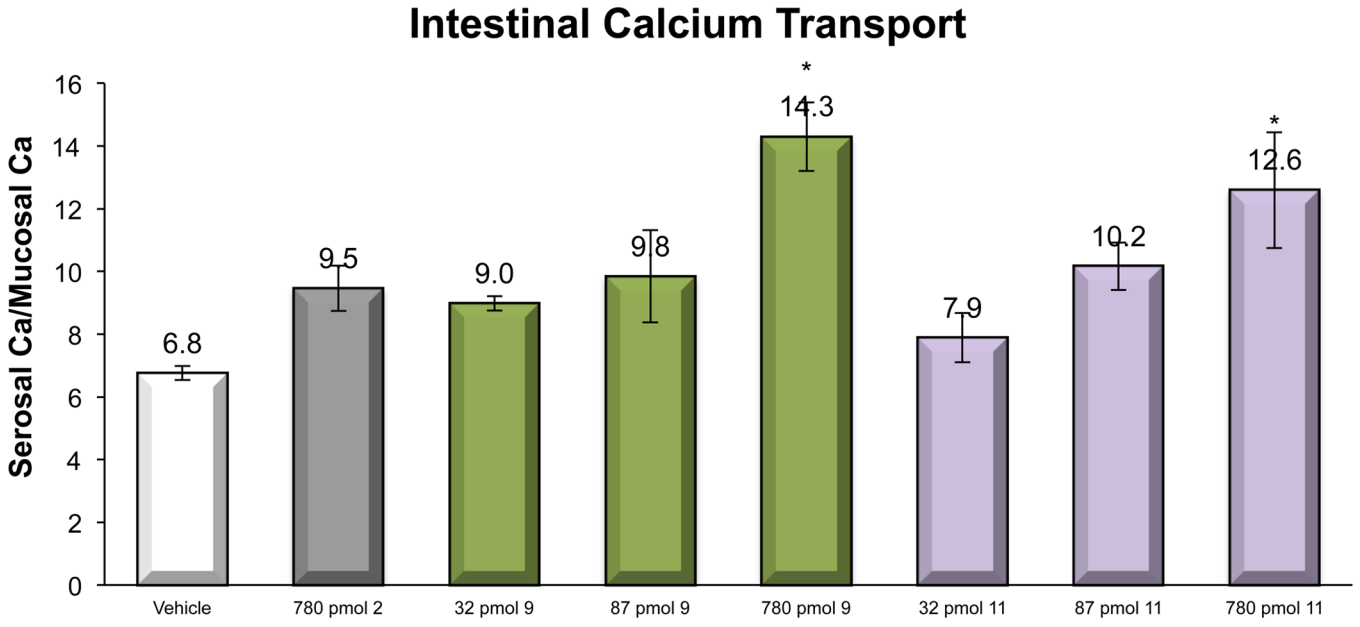

Figure 7. Intestinal calcium transport of 1α,25-$(OH)_2D_3$ (**2**) and the synthesized 25-hydroxy-2-methylene-vitamin D_3 analogues **9** and **11**. Asterisks indicate $p < 0.05$.

copper(I) iodide catalyst and diethylamine, and they yielded the expected trienynes **51** and **52**, which were further hydrogenated in the presence of Lindlar catalyst and quinoline to the respective previtamin D analogues **55** and **56**. After purification by preparative TLC they were subjected to the thermal reaction in hexane, and the protected vitamin D compounds **59** and **60** were isolated by HPLC. Finally, hydroxyl deprotection with tetrabutylammonium fluoride provided the target 25-hydroxy-2-methylene-vitamin D_3 (**8**) and its (20*S*)-epimer **9**. In their proton NMR spectra the corresponding signals, derived from A-ring and olefinic protons, were characterized by virtually identical chemical shifts. These data exclude the possibility that vitamins **8** and **9** can have different configuration at C-3 and, consequently, prove that their precursors, dienynes **12** and 13, must have had the same configuration of the hydroxyl group.

Using as substrates the dienyne **14** and both vinyl triflates **17** and **18**, (20*R*)- and (20*S*)-3-desoxy-2-methylene-1α,25-dihydroxyvitamins (**10** and **11**), respectively, were obtained via a four-step reaction sequence, applying analogous synthetic transformations as described above for the synthesis of **8** and **9**.

Biological Activity. We evaluated *in vitro* and *in vivo* biological activities of the previously (compounds **4**–**7**) and newly (compounds **8**–**11**) synthesized vitamin D analogues. In comparison with 2MD, *in vitro* activities of all tested analogues were significantly lower (Table 1). The same was true when the activities were compared with those of the natural hormone **1**, with the exception of 1α-hydroxylated compounds. As expected, analogues missing the 1α-hydroxyl showed lower

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binding activity then those hydroxylated at the C-1 position. This was reflected also in the HL-60 differentiation activity and in the induction of the 24-hydroxylase. Largely the addition of the 10-exomethylene group improved *in vitro* activity of (20*S*)-1-desoxy compounds, whereas in the (20*R*)-series only VDR binding ability was increased. No clear effect of the presence of an additional exomethylene group was found for 3-desoxy analogues. In the case of *in vivo* assays (Figures 4−7), all analogues possessed significant activity. The presence of the 2-exomethylene group improved intestinal calcium transport above that of the native hormone, 1α,25-$(OH)_2D_3$ but decreased by a factor of 10 bone calcium mobilization of the (20*R*) series. A similar reduction might also be true for the (20*S*) series. However, this is based on historical data.[9,11−13,23]

Enzymatic Hydroxylation. Since 1α-desoxy compounds with two methylene groups showed higher *in vivo* activities compared with their monosubstituted counterparts, we could assume that the 10-exomethylene group facilitates the enzymatic 1α-hydroxylation. Examination of this process using compounds **4**, **8**, and **9** showed that CYP27B1 was able to catalyze 1α-hydroxylation of all three vitamins, but the reaction rate was much lower than that of 25-OH-D_3 as substrate (Table 2). This may be due to the presence of the 2-methylene group, which could shift the juxtaposition of the A-ring relative to the enzyme active site, making it less than ideal to form the necessary bonding interactions for catalysis. The side chain, on the other hand, probably played a minor role judged by the similar k_{cat} values of **8** and **9**. Removal of the 10-methylene moiety seemed to have offset the impact of the 2-exomethylene group, slightly increasing the turnover rate of the analogue **4**. All the structural variations did not change the K_Ms of CYP27B1 for these compounds.

Table 2. Enzymatic 1α-Hydroxylation of Vitamin D Compounds 1, 4, and 8 with CYP27B1

compd	enzymatic rate constant, k_{cat} (min^{-1})	K_M (μM)	cat. efficiency, k_{cat}/K_M (M^{-1} s^{-1})
1	2.47 ± 0.05	0.48 ± 0.03	8.5×10^4
4	0.72 ± 0.14	0.44 ± 0.07	2.7×10^4
8	0.43 ± 0.02	0.42 ± 0.02	1.7×10^4
9	0.53 ± 0.05	0.45 ± 0.04	1.9×10^4

CONLUSIONS

We report here an extension of our structure−activity studies on the A-ring modified vitamin D analogues. 25-Hydroxyvitamin D_3 compounds possessing a 2-exomethylene group and a single hydroxyl substituent located at C-1 or C-3 were designed and synthesized by Sonogashira coupling of the vinyl triflates, derived from the Grundmann ketones, with the respective A-ring fragments prepared by different synthetic pathways.

The *in vitro* potencies and *in vivo* activities of the synthesized 2-methylene-25-hydroxyvitamins D_3 **8**−**11** not only proved the importance of the 1α-hydroxyl but also showed that the 10-exomethylene moiety is responsible for sustaining some of the biological activity of the compounds. Also earlier indication of easier hydroxylation of 2,10-dimethylene vitamins **8** and **9** was not proven by the enzymatic CYP27B1 oxidation experiments. The rate of this process was significantly lower and the hydroxylation proceeded even more slowly for disubstituted vitamins.

Taking into account these results, the ability of 1α-desoxy analogues to act as a prodrug is plausible since its 1α-hydroxylation could occur in a regulated manner and the half-life of the compound might be extended. However, double substitution significantly reduced the rate of the process, and by that, the usefulness of these compounds might be limited.

Further studies on the natural hormone analogues with A-ring exomethylene substituents are underway and will be reported in due course.

EXPERIMENTAL SECTION

Chemistry. Optical rotations were measured in chloroform using a PerkinElmer models 241 and 343 polarimeters at 22 °C. Ultraviolet (UV) absorption spectra were obtained on a Shimadzu UV-1800 UV spectrophotometer in 100% EtOH. All nuclear magnetic resonance spectra were recorded in deuteriochloroform using Varian Unity plus (200 MHz), Bruker DMX-400 (400 MHz), and Bruker DMX-500 (500 MHz). COSY spectra and spin decoupling, as well as NOE, DEPT 90, and DEPT 135, experiments were used to assign particular signals in the ^1H and ^{13}C NMR spectra. Chemical shifts (δ) are reported in parts per million relative to CH_3Si (δ 0.00) as an internal standard. Abbreviations used are singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). High resolution mass spectra were registered on LCT (TOF) or Mass Quattro LC spectrometers. High-performance liquid chromatography (HPLC) was performed on a Waters Associates liquid chromatograph equipped with a model 6000A solvent delivery system, model U6K universal injector, and model 486 tunable absorbance detectors. Solvents were dried and distilled following standard procedures.

The purity of final compounds was determined by HPLC, and they were judged at least 99% pure. Two HPLC columns (9.4 mm × 25 cm Zorbax-Sil and 9.4 mm × 25 cm Zorbax Eclipse XDB-C18) were used as indicated in Table 3 (Supporting Information). The purity and identity of the synthesized vitamins were additionally confirmed by inspection of their ^1H NMR and high-resolution mass spectra.

The known vinyl triflate **17**[24] was obtained according to the procedure of De Clercq et al.;[22] an analogous method was used for the preparation of the isomeric (20*S*)-triflate **18** (described below) from the protected (20*S*)-25-hydroxy Grundmann ketone **16**.[9] The starting hydroxy ester **20** was prepared from (−)-quinic acid (**19**),[11,12] whereas cyclohexanone compounds **27** and **28** were obtained from commercial 1,4-cyclohexanedione monoethylene ketal (**26**) according to the published procedures.[19,24]

*(20S)-25-[(Triethylsilyl)oxy]-8-trifluoromethanesulfonyloxy-des-A,B-cholest-8-ene (**18**).* A solution of the ketone **16** (28.5 mg, 72.19 μmol) in anhydrous THF (350 μL) was slowly added to the solution of LDA (2.0 M in THF/heptane/ethylbenzene; 40 μL, 80 μmol) in dry THF (100 μL) at −78 °C under argon. Then a solution of *N*-phenyltriflimide (28.3 mg, 79.27 μmol) in dry THF (100 μL) was added. After 2 h, the cooling bath was removed, and the reaction mixture was allowed to warm to room temperature. Stirring was continued for 30 min, and water was added. The mixture was extracted with hexane, dried over $MgSO_4$, and concentrated. The residue was applied on a silica Sep-Pak cartridge and eluted with hexane to afford the enol triflate **18** (17.2 mg, 82% considering recovered substrate) and unreacted ketone **16** (12 mg).

*(5R)-5-[(tert-Butyldimethylsilyl)oxy]-4-methylene-cyclohex-1-ene-carboxylic Acid Methyl Ester (**21**).* To a solution of hydroxy ester **20** (162.5 mg, 541 μmol) in anhydrous carbon tetrachloride (5.1 mL) at room temperature under argon was added solution of bis[α,α-bis(trifluoromethyl)benzenemethanolato]diphenylsulfur (546 mg, 812 μmol) in anhydrous carbon tetrachloride (1.8 mL). Reaction was stirred for 2 h, water was added, and the mixture was extracted with methylene chloride, dried (Na_2SO_4), and concentrated. The resulting product was applied on a silica Sep-Pak cartridge and eluted with hexane/diethyl ether (97:3) to give the desired product contaminated with dehydrating agent. Further purification was performed on preparative TLC plates (Silica Gel $60F_{254}$, 20 cm × 20 cm, 250

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nm), developed in hexane/diethyl ether (92:8), to give an unsaturated ester **21** (139 mg, 91%) as a colorless oil.

*(3aR,6R,7aR)-6-[(tert-Butyldimethylsilyl)oxy]-5-methylene-3,3a,4,5,6,7-hexahydro-indazole-7a-carboxylic Acid Methyl Ester (**22**).* Solution of diazomethane in diethyl ether [5.5 mL; prepared according to the procedure of Arndt][25] was added to the solution of unsaturated ester **21** (375 mg, 1.33 mmol) in anhydrous diethyl ether (2 mL) at room temperature. Reaction mixture was protected from light and stirred overnight. Solvent was evaporated; the residue was dissolved in hexane, applied on a silica Sep-Pak cartridge, and eluted with hexane/ethyl acetate (96:4) to give bicyclic compound **22** (423 mg, 99%) as a colorless oil.

*(5R)-5-[(tert-Butyldimethylsilyl)oxy]-2-methyl-4-methylene-cyclohex-1-enecarboxylic Acid Methyl Ester (**23**).* A solution of compound **22** (310 mg, 955 μmol) in freshly distilled anhydrous DMSO (16 mL) was stirred at 125 °C for 5 h under argon. Heating bath was removed, water added and the mixture was extracted with hexane, dried (Na_2SO_4), and concentrated. Separation by column chromatography on silica using hexane/diethyl ether (97:3) gave unsaturated ester **23** (239 mg, 84%; 98% considering recovered substrate) and unchanged adduct **22** (43 mg).

*(5′R)-5′-[(tert-Butyldimethylsilyl)oxy]-2′-methyl-4′-methylene-cyclohex-1′-enyl)-methanol (**24**).* Diisobutylaluminum hydride (1.0 M in toluene; 1.33 mL, 1.33 mmol) was slowly added to a stirred solution of the ester **23** (89 mg, 300 μmol) in toluene/methylene chloride (2:1; 8 mL) at −78 °C under argon. Stirring was continued at −78 °C for 1 h. The mixture was quenched with potassium−sodium tartrate (2 N, 4 mL), aqueous HCl (2 N, 4 mL), and H_2O and extracted with ethyl acetate. The combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated. The residue was applied on a silica Sep-Pak cartridge and eluted with hexane/ethyl acetate (95:5) to afford the alcohol **24** (78 mg, 96%).

*(5R)-5-[(tert-Butyldimethylsilyl)oxy]-2-methyl-4-methylene-cyclohex-1-enecarbaldehyde (**25**).* The mixture of alcohol **24** (77 mg, 287 μmol), and pyridinium dichromate (347 mg, 1.61 mmol) in anhydrous methylene chloride (4.6 mL) was stirred vigorously at room temperature for 16 h under argon. Then the reaction mixture was filtered through a pad of Celite (washed with methylene chloride), and solvent was removed under reduced pressure. The residue was applied on a silica Sep-Pak cartridge and eluted with hexane/ethyl acetate (98:2) to yield the aldehyde **25** (33.5 mg, 44%) as a colorless oil.

*(5R)-5-[(tert-Butyldimethylsilyl)oxy]-1-ethynyl-2-methyl-4-methylene-cyclohexene (**12**).* *n*-Butyllithium (1.6 M in hexanes; 101 μL, 161.71 μmol) was added to a solution of (trimethylsilyl)diazomethane (2.0 M in hexane, 76 μL, 151.5 μmol) in anhydrous THF (150 μL) at −78 °C under argon, and a solution of aldehyde **25** (33.5 mg, 125.7 μmol) in dry THF (100 μL +100 μL) was added via cannula. The cooling bath was removed after 1 h, and stirring was continued at room temperature overnight. Water was added, and the mixture was extracted with hexane, dried (Na_2SO_4), and concentrated. The residue was applied on a silica Sep-Pak cartridge and eluted with hexane to afford the dienyne **12** (16 mg, 52%) and the recovered substrate (1.5 mg).

*(2R,4R,5R)- and (2R,3R,4S)-2-Hydroxy-5-methyl-4-pivaloyloxy-cyclohexanone (**29**, **31**) and (2R,4S,5S)-2-Hydroxy-5-methyl-4-pivaloyloxy-cyclohexanone (**30**).* To a stirred solution of racemic mixture of **27** and **28** (551 mg, 2.59 mmol) and L-proline (143.6 mg, 1.25 mmol) in chloroform (5 mL), a solution of nitrosobenzene (485 mg, 4.53 mmol) in chloroform (10 mL) was slowly added by a syringe pump at 4 °C over 24 h. Then the mixture was stirred at room temperature for additional 2 h. Reaction was quenched with brine and extracted with ethyl acetate, dried ($MgSO_4$), and concentrated. The residue was separated by column chromatography on silica using hexane/ethyl acetate (9:1) to give the isomeric α-hydroxy ketones (in the elution order) **29**, **30**, and **31** (ratio of 1.14:1:1.17; 380 mg, 64%). The compounds were sufficiently pure to be used for the next synthetic steps without further purification.

*(2R,4R,5R)-2-[(tert-Butyldiphenylsilyl)oxy]-5-methyl-4-pivaloyloxy-cyclohexanone (**32**).* *t*-Butylchlorodiphenylsilane (1.16 mL, 4.53 mmol) was added to a solution of α-hydroxy ketone **29** (765 mg, 3.6 mmol) and silver nitrate (1.72 g, 10.14 mmol) in anhydrous DMF (16 mL) under argon at room temperature; white precipitate formed immediately. The reaction was stirred for 17 h and then quenched with water. The mixture was extracted with hexane, dried ($MgSO_4$), and concentrated. Purification by column chromatography on silica (1% → 4% diethyl ether/hexane) gave protected α-hydroxy ketone **32** (1.2 g, 97%).

*(2R,4S,5S)-2-[(tert-Butyldiphenylsilyl)oxy]-5-methyl-4-pivaloyloxy-cyclohexanone (**38**).* Silylation of the hydroxy ketone **30** was performed according to procedure described above. The crude product was purified by column chromatography on silica. Elution with hexane/diethyl ether (98:2) gave an oily protected compound **38** in 95% yield.

*(2R,3R,4S)-2-[(tert-Butyldiphenylsilyl)oxy]-3-methyl-4-pivaloyloxy-cyclohexanone (**44**).* Silylation of the hydroxy ketone **31** with *t*-BDPSCl was performed as described for **29**, and it was completed after 30 h. Purification by column chromatography on silica gave protected compound **44** in 73% yield.

*(2R,4R,5R)-2-[(tert-Butyldiphenylsilyl)oxy]-5-methyl-1-methylene-4-pivaloyloxy-cyclohexane (**33**).* *n*-Buthyllithium (1.6 M in hexanes; 430 μL, 688 μmol) was added dropwise to methyltriphenylphosphonium bromide (122 mg, 342 μmol) in anhydrous THF (1.4 mL) at 0 °C. After 15 min, another portion of phosphonium salt (122 mg, 342 μmol) was added, and the solution was stirred at 0 °C for 10 min and at room temperature for 20 min. The orange-red mixture was then cooled to −78 °C and siphoned to the precooled (−78 °C) solution of the ketone **32** (160 mg, 344 μmol) in anhydrous THF (350 μL). The reaction mixture was stirred at −78 °C for 4 h and then at room temperature for 1 h. The mixture was poured into brine and extracted with hexane. The organic layer was dried ($MgSO_4$) and evaporated to give an orange oily residue, which was applied on a Waters silica Sep-Pak cartridge. Elution with hexane/diethyl ether (97:3) gave pure olefinic compound **33** (153 mg, 97%) as a colorless oil.

*(2R,4S,5S)-2-[(tert-Butylodiphenylsilyl)oxy]-5-methyl-1-methylene-4-pivaloyloxy-cyclohexane (**39**).* Wittig reaction of the ketone **38**, performed according to procedure described above, gave an oily olefinic compound **39** in 96% yield.

*(2R,3R,4S)-2-[(tert-Butyldiphenylsilyl)oxy]-3-methyl-1-methylene-4-pivaloyloxy-cyclohexane (**45**).* Wittig reaction of the ketone **44** was performed as described for **32**. The product was purified on a silica Sep-Pak cartridge. Elution with hexane/diethyl ether (98:2) gave an oily olefinic compound **45** in 79% yield.

*(1R,2R,5R)-5-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-cyclohexanol (**34**).* Diisobutylaluminum hydride (1.0 M in toluene; 6.12 mL, 6.12 mmol) was slowly added to a stirred solution of the ester **33** (710 mg, 1.53 mmol) in toluene/methylene chloride (2:1, 45 mL) at −78 °C under argon. Stirring was continued at −78 °C for 1 h and at −40 °C for 30 min. The mixture was quenched with potassium−sodium tartrate (2 N, 4 mL), aqueous HCl (2 N, 4 mL), and H_2O (14 mL) and extracted with ethyl acetate. The organic phase was washed with brine, dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography on silica using hexane/ethyl acetate (9:1) to give alcohol **34** (558 mg, 96%).

*(1S,2S,5R)-5-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-cyclohexanol (**40**).* Reduction of the ester **39** with diisobutylaluminum hydride, performed according to the procedure described above, gave the alcohol **40** in 96% yield.

*(1S,2R,3R)-3-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-cyclohexanol (**46**).* Reduction of the ester **45** with diisobutylaluminum hydride, performed as described for **33**, gave the alcohol **46** in 97% yield.

*(2R,5R)-5-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-cyclohexanone (**35**).* Dess−Martin periodinane (529 mg, 1.25 mmol) was added to a stirred solution of alcohol **34** (396 mg, 1.04 mmol) in anhydrous methylene chloride (20 mL) at room temperature under argon. Stirring was continued for 1 h and saturated $NaHCO_3$ was slowly added. The mixture was extracted with methylene chloride, dried ($MgSO_4$), and concentrated. The residue was applied on a silica Sep-Pak cartridge and eluted with hexane/diethyl ether (98:2) to afford ketone **35** (389 mg, 95%) as a colorless oil.

*(2S,5R)-5-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-cyklohexanone (**41**).* Oxidation of the alcohol **40** with Dess−Martin

periodinane, performed according to procedure described above, gave the oily ketone **41** in 96% yield.

*(2S,3R)-3-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-cyclohexanone (**47**).* Oxidation of the alcohol **46** with Dess−Martin periodinane, performed as described for **34**, gave the oily ketone **47** in 95% yield.

*(1S,2R,5R)-5-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-1-[(trimethylsilanyl) ethynyl]-cyclohexanol (**36**).* A solution of *n*-BuLi (1.6 M in hexanes, 325 μL, 520 μmol) was added dropwise to a solution of trimethylsilylacetylene (76 μL, 537 μmol) in anhydrous THF (2 mL) under argon at 0 °C. The solution was stirred for 30 min and cooled to −78 °C; then a precooled (−78 °C) solution of the ketone **35** (158 mg, 417.3 μmol) in dry THF (2 mL) was slowly added. After 15 min, the mixture was warmed to 0 °C and stirred for additional 30 min. Then it was quenched with water, extracted with ether, dried ($MgSO_4$), and concentrated. The resulting product was applied on a silica Sep-Pak cartridge and eluted with hexane/ethyl acetate (98:2) to afford alcohol **36** (190 mg, 96%) as a colorless oil.

*(1S,2S,5R)-5-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-1-[(trimethylsilanyl) ethynyl]-cyclohexanol (**42**).* Reaction of the ketone **41** with an anion of trimethylsilylacetylene, performed according to procedure described above, gave an oily alcohol **42** in 94% yield.

*(1R,2S,3R)-3-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-1-[(trimethylsilanyl) ethynyl]-cyclohexanol (**48**).* Reaction of the ketone **47** with an anion of trimethylsilylacetylene, performed as described for **35**, gave an oily alcohol **48** in 99% yield.

*(5R)-5-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-1-[(trimethylsilanyl)ethynyl]-cyclohexene (**37**).* Mesyl chloride (170 μL, 2.19 mmol) was slowly added to a stirred solution of alcohol **36** (174 mg, 364 μmol) and TEA (310 μL, 2.21 mmol) in dry methylene chloride (6 mL) at room temperature under argon. The reaction was quenched after 1 h with 5% HCl and extracted with methylene chloride. The organic phase was washed with saturated $NaHCO_3$, dried ($MgSO_4$), and concentrated. The resulting product was applied on a silica Sep-Pak cartridge and eluted with hexane/diethyl ether (99:1) to afford dienyne **37** (64 mg, 38%) as a colorless oil.

*(3R,6S)-3-[(tert-Butyldiphenylsilyl)oxy]-6-methyl-4-methylene-1-[(trimethylsilanyl)ethynyl]-cyklohexene (**43**).* For method a, dehydration of the alcohol **42**, performed according to procedure described above, gave an oily dienyne **43** in 97% yield.

For method b, to a solution of the alcohol **42** (21 mg, 44 μmol) in anhydrous carbon tetrachloride (0.5 mL) at room temperature under argon was added solution of bis[α,α-bis(trifluoromethyl)-benzenemethanolato]diphenylsulfur (44 mg, 65.4 μmol) in anhydrous carbon tetrachloride (2 mL). Reaction was stirred for 6 h, and during this time dehydrating reagent was added twice (in ca. 15 mg portions). Water was added, and the mixture was extracted with methylene chloride, dried (Na_2SO_4), and concentrated. The resulting product was applied on a silica Sep-Pak cartridge and eluted with hexane/diethyl ether (98:2) to afford the product contaminated with dehydrating agent. Further purification was performed on preparative TLC plate (Silica Gel $60F_{254}$, 20 cm × 20 cm, 250 μm), developed in hexane/diethyl ether (92:8), to give the dienyne **43** (19.8 mg, 98%).

For method c, a solution of alcohol **42** (30 mg, 62.9 μmol) and methyl *N*-(triethylammoniumsulfonyl)carbamate (45 mg, 188 μmol) in anhydrous toluene (2.2 mL) was stirred at room temperature for 5 h and at 40 °C for 14 h under argon. The reaction mixture was cooled to room temperature; then water and brine were added. The mixture was extracted with diethyl ether, dried (Na_2SO_4), and concentrated. The residue was applied on a silica Sep-Pak cartridge. Elution with hexane/diethyl ether (98:2) afforded the dienyne **43** (6 mg, 21%).

*(5R,6R)-5-[(tert-Butyldiphenylsilyl)oxy]-6-methyl-4-methylene-1-[(trimethylsilyl)ethynyl]-cyclohexene (**49**) and (3S)-3-[(tert-Butyldiphenylsilyl)oxy]-2-methyl-4-methylene-1-[(trimethylsilyl)-ethynyl]-cyclohexene (**50**).* Reaction of the alcohol **48** with the Martin sulfurane dehydrating agent, performed as described for **42**, gave products, which were purified on a silica Sep-Pak cartridge. Elution with hexane/diethyl ether (98:2) afforded the oily isomeric dienynes **49** and **50** (62 mg, 81%; isomer ratio of 2:1).

*(5R)-5-[(tert-Butyldiphenylsilyl)oxy]-1-ethynyl-2-methyl-4-methylene-cyclohexane (**13**).* Anhydrous potassium carbonate (34.5 mg, 250 μmol) was added to a stirred solution of protected acetylene **37** (58 mg, 126 μmol) in anhydrous THF/MeOH (1:1, 6 mL) at room temperature under argon. The stirring was continued for 19 h, and then water and saturated NH_4Cl were added; the mixture was extracted with hexane, dried ($MgSO_4$), and concentrated. The residue was applied on a silica Sep-Pak cartridge and eluted with hexane to afford compound **13** (47 mg, 96%).

*(3S)-3-[(tert-Butyldiphenylsilyl)oxy]-1-ethynyl-2-methyl-4-methylene-cyclohexane (**14**).* Treatment of a mixture of protected enynes **49** and **50** (ratio of 1:2) with potassium carbonate in anhydrous THF/MeOH, performed according to the procedure described above, gave products, which were separated by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using hexane as an eluent. Pure dienyne **14** (60% yield from **50**) was collected at R_V = 22 mL.

*3β-[(tert-Butyldiphenylsilyl)oxy]-2-methylene-25-[(triethylsilyl)-oxy]-9,10-secocholesta-5(10),8-dien-6-yne (**51**).* To a solution of the dienyne **13** (47 mg, 121.6 μmol) and vinyl triflate **17** (50 mg, 95 μmol) in anhydrous DMF (0.95 mL) were added CuI (2.7 mg, 14.2 μmol), $(PPh_3)_2Pd(OAc)_2$ (2.0 mg, 2.7 μmol), and Et_2NH (945 μL) at room temperature under argon. After 20 min, the reaction mixture turned deep reddish-brown. Water was added, and the mixture was extracted with hexane, dried ($MgSO_4$), and concentrated. The residue was applied on a silica Sep-Pak cartridge and eluted with hexane to afford compound **51** (63 mg, 87%).

*(20S)-3β-[(tert-Butyldimethylsilyl)oxy]-2-methylene-25-[(triethylsilyl)oxy]-9,10-secocholesta-5(10),8-dien-6-yne (**52**).* Sonogashira coupling of the dienyne **12** and vinyl triflate **18**, performed according to the procedure described above, gave the trienyne **52** in 93% yield.

*3-Desoxy-1α-[(tert-butyldiphenylsilyl)oxy]-2-methylene-25-[(triethylsilyl)oxy]-9,10-secocholesta-5(10),8-dien-6-yne (**53**).* Sonogashira coupling of the dienyne **14** and vinyl triflate **17**, performed as described for preparation of **51**, gave the trienyne **53** in 74% yield.

*(20S)-3-Desoxy-1α-[(tert-butyldiphenylsilyl)oxy]-2-methylene-25-[(triethylsilyl)oxy]-9,10-secocholesta-5(10),8-dien-6-yne (**54**).* Sonogashira coupling of the dienyne **14** and vinyl triflate **18**, performed as described for preparation of **51**, gave the trienyne **54** in 58% yield.

*2-Methylene-25-[(triethylsilyl)oxy]-vitamin D_3 tert-Butyldiphenylsilyl Ether (**59**).* To a solution of the trienyne **51** (63 mg, 82.5 μmol) in hexane (7.6 mL) and quinoline (13.5 μL) was added Lindlar catalyst (189 mg), and the mixture was stirred at room temperature under a positive pressure of hydrogen. Lindlar catalyst was added four times during 8 h (in 30 mg portions), and then the mixture was applied on a silica Sep-Pak cartridge and eluted with hexane/ether (99.7:0.3) to yield a mixture of previtamin D product and unreacted substrate. Further purification by preparative TLC (Silica Gel $60F_{254}$, 20 cm × 20 cm, 250 μm layer) with hexane/ether (98:2) gave the previtamin **55** (12.7 mg, 20%; 25% based on recovered substrate) and 12.1 mg of the unchanged trienyne. Previtamin compound was then dissolved in anhydrous hexane (6 mL) and stirred at 65 °C for 5 h and at 40 °C overnight under argon. Solvent was evaporated, and the residue was purified by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using hexane/ethyl acetate (99:1) solvent system. Pure protected vitamin **59** (9.7 mg, 76%) was eluted at R_V 15.5 mL.

*(20S)-2-Methylene-25-[(triethylsilyl)oxy]-vitamin D_3 tert-Butyldimethylsilyl Ether (**60**).* Hydrogenation of the trienyne **52**, performed according to procedure described above, gave after purification on a silica Sep-Pak cartridge the previtamin **56** in 80% yield. The previtamin compound was then subjected to thermal isomerization to give protected vitamin **60** in 87% yield.

*3-Desoxy-1α-[(tert-butyldiphenylsilyl)oxy]-2-methylene-25-[(triethylsilyl)oxy]-vitamin D_3 (**61**).* Hydrogenation of the trienyne **53**, performed as described for **51**, gave after purification on a silica Sep-Pak cartridge the previtamin **57** in 80% yield. The previtamin compound was then subjected to thermal isomerization to give the product that was purified by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using hexane/ethyl acetate (99:1) solvent system. Pure protected vitamin **61** (96%) was eluted at R_V 16.3 mL.

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*(20S)-3-Desoxy-1α-[(tert-butyldiphenylsilyl)oxy]-2-methylene-25-[(triethylsilyl)oxy]-vitamin D_3 (**62**).* Hydrogenation of the trienyne **54**, performed as described for **51**, gave after purification on a silica Sep-Pak cartridge the previtamin **58** in 80% yield. The previtamin compound was then subjected to thermal isomerization to give the product that was purified on a silica Sep-Pak cartridge. Elution with hexane gave pure protected vitamin **62** in 64% yield.

*25-Hydroxy-2-methylene-vitamin D_3 (**8**).* To a solution of protected vitamin **59** (8.7 mg) in THF (0.7 mL) was added tetrabutylammonium fluoride (1.0 M in THF; 546 μL, 546 μmol) at room temperature under argon. The stirring was continued for 18 h, brine was added, and the mixture was extracted with ethyl acetate. The organic extracts were dried ($MgSO_4$) and evaporated. The residue was purified by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using hexane/2-propanol (97:3) solvent system; vitamin **8** (1.357 mg, 29%) was collected at R_V 35 mL. Analytical sample of the vitamin was obtained after HPLC (9.4 mm × 25 cm Zorbax Eclipse XDB-C18 column, 4 mL/min) using methanol/water (93:7) solvent system (R_V 28 mL).

*(20S)-25-Hydroxy-2-methylene-vitamin D_3 (**9**).* Hydroxyl deprotection in the silylated vitamin **60**, performed according to procedure described above, gave product that was purified by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using hexane/2-propanol (96:4) solvent system; vitamin **9** (23% yield) was collected at R_V 39 mL. An analytical sample of the vitamin was obtained after HPLC (9.4 mm × 25 cm Zorbax Eclipse XDB-C18 column, 4 mL/min) using methanol/water (93:7) solvent system (R_V 35 mL).

*3-Desoxy-1α,25-dihydroxy-2-methylene-vitamin D_3 (**10**).* Hydroxyl deprotection in the silylated vitamin **61**, performed as described above for **59**, gave product that was purified by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using hexane/2-propanol (97:3) solvent system; vitamin **10** (33% yield) was collected at R_V 25 mL. An analytical sample of the vitamin was obtained after HPLC (9.4 mm × 25 cm Zorbax Eclipse XDB-C18 column, 4 mL/min) using methanol/water (93:7) solvent system (R_V 42 mL).

*(20S)-3-Desoxy-1α,25-dihydroxy-2-methylene-vitamin D_3 (**11**).* Hydroxyl deprotection in the silylated vitamin **62**, performed as described above for **59**, gave product that was purified by HPLC (9.4 mm × 25 cm Zorbax-Sil column, 4 mL/min) using hexane/2-propanol (97:3) solvent system; vitamin **11** (56% yield) was collected at R_V 36 mL. An analytical sample of the vitamin was obtained after HPLC (9.4 mm × 25 cm Zorbax Eclipse XDB-C18 column, 4 mL/min) using methanol/water (91:9) solvent system (R_V 56 mL).

Biological Studies. *In Vitro Studies.* VDR binding, HL-60 differentiation, and 24-hydroxylase transcription assays were performed as previously described.[26,27]

CYP27B1 Activity Assay. 25-Hydroxyvitamin D_3 was purchased from SAFC-Pharma (Madison, WI). Mouse CYP27B1, bovine adrenodoxin (Adx), and adrenodoxin reductase (AdR) were purified as described previously.[28] The reconstituted CYP27B1 enzymatic assay was carried out in a buffer containing 20 mM Tris (pH 7.7), 125 mM NaCl, 0.1% CHAPS, 2 μM Adx, 0.2 μM AdR, 0.05 μM CYP27B1, and 0−5 μM vitamin D substrate. All the vitamin D analogues were dissolved in ethanol, and the concentrations were determined based on UV absorption at appropriate wavelengths (ε_{254} = 42 mM^{-1} cm^{-1} for **4**; ε_{265} = 18.2 mM^{-1} cm^{-1} for 25-OH-D_3; ε_{270} = 18.0 mM^{-1} cm^{-1} for **8** and **9**). The reactions were initiated by the addition of NADPH at a final concentration of 1 mM and incubated at 37 °C for 5−20 min to keep substrate conversion ≤20%. Then the reactions were quenched and extracted with dichloromethane, and the organic layer was collected and dried under ambient conditions. After being redissolved in hexane/2-propanol (93:7) solvent system, the mixture was analyzed by HPLC (Zorbax Rx-Sil, 9.4 mm × 25 cm, 4 mL/min) at 265 nm. The initial rates calculated from product formation were fitted to the Michaelis−Menten equation $V = k_{cat}[E]_0[S]/(K_M + [S])$ using GraphPad Prism 5 to obtain the k_{cat} and K_M values.

In Vivo Studies. Bone Calcium Mobilization and Intestinal Calcium Transport. Male weanling Sprague−Dawley rats were purchased from Harlan (Indianapolis, IN). The animals were group housed and placed on Diet 11 (0.47% Ca) + AEK oil for 1 week followed by Diet 11 (0.02% Ca) + AEK oil for 3 weeks. The rats were then switched to a diet containing 0.47% Ca[29] for 1 week followed by 2 weeks on a diet containing 0.02% Ca. Dose administration began during the last week on 0.02% Ca diet. Four consecutive intraperitoneal doses were given approximately 24 h apart. Twenty-four hours after the last dose, blood was collected from the severed neck, and the concentration of serum calcium was determined as a measure of bone calcium mobilization. The first 10 cm of the intestine was also collected for the intestinal calcium transport analysis using the everted gut sac method.[26]

Molecular Modeling. The molecular mechanics studies were used to establish the energy-minimized structures of the synthesized compounds shown in Figure 3. The calculation of optimized geometries and steric energies was initially carried out using the algorithm from the MM^+ HyperChem (release 8.0) software package (Autodesk, Inc.). MM^+ is an all-atom force field based on the MM2 functional form. The procedure used for finding the global minimum structures involved the Conformational Search module; these structures were further energy-minimized using the semiempirical AM1 method. Finally, the respective vicinal proton−proton coupling constants in the preferred energy-minimized conformations were calculated using PCMODEL (release 9.0) software package (Serena Software).

■ ASSOCIATED CONTENT

Supporting Information

Purity criteria of the vitamin D analogues **8**−**11**, their ^1H and ^{13}C NMR spectra, competitive binding curves or dose−response curves derived from cellular differentiation and transcriptional assays, and spectral data of the all synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

$1\alpha,25\text{-}(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 2MD, (20*S*)-1α,25-dihydroxy-2-methylene-19-norvitamin D_3; VDR, vitamin D receptor

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