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Synthesis, Conformational Analysis, and Biological Activity of the 1α,25-Dihydroxy-10,19-Dihydrovitamin D_3 Isomers

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The synthesis of the four 1α,25-dihydroxy-10,19-dihydrovitamin D_3 stereoisomers **(8–11)** is described starting from 25-hydroxyvitamin D_3 **(1c)**. Acetic acid-catalyzed cycloreversion of 1α-hydroxylated 3,5-cyclovitamin D compound **14,** produced by allylic oxidation of the intermediate cyclovitamin **13,** afforded 1α,25-dihydroxyvitamin D_3 3-acetate **(16)** and its 5E-isomer **17.** Catalytic hydrogenation of **16** produced 10,19-dihydrovitamin acetates **20** and **21,** whereas the same reaction of **17** resulted in the formation of 5E-isomers **22** and **23.** The analogous saturation of the 10,19-double bond in **14** gave 10(*S*), 19- and 10(*R*), 19-dihydrocyclovitamins **18** and **19** which after cycloreversion with acetic acid yielded different stereoisomeric pairs of 10,19-dihydrovitamin acetates **21, 23, 20,** and **22,** respectively. The stereochemistry and solution conformations of the A-ring of the 3-acetates **20–23** and their parent alcohols **8–11** were studied using ^{1}H NMR data. The A-ring chair population ratios of these stereoisomers were determined by the method of correlation of the observed coupling constants with the limiting values derived from cyclohexanol. The obtained results were confirmed by evaluation of interaction energies introduced by A-ring substituents and calculation of the free energy differences between the respective dihydrovitamin conformers. Conformational analyses of 10,19-dihydrovitamins were also carried out on model compounds **24–27** by using force-field calculations. Biological activity *in vivo* revealed that the 1α,25-dihydroxy-10(*S*), 19-dihydrovitamin D_3 **(9)** followed by the 1α,25-dihydroxy-10(*S*), 19-dihydro-(5*E*)-vitamin D_3 **(11)** to be the most active, while the 10(*R*)-isomers **8** and **10** possessed little or no activity. *In vitro,* the compounds possessing the most equatorial 1-hydroxyl, i.e., the 10(*R*)-isomers, were found most active, and the least equatorial were the least active.

Extensive studies (*1–3*) on the metabolism of vitamin D_3 **(1a)** (Fig. 1) and parallel work carried out with vitamin D_2 **(1b)** have shown that they undergo hydroxylation at C-25 in the liver to the corresponding 25-hydroxyvitamin D_3 [25-OH-D_3] **(1c)** (*4*) and 25-hydroxyvitamin D_2 [25-OH-D_2] **(1d)** (*5*) which are then further C-1-hydroxylated in the kidney to the active hormonal forms, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] **(2c)** (*6*) and 1α,25-dihydroxyvitamin D_2 [1,25-$(OH)_2D_2$] **(2d)** (*7*), respectively. The primary function of the most active renal metabolites **2c** and **2d** is to increase serum calcium and phosphorus concentration to supersaturating levels that can support normal bone formation (*2*). Many other vitamin D metabolites and analogs have been obtained and tested with the aim of

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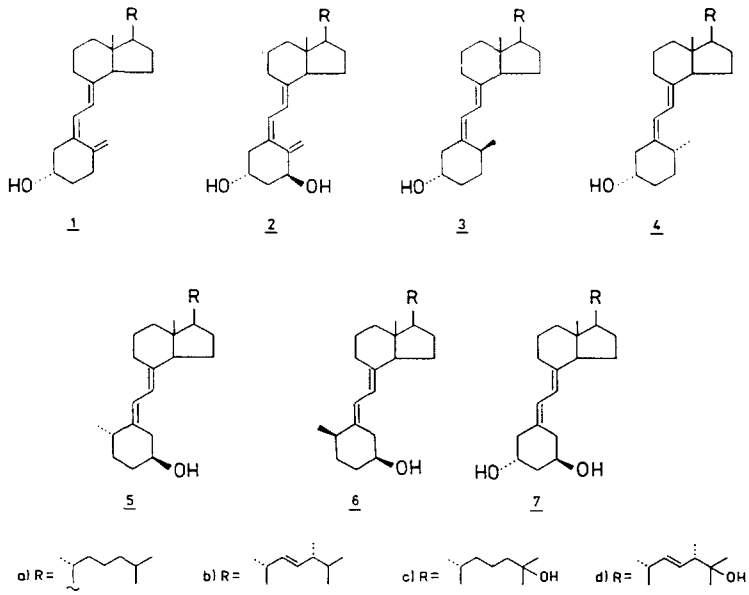


FIG. 1. Structure of vitamin D metabolites and analogs.

establishing structural and stereochemical features necessary for their biological activity as calcium regulators (*1–3, 8*). Systematic studies of structure–activity relationships have illustrated the key importance of the hydroxy functions at C-1 and C-25. The C-3 hydroxy group is usually less important, although it enhances considerably the biological potency of 5*E*-vitamins D due to transposition into a pseudo-1α-hydroxy group (*8–10*). The discovery of Suda *et al.* (*11–13*) that 1,25-$(OH)_2D_3$ **(2c)** also induces monocytic differentiation of human promyelocytic leukemia cells (HL-60) stimulated new interest in establishing the importance of specific structural units of the vitamin D molecule for expression of this unusual activity. Preliminary studies on activity comparisons between various vitamin D metabolites have shown that **2c** is the most potent in cellular differentiation and the order of activity correlates with calcemic activity of the compounds (*14, 15*). Although our extensive and systematic study of structure–activity relationships (*16, 17*) supports the crucial role of the hydroxy groups at C-1 and C-25, it also indicates the structural modifications of the vitamin D molecule which result in preferential differentiating activity of the analogs. Since the structural requirements referred mainly to steroidal side chain, we have turned our attention toward the synthesis and biological evaluation of the vitamin D analogs modified in the triene system, namely the 10,19-dihydrovitamins.

The products of reductions of the C(10)–C(19) double bond in vitamin D_2 and D_3 were described previously in the literature and two of them [DHT_3, **5a** (*18*) and DHT_2, **5b** (*19, 20*)] found clinical application. However, the stereochemistry at C-10 was assigned much later. The four possible 10,19-dihydrostereoisomers **(3a–**

6a) (*21*), resulting from the reduction of the natural vitamin D_3 **(1a),** and the corresponding compounds **(3b–6b)** (*22*) in the related vitamin D_2 series, have been synthesized and fully characterized. Epimeric 5*Z* compounds in the vitamin D_3 side chain series **3a** and **4a** failed to exhibit any biological activity in terms of intestinal calcium transport, intestinal calcium absorption, and bone calcium mobilization (*in vivo* in the chick) (*23*), whereas 5*E*-epimers **5a** and **6a** were found to be active (*24*). Dihydrotachysterols **5a** and **5b** are known to be metabolized (hydroxylation at C-25) to **5c** (*25–27*) and **5d** (*28*), respectively. Moreover, it has recently been established that a further metabolic pathway (hydroxylation at C-1) can also occur in the D_2 and D_3 (*29–31*) series; the evidence has been provided for hydroxylation of 9,10-seco steroids at pseudo-C-3 position. In view of this finding and the observation of highly selective activity in the HL-60 system of 1α,25-dihydroxy-19-nor-vitamin D_3 **(7c)** recently synthesized in our laboratory (*32*), we were encouraged to pursue studies on the related compounds possessing 1,3-diene moiety at C-5 and C-8 and all hydroxyl groups (1α, 3β, 25) which seemed to be important for differentiating potency. This paper describes our synthetic route to the stereoisomeric 1α,25-dihydroxy-10,19-dihydrovitamin D_3 analogs (*8–11*) (Fig. 2) and the detailed conformational analysis of these A-ring compounds as well as their biological activity.

RESULTS

The starting material, 25-OH-D_3 **(1c),** was converted to 1α-hydroxycyclovitamin compound **14** utilizing the method of Paaren *et al.* (*33*) and the procedure analogous to that described by us (*34*) for D_2 analogs (Fig. 2). Tosylation of **1c** followed by bicarbonate-buffered methanolysis of 3β-tosylate **12** afforded the cyclovitamin **13** which was, in turn, oxidized with selenium dioxide and *tert*-butyl hydroperoxide system. The 1α-hydroxylated cyclovitamin **14** (40% yield from **12**) was separated from the minor component, 1-ketocyclovitamin **15,** by column chromatography. Acetic acid-catalyzed cycloreversion (*33*) of **14** yielded 1,25-$(OH)_2D_3$ 3-acetate **16** and its 5*E*-isomer **17** in the ratio 2.5 : 1. Repeated HPLC separation of the mixture and purification of **16** by a maleic anhydride procedure (*35*) furnished pure geometrical isomers. Cyclovitamin **14** was also subjected to homogeneous catalytic hydrogenation using tris(triphenylphosphine)rhodium chloride (Wilkinson's catalyst) (*36*). The two epimeric 10,19-dihydrocyclovitamins **18** and **19** (15 and 55% yield, respectively) were readily distinguished by their ^1H NMR spectra. A deshielding effect in **19** was observed on the signal due to a hydrogen at C-6 ($\Delta\ \delta$ 0.39) whereas 10-methyl and (6*R*)-methoxy groups were more deshielded ($\Delta\ \delta$ ca. 0.08 for both signals) in **18.** Similar shift differences have been reported for 10(*R*)- and 10(*S*)-methyl cyclovitamin pairs (1- and 25-deoxy analogs of **19** and **18**) synthesized in our laboratory (*37*). All compounds **16–19,** easily obtained from cyclovitamin **14,** were the direct precursors of the desired 10,19-dihydrovitamins. Thus, the homogeneous catalytic hydrogenation of **16** resulted in the selective reduction of 10,19 double bond and formation of C-10 epimeric acetoxy dienes **20** and **21** in the ratio of 1 : 10 and 86% yield. The analo-

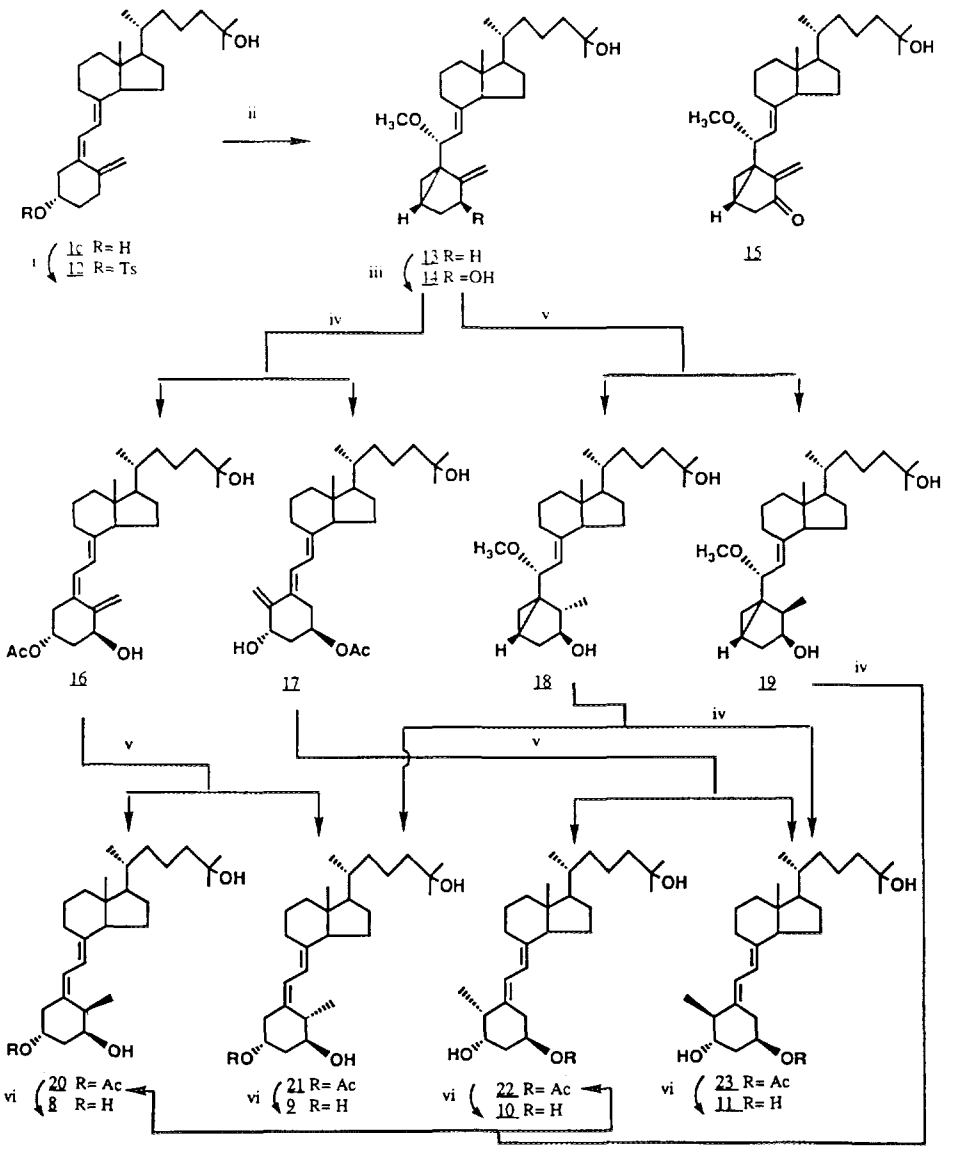


FIG. 2. Structures and reaction schemes pertaining to synthesis and biological activity measurements. Reagents and conditions: (i) *p*-TsCl, py, 4°C; (ii) $KHCO_3$, MeOH, 55°C; (iii) *t*-BuOOH, SeO_2, CH_2Cl_2, py; (iv) AcOH, 55°C; (v) H_2, $[(C_6H_5)_3P]_3$ RhCl, C_6H_6; (vi) KOH, MeOH–EtOH.

gous reduction of **17** provided the corresponding dihydrovitamins **22** and **23** (ratio of 1 : 2.5, 71% yield) in 5*E* series. The intense uv spectra of vitamin acetates **20–23** and their parent 3β-hydroxy analogs **8–11,** obtained by hydrolysis with methanolic KOH, exhibited a characteristic triplet (λ_{max} ca. 243, 251, and 260 nm), indicating a presence of a planar transoidal C-5 and C-8, 1,3-diene chromophore

TABLE I

The Chemical Shifts and Multiplet Structure of the Signals in ^{1}H NMR Spectra[a,b] of 1α,25-Dihydroxy-10,19-dihydrovitamin D_3 Stereoisomers

Assignment	Compound: **8**	**20**	**9**	**21**	**10**	**22**	**11**	**23**
18-H_3	0.55 s	0.55 s	0.54 s	0.54 s	0.55 s	0.54 s	0.55 s	0.54 s
21-H_3	0.94 d(6.5)	0.94 d(6.0)	0.94 d(6.4)	0.94 d(6.1)	0.94 d(6.3)	0.94 d(6.3)	0.94 d(6.2)	0.94 d(6.3)
19-H_3	1.06 d(7.0)	1.05 d(7.0)	1.09 d(7.2)	1.10 d(6.8)	1.15 d(6.9)	1.15 d(7.1)	1.19 d(6.6)	1.20 d(6.8)
26-H_3, 27-H_3	1.22 s	1.22 s	1.22 s	1.22 s	1.22 s	1.22 s	1.22 s	1.22 s
3β-OAc		2.00 s		2.04 s		2.02 s		1.99 s
10-H	3.26 dq(4.5,7.0)	3.26 dq(4.5,7.0)	2.99 dq(1.5,7.2)	3.02 dq(1.9,6.8)	2.43 m	2.46 dq(2.4,7.1)	2.23 quint(6.6)	2.22 quint(6.8)
4α-H	2.61 br d(~14.5)	2.50 br d(~15.5)	2.42 dd(12.7,4.5)	2.50 dd(12.8,4.5)	3.04 dd(13.0,3.7)	2.93 dd(13.1,3.8)	2.58 dd(13.4,3.5)	2.40 dd(13.7,3.1)
4β-H	2.08 br d(~14.5)	2.23 br d(~15.5)	2.34 br t(~12.0)	2.37 br t(~12.2)	~2.0 n	~2.15 n	2.45 dd(13.4,7.1)	2.71 dd(13.7,7.0)
9β-H	2.80 br d(~13.0)	2.80 br d(~12.5)	2.81 br d(~12.3)	2.80 br d(~12.5)	2.82 br d(~12.5)	2.81 br d(~13.0)	2.81 br d(~12.6)	2.81 br d(~12.5)
1β-H	4.05 m($w/2$~22)	4.04 m($w/2$~23)	3.94 m($w/2$~10)	3.94 m($w/2$~9)	~3.95 m	4.00 m($w/2$~15)	3.71 m($w/2$~18)	3.66 m($w/2$~18)
3α-H	4.13 quint(2.8)	5.11 quint(2.7)	3.99 tt(11.0,4.5)	5.02 tt(11.5,4.5)	~3.95 m	4.98 tt(8.8,3.8)	4.05 m($w/2$~22)	5.07 tt(7.0,3.1)
7-H	5.89 d(11.2)	5.88 d(11.2)	5.82 d(11.1)	5.81 d(11.3)	5.91 d(11.0)	5.85 d(11.1)	5.87 d(11.2)	5.78 d(11.0)
6-H	6.20 d(11.2)	6.11 d(11.2)	6.34 d(11.1)	6.36 d(11.3)	6.24 d(11.0)	6.25 d(11.1)	6.36 d(11.2)	6.30 d(11.0)

[a] ppm δ values, the coupling constants (in parentheses) and halfwidths are given in Hz, 500 MHz, solutions in $CDCl_3$ with Me_4Si as an internal standard.

[b] br, broad; d, doublet; m, multiplet; n, not observable (overlapped with other signals); q, quartet; quint, quintet; s, singlet; t, triplet; $w/2$, halfwidth.

(*38*). The structures of the isolated products have been initially assigned on the basis of a careful comparison of their ^{1}H NMR spectra (Table 1) with the corresponding spectral parameters of the 10(*R*),19- and 10(*S*),19-dihydrovitamin D_3 compounds **3a–6a** reported in the literature (*21*). Further confirmation of the ascribed configurations came from the results of the cycloreversion process of 10,19-dihydrocyclovitamins **18** and **19.** Thus, cycloreversion reaction of **18** performed in acetic acid produced a mixture of 3β-acetoxy 5*Z*-vitamin **21** and its 5*E*-isomer **23** in the ratio of 4.4 : 1 (67% yield), whereas the same process for **19** gave only traces of the product with 5*Z* configuration (50 : 1 ratio of **22** and **20,** 88% yield).

The stereoselective formation of almost exclusively one of the two possible acetoxy vitamins in the acetolysis (*39*) of the cyclovitamin **19** strongly indicates

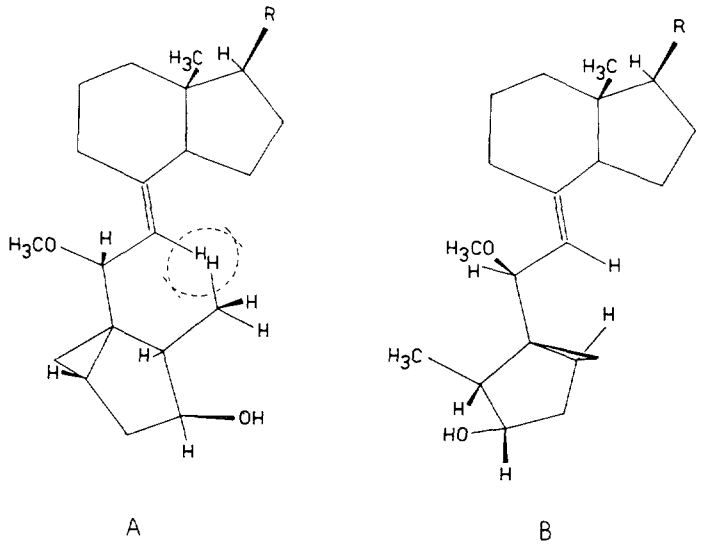


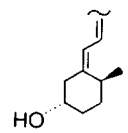
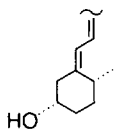
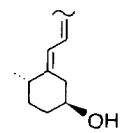
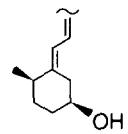
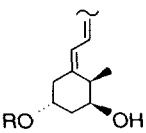
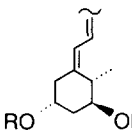
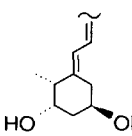
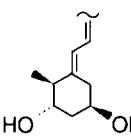
FIG. 3. Perspective formulas of 6*R*-substituted 10(*R*)-methylcyclovitamin in the conformation (A) suitable for formation of 5*Z*-vitamin and an epimeric 6*S*-substituted cyclovitamin in the conformation (B) suitable for formation of 5*E*-vitamin.

the creation of an intermediate with a high degree of carbonium ion character (*40, 41*). It is well known that cycloreversion of 6*R*-substituted cyclovitamin can only yield 5*Z*-vitamin system by either a concerted S_N2 or a solvolytic S_N1 process (*33*). However, in the case of cyclovitamin **19,** it can be postulated that the molecule cannot adopt a conformation suitable for creation of a low energy concerted transition state (Fig. 3A) due to severe interaction of C-10 methyl protons with the hydrogen at C-7. It is, therefore, likely that protonated (6*R*)-methoxy group dissociates from the cyclovitamin and the resulting cyclopropylcarbinyl cation recombines with a methanol molecule to give epimeric (6*S*)-methyl ether. Contrary to cyclopropylcarbinyl cation (*42, 43*), 6*S*-substituted cyclovitamin can rotate about the 5,6-double bond and easily achieve the geometry (Fig. 3B) necessary for the C(3) and C(5) bond participation in the transition state leading to 5*E*-vitamin **22.** Examination of Dreiding models indicated that 10(*S*)-methyl group in the cyclovitamin **18** does not introduce any steric hindrance, and it was not, therefore, surprising that cycloreversion of **18** accorded predominantly acetoxy alcohol with 5*Z* geometry of the diene system.

However, detailed literature studies indicated that stereochemical course of 10,19-double bond reduction established by us for vitamins **16** and **17** contrasts with some published data. Barton and Hesse (*44*) described hydrogenation of a number of vitamin D compounds using tris(triphenylphosphine)rhodium chloride and they observed decreased rate of the reactions of 1α-hydroxylated compounds. Authors claimed that the presence of a free 1α-hydroxy group resulted also in a stereospecific formation of isomers having 3β-hydroxy and C-10-methyl group in

TABLE 2

Equilibrium Populations of the Ring A Conformers of 10,19-Dihydrovitamin D Stereoisomers

Compound	Compound no.	R	$J_{3\alpha,4\beta}$ (Hz)[a]	$J_{3\alpha,4\alpha}$ (Hz)[a]	N_J (%)[b]	ΔG_e° (kcal/mol)[c,d]	ΔG_a° (kcal/mol)[d,e]	ΔG_{ae}° (kcal/mol)[d,f]	N_T (%)[g]
	**3a**		~3[h]	~3[h]	0 ± 6[h]	4.84	2.22	2.62	1
	**4a**		10.5[h]	4.0[h]	94 ± 6[h]	1.70	5.36	−3.66	100
	**5a**		10.0[h]	4.1[h]	88 ± 6[h]	1.25	2.22	−0.97	84
	5b		9.7[i]		84 ± 8[i]				
	**6a**		7.0[h]	3.5[h]	50 ± 5[h]	1.70	1.77	−0.07	53
	**8**	H	2.8	2.8	1 ± 4	5.71	2.57	3.14	1
	20	Ac	2.7	2.7	0 ± 4	5.71	2.65	3.06	1
	**9**	H	11.0	4.5	99 ± 5	2.22	5.71	−3.49	100
	21	Ac	11.5	4.5	100 ± 4	2.22	5.79	−3.57	100
	**10**	H		3.7		2.12	2.57	−0.45	68
	22	Ac	8.8	3.8	71 ± 7	2.12	2.65	−0.53	71
	**11**	H	7.1	3.5	52 ± 4	2.22	2.12	0.10	46
	23	Ac	7.0	3.1	46 ± 6	2.22	2.20	0.02	49

[a] The coupling constants are considered to be accurate to ± 0.1 Hz.

[b] Percentage of the conformer with an equatorial substituent at C-3 calculated using the limiting coupling constants taken from Ref. (*52*).

[c] The free energy differences between the 3β-equatorially substituted vitamin conformer and an isomeric hypothet-

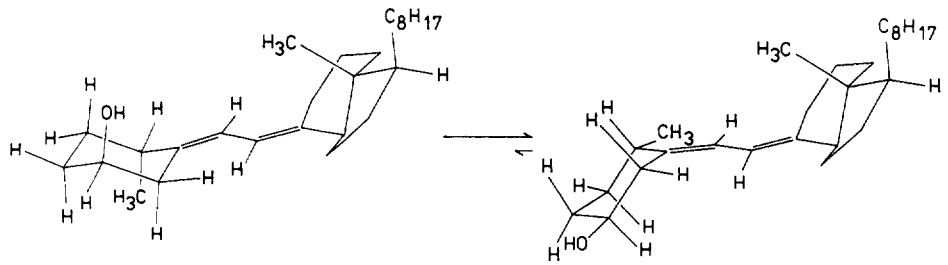


FIG. 4. Conformational equilibrium in dihydrotachysterol$_3$ (DHT_3, **5a**).

anti-relationship (*45*). Although we believed that ^{1}H NMR chemical shift comparisons lend considerable confidence to the C-10 configurational assignments given by us to the four target compounds **8–11,** we decided to acquire additional evidence with a detailed conformational analysis of the dihydrovitamin system in view of these divergent data.

From ^{1}H NMR studies of vitamin D_3 **(1a)** (*46*) and D_2 **(1b)** (*47*), it was determined that these B-ring secosteroids exist in solution as a mixture of approximately equal amounts of two rapidly equilibrated A-ring chair conformers. A similar conformational equilibrium has been also found for **1c** and **2a,** the nature hormone **2c** (*46, 48*), and other ring-A-substituted vitamin D derivatives (*48–50*), as well as for the 10,19-dihydrovitamin D_3 analogs (*21, 51*). The dynamic equilibration between two chair forms of the A-ring (exemplified in Fig. 4 for the clinically useful DHT_3, **5a**) can be deduced from an analysis of a multiplet pattern of the methine 3α-proton, namely from the magnitudes of its degenerate couplings to the protons at C-2 and C-4. Analysis of the observed coupling constants of the 3α-proton in **5a** established a ratio of the two conformers to be 12 : 88 in favor of the 3β-OH equatorial conformer (*21*); a highly similar ratio was found for the analog **5b** with ergosterol side chain (*51*). The corresponding vicinal couplings for the proton at C-3 and the calculated proportions of the 3β-equatorial conformers reported for the 10,19-dihydrovitamin D isomers are listed in Table 2.

The simplicity of the coupling constant method giving in most cases results comparable with those of a computer analysis of the lanthanide shifted spectra

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ical vitamin whose A-ring substituents are all equatorial, and they do not interact with each other as well as with the olefinic protons.

[d] Value is calculated on the basis of energy considerations and refers to room temperature (25°C).

[e] The free energy differences between the 3β-axially substituted vitamin conformer and an isomeric hypothetical vitamin whose A-ring substituents are all equatorial, and they do not interact with each other as well as with the olefinic protons.

[f] The free energy difference for the equilibrium: 3β-axially substituted $\rightleftharpoons$ 3β-equatorially substituted vitamin conformer.

[g] Percentage of the conformer with an equatorial substituent at C-3 computed from the free energy difference ΔG°_{ae}.

[h] Value taken from Ref. (*21*).

[i] Value taken from Ref. (*51*).

(*21, 46*) encouraged us to attempt a similar conformational analysis of 1α,25-dihydroxy-10,19-dihydrovitamin D_3 analogs (*8–11*). Assignments of the NMR signals to the particular A-ring protons were established from the ^1H, ^1H COSY spectra of vitamins. The multiplet structure of the methine C-3 proton was sufficiently resolved in the spectrum of dihydrovitamins **8** and **9** only (Table 1); the corresponding vicinal couplings for 3α-H in compound **11** were found in its 4α- and 4β-H signals. From the observed larger *trans*-vicinal couplings of the 3α-proton $J_{3\alpha,4\beta}$ representing average axial–axial and equatorial–equatorial values, and the vicinal coupling constant data reported by Anet (*52*) for 3,3,4,5,5-pentadeuterio-4-*tert*-butylcyclohexanol ($J_{a,a}$ = 11.1 Hz, $J_{e,e}$ = 2.7 Hz), the A-ring conformational populations were calculated. Standard deviations in $J_{a,a}$, $J_{e,e}$, and J_{obsd} of 0.1 Hz each gave an estimated standard deviation in the 3β-equatorial conformer populations N_J (Table 2) computed from the relation

$$J_{3\alpha,4\beta} = [N_J J_{a,a} + (100 - N_J)J_{e,e}]/100.$$

For the corresponding 3β-acetates **20–23,** exhibiting well-resolved 3α-H resonances, the coupling constants found for 3,3,4,4,5,5-hexadeuteriocyclohexanol acetate (*52*) ($J_{a,a}$ = 11.4 Hz, $J_{a,e}$ = 4.2 Hz, average of $J_{e,a}$ and $J_{e,e}$ = 2.7 Hz) were applied; the calculated N_J values were close to those of the parent hydroxy compounds. A comparison of the data obtained from the coupling constant analysis shows that the population of 3β-equatorially substituted vitamin conformers in dihydrovitamin D_3 compounds and their 1α,25-dihydroxylated counterparts (**3a, 8; 4a, 9; 6a, 11** pairs) are similar except the notable difference between tachysterol compounds **5a,b** and **22.** Ring-A conformational equilibria in 1,25-dihydroxy-10,19-dihydrovitamin D_3 compounds are shown in Fig. 5.

Although these results of conformational analysis seemed to support the configurational assignments given for the target vitamins **8–11,** we decided to get additional information by considering conformational energies of the respective molecules. Since the equilibrium between the two interconverting vitamin conformers is dependent on the nonbonded interactions present in both forms, we attempted to estimate the corresponding conformational energies. Only the nonbonded interactions caused by acetoxy, hydroxy, and methyl substituents of the A-ring and the olefinic protons at C-6 and C-7 were considered, assuming that the conformation of the rest of the molecule (C/D rings and side chain) remains the same in both equilibrating forms. We also assumed that the occurrence of one interaction in the compound does not influence the magnitude of another.

The calculations were done in the following way. The corresponding energies of each nonbonded interaction in each conformer were estimated and added up. Neglecting the entropy contribution, the sum estimates the free energy content of the given conformer, calculated relative to the hypothetical isomeric vitamin molecule whose A-ring substituents are all equatorial, and they do not interact with each other or with the diene part of the molecule. The difference between the free energies for each conformer and its corresponding counterpart, which is calculated in this way, is assumed to represent the free energy difference between the equilibrating forms. The following interaction energies were used for the calcula-

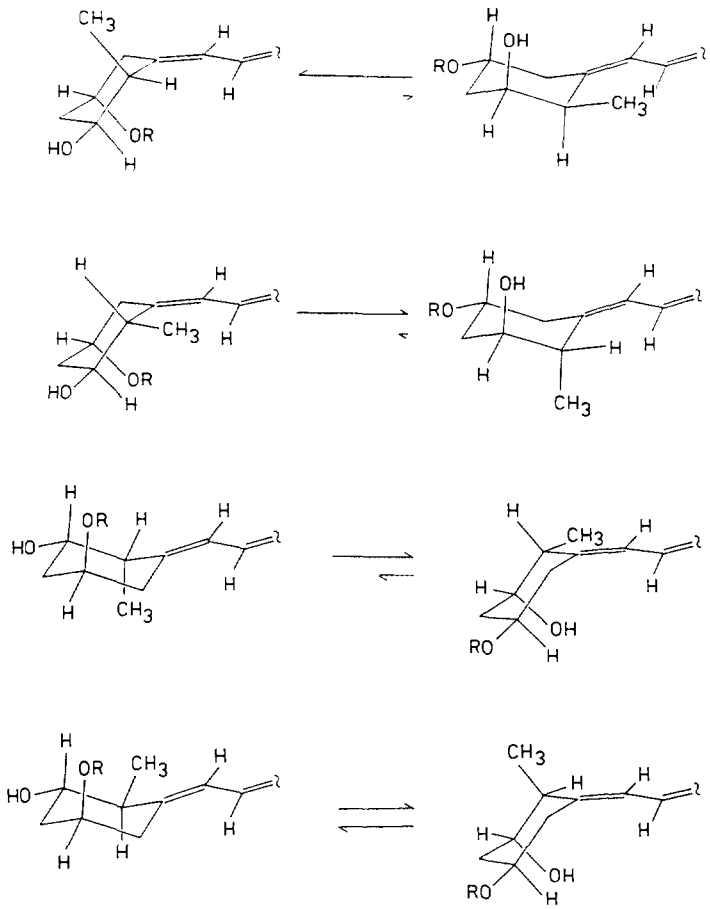


FIG. 5. Conformational equilibria between 3β-axial (left side) and 3β-equatorial (right side) A-ring chair conformers of 1α,25-dihydroxy-10,19-dihydrovitamin D_3 stereoisomers [top to bottom: **8(20), 9(21), 10(22), 11(23);** R = H (Ac)].

tions: (a) conformational energies (*53*) of acetoxy (0.60 kcal/mol), hydroxy (0.52 kcal/mol), and methyl (1.70 kcal/mol) substituents of the cyclohexane-A chair-like ring; (b) syn-clinal interaction between 10-methyl and 1α-hydroxy group (0.35 kcal/mol) (*54*); (c) $A^{1,3}$-strain interaction (*55*) between an equatorial 10-methyl and the vinyl hydrogen at C-7 (4.84 kcal/mol) present in the compounds with 5*Z*-configuration (*56, 57*); (d) 1 : 3 peri interaction between an equatorial 10-methyl and the vinyl hydrogen at C-6 (1.25 kcal/mol) occurring in the compounds of 5*E* series (*56, 57*). The results of these computations, performed for all 10,19-dihydrovitamins including 1-deoxy forms **3–6** are summarized in Table 2. The most important value, ΔG°_{ae}, is defined as the free energy difference for the equilibrium between chair conformer possessing the axial 3β-hydroxyl (or acetoxyl) and the conformer with equatorial 3β-substituent. The values for the 3β-equatorial con-

former populations (N_T, Table 2) calculated from the relation

$$\Delta G^\circ_{ae} = -RT \ln(N_T/100 - N_T)$$

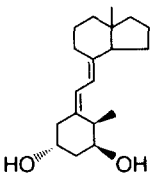
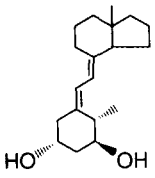
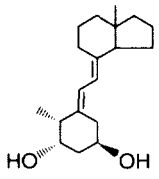
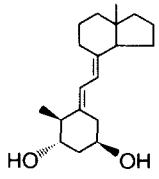
were found to be in surprisingly good agreement with those (N_J) derived from the 3α-proton coupling constant data.

These findings encouraged us to extend the conformational analysis of 10,19-dihydrovitamins by testing one of molecular mechanics methods (*58*) which have recently been used extensively for the determination of the structures and energies of different molecules (*59*). Our choice fell on the MM+ (*60*) molecular mechanics program (an enhanced version of MM2) (*61*), and force-field calculations were carried out on model compounds lacking side chain, i.e., 1α-hydroxy-10,19-dihydro-20,21,22,23,24,25,26,27-octanorvitamin D_3 stereoisomers **(24–27).** The estimated values of steric energies (Table 3) represent the difference in energy between the "real" vitamin conformers with equatorially (E_e) or axially (E_a) oriented 3β-hydroxy groups and the hypothetical molecules where all the structural parameters (bond lengths, bond angles, dihedral angles, etc.) have preferential "ideal" values. Thus, the difference in the steric energy for both geometries of the same molecule (ΔE_{ae}) is suitable for calculation of the preferred A-ring conformation. The estimated populations of 3β-equatorially oriented conformers (N_M, Table 3) show close similarity with the corresponding N_J values (Table 2) obtained from proton coupling constant analysis of the analogous compounds with the same configurations of the A-ring substituents and diene system. From the above observations, it follows that simple calculations of interaction energies as well as a molecular mechanics approach can be satisfactorily used to predict conformational equilibria in vitamin analogs lacking the exocyclic 10,19-double bond. Evidently, in the case of 5*Z* compounds a destabilizing effect of the severe steric repulsion between the equatorial methyl substituent at C-10 and the proton at C-7 plays a crucial role shifting the equilibrium far to the side of the conformer possessing an axially oriented 10-methyl group. Thus for example for the structure **8(20),** it results in stabilization of the chair conformation (see Fig. 5) with 1α-OH and 3β-OH (OAc) groups in equatorial and axial orientation, respectively. This fact in turn is reflected in the corresponding ^{1}H NMR spectra showing, as in the case of 1-deoxy analogs **3a,** similar small couplings of 3α-H to both 4-proton ($J_{e,e} \approx J_{e,a} \approx 3$ Hz). It is certain, therefore, that we did synthesize the compound of structure **8(20)** and all structural assignments given by us to the remaining stereoisomeric vitamins **9–11** are correct.

The fact that A-ring conformational population in the vitamin D analogs **8–11** can be relatively easily predicted and established introduced another intriguing problem, i.e., the possibility of correlation between the preferred geometries of vitamins and their biological activity. In 1974, it was proposed (*9*) that calcium regulation ability of vitamins D is limited to the compounds that can assume A-ring chair conformation in which 1α-OH (or pseudo-1α-OH) occupies the equatorial orientation. Thus, the 1α, 25-dihydroxy-10,19-dihydrovitamin D_3 isomers **8, 10, 11,** and **9** form a series exhibiting decreasing equatorial character of 1α-OH **(8, 9)** or pseudo-1α-OH **(10, 11).** These stereoisomers contain ≈100, 70, 50, and 0%,

TABLE 3

A-Ring Conformational Populations of Model 10,19-Dihydrovitamin D Stereoisomers Lacking Side Chain

Compound	Compound no.	E_e (kcal/mol)[a,b]	E_a (kcal/mol)[a,c]	ΔE_{ae} (kcal/mol)[d]	N_M (%)[e]
	**24**	38.00	34.80	3.20	0
	**25**	34.63	37.80	−3.17	100
	**26**	34.02	34.66	−0.64	74
	**27**	34.04	34.20	−0.16	57

[a] The energies calculated for model 1α-hydroxy-10,19-dihydro-octanorvitamin D_3 compounds by MM$^+$ force-field method (Ref. *60*).

[b] Steric energy of 3β-equatorially substituted vitamin conformer.

[c] Steric energy of 3β-axially substituted vitamin conformer.

[d] The steric energy difference between 3β-equatorially and 3β-axially substituted vitamin conformers.

[e] Percentage of the conformer with an equatorial substituent at C-3 computed from the steric energy difference ΔE_{ae}; value calculated for room temperature (25°C).

respectively, of such "required" equatorial hydroxyl, and it was interesting to determine whether their biological activity follows the same order.

An initial investigation of biological activity was carried out by determining the ability of the four isomers to displace 1,25-$(OH)_2[^3H]D_3$ from the porcine nuclear 1,25-$(OH)_2D_3$ receptor. The results shown in Figs. 6A and 6B demonstrate that compounds **10** and **8** are equally active in binding to the receptor, whereas com-

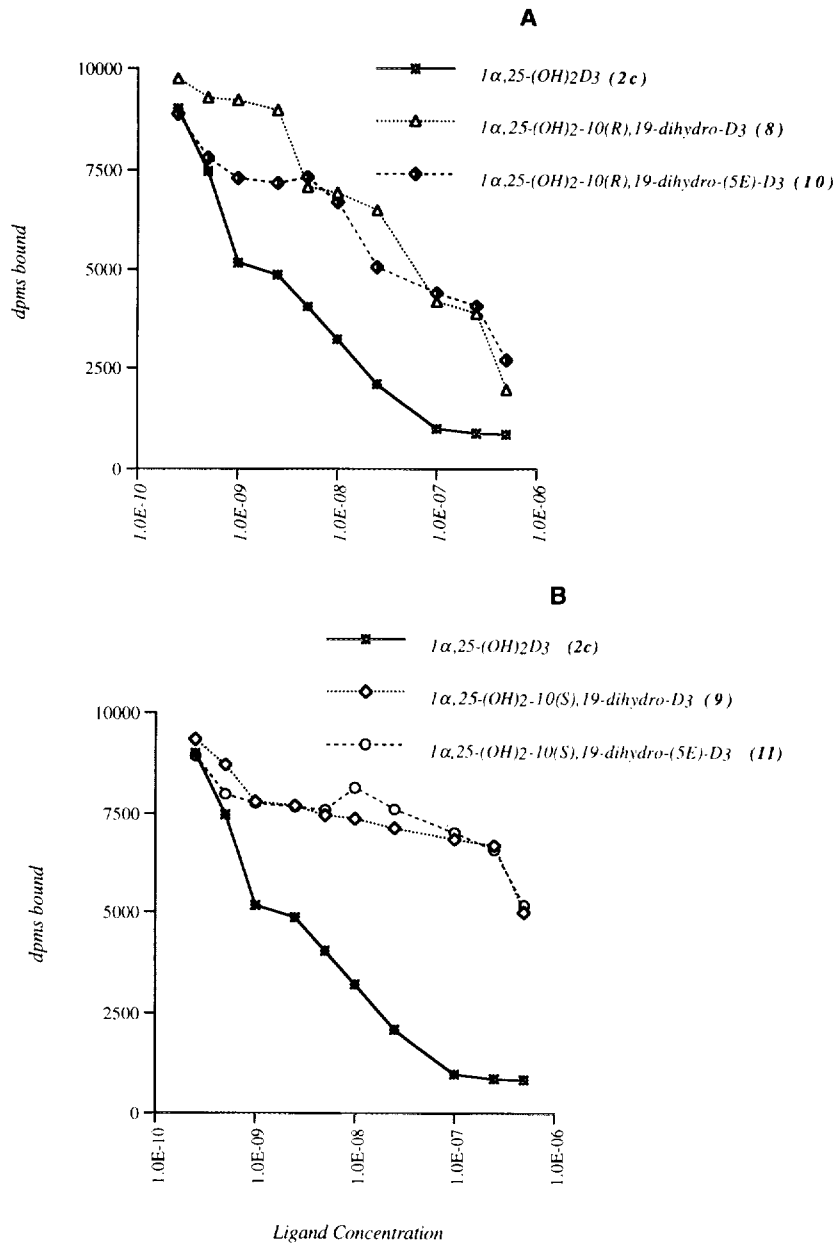


FIG. 6. Displacement of [26,27-^{3}H] 1α,25-$(OH)_2D_3$ from the 1,25-$(OH)_2D_3$ porcine intestinal nuclear receptor by 10,19-dihydro analogs of 1,25-$(OH)_2D_3$. (A) 10(*R*)-methyl analogs; (B) 10(*S*)-methyl analogs.

pounds **9** and **11** show little activity in this regard. Cellular activity was determined by studying the differentiation of HL-60 cells into monocytes. In this system, compound **10** proved to be the most active, followed by compound **8**, whereas compounds **9** and **11** again showed little activity (Figs. 7A and 7B).

It was indeed surprising that compounds **8** and **10**, when given a 1 μg/day/7 days (a very high dose), gave no significant response in either intestinal calcium

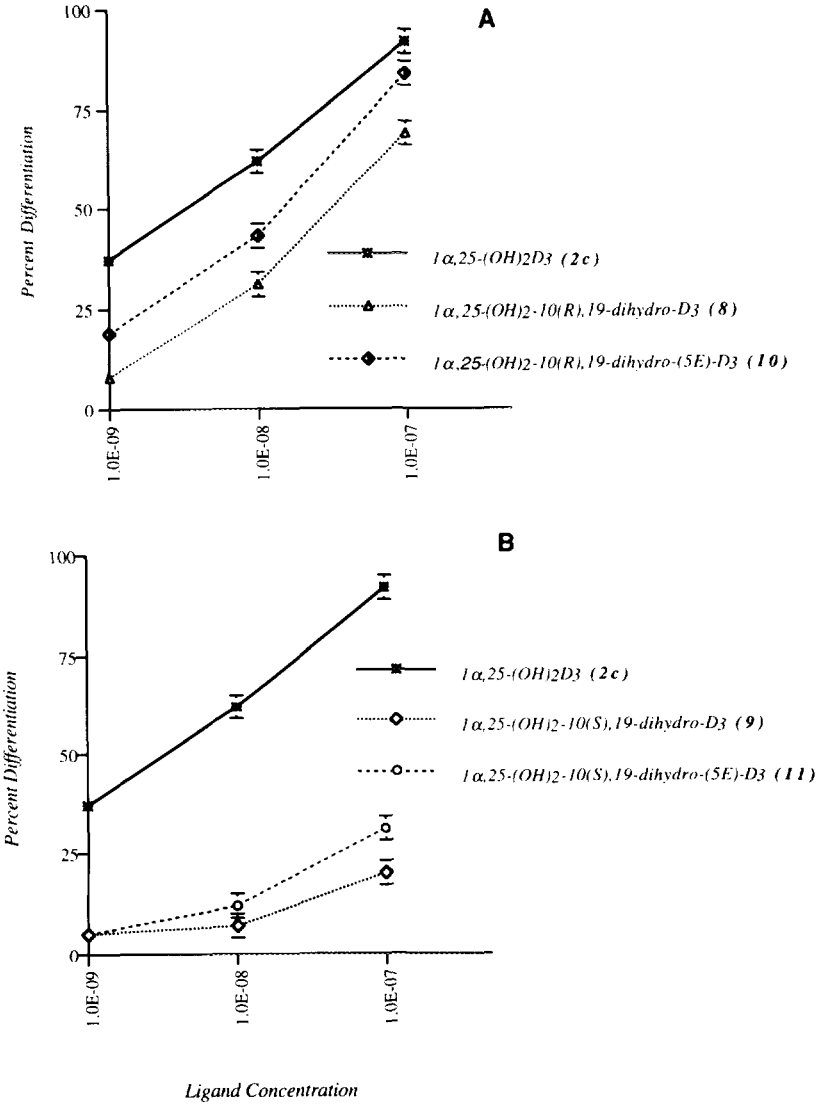


FIG. 7. Induction of differentiation of HL-60 promyelocytes to monocytes by the 10,19-dihydro analogs. (A) 10(*R*)-methyl analogs; (B) 10(*S*)-methyl analogs. Differentiation state was determined by measuring percentage cells reducing nitro blue tetrazolium (NBT).

TABLE 4

Intestinal Calcium Transport and Bone Calcium Mobilizing Activity of the 1α,25-Dihydroxy-10,19-Dihydrovitamin D_3 Compounds

Compound	Compound no.	Intestinal calcium transport serosal Ca/mucosal Ca	Bone calcium mobilization (serum calcium)
None (deficient control)	—	3.4 ± 0.4	4.3 ± 0.07
1α,25-$(OH)_2D_3$	**2c**	10.0 ± 0.6[a]	5.7 ± 0.07[a]
1α,25-Dihydroxy-10(*R*),19-dihydro-(5*E*)-vitamin D_3	**10**	4.4 ± 0.4	4.3 ± 0.06
1α,25-Dihydroxy-10(*R*),19-dihydrovitamin D_3	**8**	4.1 ± 0.4	4.4 ± 0.2
1α,25-Dihydroxy-10(*S*),19-dihydrovitamin D_3	**9**	8.0 ± 0.8[a]	4.6 ± 0.05
1α,25-Dihydroxy-10(*S*),19-dihydro-(5*E*)-vitamin D_3	**11**	6.0 ± 0.7[b]	4.4 ± 0.09

[a] Significantly higher than deficient control, $P < 0.001$ (or [b]$P < 0.05$). All other values not significantly different from deficient control. Values are the mean ± standard error of the mean.

transport or bone calcium mobilization (Table 4). On the other hand, compounds **9** and **11** had significant activity on intestinal calcium transport. Thus, the 10(*S*)-configuration having the least 1α-OH-equatorial configuration provided the greatest activity. No significant elevation of serum calcium or bone calcium mobilization was seen with any of the four analogs at this dose level. 1,25-$(OH)_2D_3$ given at $\frac{1}{10}$th the dose gave very high intestinal calcium transport activity and very high bone calcium mobilizing activity. These results show that the most active of the 10,19-dihydrovitamin D compounds is less than $\frac{1}{10}$th as active as 1,25-$(OH)_2D_3$. In any case, the *in vivo* biological activity gave quite the opposite picture than predicted based on decreasing equatorial character of the 1α-hydroxyl group. Very likely these compounds differ in their ability to bind to the vitamin D transport protein in blood or may differ in their metabolism or some other factor *in vivo*. In any case, *in vivo* results do not support the concept that the greatest biological activity is provided by compounds having an equatorial 1α-hydroxyl. It is interesting that at least in part the prediction that the more favored equatorial hydroxyl compound is the most active was found in the cultures of HL-60 cells *in vitro* or in binding to the receptor.

Although the biological results do not support the idea that the most equatorially favored 1-hydroxyl compound would be the most biologically active, compounds which we prepared do have other structural changes which could easily play a role in determining target response (presence of bulky 10-methyl substituent changing the arrangement of the A-ring in relation to the rest of the vitamin molecule). This may be more detrimental than the favorable equatorial nature of the 1-hydroxyl. Thus, it would appear that the *in vitro* culture system and the receptor binding data cannot by themselves be used in a predictive way for *in vivo* biological responses. The results also demonstrate that the *in vivo* responses are complex, representing the drug interacting with a variety of components, making

prediction of the biological outcome very difficult. The present study, therefore, does not allow the conclusion that equatorial 1α-hydroxyl is an important aspect of biological activity of 1,25-$(OH)_2D_3$.

EXPERIMENTAL

General. Proton NMR spectra were recorded with Bruker AM-500 FT spectrometer ($CDCl_3$ solution with internal tetramethylsilane at δ 0.00). Ultraviolet (uv) spectra were taken in ethanol on a Hitachi Model 100-60-spectrophotometer. Mass spectra (MS) were obtained at 110–120°C above ambient temperature, at 70 eV, with a Kratos MS-50 TC instrument equipped with a Kratos DS-55 data system. High-resolution data were obtained by peak matching. Column (flash) chromatography was performed on silica gel Merck (230–400 mesh). Thin-layer chromatography (TLC) was performed using a precoated aluminum silica gel sheet with a uv indicator from EM Science (Gibbstown, NJ); R_f values are given for ethyl acetate–hexane 1 : 1 solvent system. High-performance liquid chromatography (HPLC) was performed on a Waters Associates Model ALC/GPC 204 using a Zorbax silica (Dupont) column; R_v values are given for 6% 2-propanol in hexane (solvent system A), 10% 2-propanol in hexane (system B), 30% ethyl acetate in hexane (system C), and ethyl acetate–hexane 1 : 1 (system D). Crystalline 25-OH-D_3 was purchased from Tetrionics, Inc. (Madison, WI).

Preparation of (7E)-(1S,3R,5R,6R)-6-methoxy-3,5-cyclo-9,10-seco-7,10(19)-cholestadiene-1,25-diol ***(14).*** 25-OH-D_3 **(1c)** was converted to **14** in a manner analogous to that described for 25-OH-D_2 **(1d)** (*34*). Reaction of **1c** with *p*-toluenesulfonyl chloride in dry pyridine gave a crystalline C-3 tosylate **12** in 93% yield. The buffered methanolysis of **12** afforded the oily cyclovitamin **13** (TLC, R_f 0.50) which was sufficiently pure for the following oxidation step. Crude compound **13** was oxidized with selenium dioxide and *tert*-butyl hydroperoxide in CH_2Cl_2 containing pyridine. Products were separated by flash chromatography. Elution with ethyl acetate-hexane (1 : 1) gave **14** as its main oxidation product (40% overall yield from **12**) exhibiting physical data essentially identical to those of the 1,25-dihydroxy-3,5-cyclovitamin D_3 prepared previously in this laboratory (*33*). Also isolated was 1-oxo-cyclovitamin **15** (12%).

15: TLC, R_v 0.40; uv λ_{max} 244 nm (ε 4200), λ_{min} 223 nm; NMR δ 0.50 (3H, s, 18-H_3), 0.93 (3H, d, J = 6.5 Hz, 21-H_3), 1.22 (6H, s, 26-H_3 and 27-H_3), 3.31 (3H, s, 6R-OCH_3), 4.07 (1H, d, J = 9.6 Hz, 6-H), 5.03 (1H, d, J = 9.6 Hz, 7-H), 5.62 and 6.04 (2H, each s, 19-H_2); MS *m/e* (rel. intensity) 428 (M^+, 14), 410 (20), 396 (39), 378 (25), 245 (39), 133 (100), 59 (71).

Cycloreversion of cyclovitamin ***14;*** *preparation of (5Z,7E,1S,3R)- and (5E,7E,1S,3R)-3-acetoxy-9,10-seco-5,7,10(19)-cholestatriene-1,25-diols* ***(16 and 17).*** A solution of cyclovitamin **14** (20 mg, 46 μmol) in glacial acetic acid (0.5 ml) was heated to 55°C for 15 min, cooled, and poured carefully over ice-saturated $NaHCO_3$. The neutralized mixture was extracted with benzene and ether, and the combined extracts were washed with saturated $NaHCO_3$ and water, dried (Na_2SO_4), and concentrated *in vacuo*. The residue, consisting of a mixture of **16**

and its 5*E*-isomer **17** (ratio 2.5 : 1) was subjected to HPLC (6.2-mm × 25-cm column, system A) to give partially separated isomers **16** (R_v 35 ml) and **17** (R_v 38 ml). Rechromatography of the latter compound in the same solvent system (recycling mode) gave the analytically pure vitamin acetate **17** (4.0 mg, 19%). Pure 5*Z*-isomer **16** (8.5 mg, 40%) was obtained by the maleic anhydride procedure worked out in this laboratory (*35*). Spectral data of **16** and **17** were essentially identical to those of the acetoxy vitamins prepared by Paaren *et al.* (*33*).

Hydrogenation of ***14****; preparation of (7E,1S,3R,5S,6R,10S)- and (7E,1S,3R,5S, 6R,10R)-6-methoxy-3,5-cyclo-9,10-seco-7-cholestene-1,25-diols (****18*** *and* ***19****).* Tris(triphenylphosphine)rhodium chloride {$[(C_6H_5)_3P]_3RhCl$; 102 mg, 0.11 mmol} was added to dry benzene (30 ml, freshly distilled from P_2O_5) presaturated with hydrogen. The mixture was stirred at room temperature under hydrogen until a homogeneous solution was formed (ca. 30 min). A solution of cyclovitamin **14** (47 mg, 0.11 mmol) in dry benzene (5 ml) was then added and the reaction was allowed to proceed under a continuous stream of hydrogen for 8 h. Benzene was removed under vacuum and the residue was separated by silica gel flash chromatography with ethyl acetate–hexane (1 : 1). Final purification of the two epimers by HPLC (9.4-mm × 25-cm column, system D) afforded dihydrocyclovitamins **18** (7 mg, 15%) and **19** (26 mg, 55%).

18: HPLC, R_v 65 ml; NMR δ 0.55 (3H, s, 18-H_3), 0.94 (3H, d, J = 6.5 Hz, 21-H_3), 1.15 (3H, d, J = 6.6 Hz, 19-H_3), 1.22 (6H, s, 26-H_3 and 27-H_3), 3.25 (3H, s, 6R-OCH3), 3.36 (1H, m, 1-H), 3.90 (1H, d, J = 9.2 Hz, 6-H), 4.87 (1H, d, J = 9.2 Hz, 7-H); MS *m/e* (rel intensity), 432 (M^+, 64), 400 (69), 382 (25), 271 (47), 135 (48), 59 (100); exact mass calcd for $C_{28}H_{48}O_3$ 432.3603, found 432.3596.

19: HPLC, R_v 39 ml; NMR δ 0.54 (3H, s, 18-H_3), 0.95 (3H, d, J = 6.4 Hz, 21-H_3), 1.08 (3H, d, J = 6.8 Hz, 19-H_3), 1.22 (6H, s, 26-H_3 and 27-H_3), 3.17 (3H, s, 6R-OCH_3), 3.97 (1H, m, 1-H), 4.29 (1H, d, J = 9.6 Hz, 6-H), 4.55 (1H, d, J = 9.6 Hz, 7-H); MS *m/e* (rel intensity), 432 (M^+, 100), 400 (26), 382 (12), 271 (34), 135 (32), 59 (75); exact mass calcd for $C_{28}H_{48}O_3$ 432.3603, found 432.3589.

Hydrogenation of ***16****; preparation of (5Z,7E,1S,3R,10R)- and (5Z,7E,1S,3R, 10S)-3-acetoxy-9,10-seco-5,7-cholestadiene-1,25-diols (****20*** *and* ***21****).* Vitamin acetate **16** (5 mg, 11 μmol) in dry benzene (2 ml) was added to the benzene solution (12 ml) of catalyst prepared from $[(C_6H_5)_3P]_3RhCl$ (12 mg, 13 μmol) as described in the preceding experiment. The stirred mixture was hydrogenated under atmospheric pressure for 3 h at room temperature. Benzene was evaporated and the residue was filtered through a silica gel Sep-Pak cartridge in ethyl acetate–hexane (1 : 1, ca. 10 ml). Separation of hydrogenation mixture by HPLC (6.2-mm × 25-cm column, system A) gave pure products **20** (0.38 mg, 8%) and **21** (3.9 mg, 78%).

20: TLC, R_f 0.34; HPLC, R_v 46 ml; uv λ^{max} 242.5 nm, 250.5, 260.5 ($A_{242}/A_{250}/A_{260}$ = 0.85 : 1 : 0.67); NMR (Table 1); MS *m/e* (rel intensity), 460 (M^+, 4), 400 (100), 382 (27), 271 (18), 176 (17); exact mass calcd for $C_{29}H_{48}O_4$ 460.3552, found 460.3536.

21: TLC, R_f 0.43; HPLC, R_v 27 ml; uv λ_{max} 242.5 nm (ε 28,800), 250.5 (33,100), 260.0 (22,300); NMR (Table 1); MS *m/e* (rel intensity), 460 (M^+3), 400 (100), 382 (11), 271 (14), 176 (33); exact mass calcd for $C_{29}H_{48}O_4$ 460.3552, found 460.3554.

Hydrogenation of ***17****; preparation of (5E,7E,1S,3R,10R)- and (5E,7E,1S,3R,*

*10S)-3-acetoxy-9,10-seco-5,7-cholestadiene-1,25-diols (**22** and **23**).* Vitamin acetate **17** (4 mg, 9 μmol) in benzene (2 ml) was added to the benzene solution (10 ml) of the catalyst prepared from $[(C_6H_5)_3P]_3RhCl$ (9 mg, 10 μmol) as described for **14.** The mixture was hydrogenated for 3 h at room temperature, benzene was evaporated, and the residue was filtered through a silica gel Sep-Pak cartridge in ethyl acetate–hexane (1 : 1, ca. 10 ml) and separated by HPLC (6.2-mm × 25-cm column, system A) to give pure products **22** (0.7 mg, 20%) and **23** (1.8 mg, 51%).

22: TLC, R_f 0.41; HPLC, R_v 35 ml; uv λ_{max} 242.5 nm (ε 25,500), 250.5 (29,800), 260.5 (19,600); NMR (Table 1); MS *m/e* (rel intensity), 460 (M^+, 7), 400 (100), 382 (18), 271 (12), 176 (36); exact mass calcd for $C_{29}H_{48}O_4$ 260.3552, found 460.3554.

23: TLC, R_f 0.34; HPLC, R_v 49 ml; uv λ_{max} 242.5 nm (ε 30,000), 250.5 (34,300), 260.5 (22,800); NMR (Table 1); MS *m/e* (rel intensity), 460 (M^+, 3), 400 (100), 382 (25), 271 (14), 176 (23); exact mass calcd for $C_{29}H_{48}O_4$ 460.3552, found 460.3552.

*Cycloreversion of dihydrovitamins **18** and **19**.* Glacial acetic acid-catalyzed cycloreversion of **18** (4.3 mg, 10 μmol) was performed under conditions identical to these described for cyclovitamin **14.** The two vitamin acetates were separated by HPLC (6.2-mm × 25-cm column, system A) to yield pure compound **21** (R_v 27 ml; 2.4 mg, 52%) and its isomer **23** (R_v 49 ml; 0.70 mg, 15%). Cycloreversion of dihydrocyclovitamin **19** (18.6 mg, 43 μmol) was carried out in the same manner as described above. Preparative HPLC (6.2-mm × 25-cm column, system D) of the resulted product mixture gave pure vitamin acetate **22** (R_v 40 ml; 17.0 mg, 86%) and a slightly impure compound **20** (R_v 57 ml). Final purification of the latter product by HPLC (6.2-mm × 25-cm column, system A) afforded the analytically pure isomer **20** (R_v 46 ml; 0.34 mg, 2%).

*Hydrolysis of 3β-acetoxyvitamins **20–23**.* All of the hydrolyses were performed under identical conditions. The acetoxyvitamin (0.5–1.8 mg) in ethanol (0.5 ml) was treated with 10% methanolic KOH (1 ml) for 1 h at 50°C. The mixture was poured into brine and extracted successively with ether, benzene and CH_2Cl_2. The extracts were washed with brine, dried (Na_2SO_4), collected, and evaporated. HPLC (6.2-mm × 25-cm column, system B) provided analytically pure 9,10-seco-5,7-cholestadiene-1,3,25-triols: **8** (80%), **9** (86%), **10** (68%), and **11** (80%).

(5*Z*,7*E*,1*S*,3*R*,10*R*)-**8:** HPLC, R_v 56 ml; uv λ_{max} 242.5 nm, 251.0, 261.0 ($A_{242}/A_{252}/A_{261}$ = 0.86 : 1 : 0.67); NMR (Table 1); MS *m/e* (rel intensity), 418 (M^+, 100), 400 (33), 289 (24), 245 (33); exact mass calcd for $C_{27}H_{46}O_3$ 418.3447, found 418.3457.

(5*Z*,7*E*,1*S*,3*R*,10*S*)-**9:** HPLC, R_v 53 ml; uv λ_{max} 242.5 nm, 250.5, 260.5 ($A_{242}/A_{250}/A_{260}$ = 0.87 : 1 : 0.67); NMR (Table 1); MS *m/e* (rel intensity), 418 (M^+, 100), 400 (34), 289 (25), 245 (29); exact mass calcd for $C_{27}H_{46}O_3$ 418.3447, found 418.3439.

(5*E*,7*E*,1*S*,3*R*,10*R*)-**10:** HPLC, R_v 52 ml; uv λ_{max} 242.5 nm (ε 30,300), 251.0 (35,000), 261.0 (23,300); NMR (Table 1) MS *m/e* (rel intensity), 418 (M^+, 100), 400 (58), 289 (29), 245 (42); exact mass calcd for $C_{27}H_{46}O_3$ 418.3447, found 418.3447.

(5*Z*,7*E*,1*S*,3*R*,10*S*)-**11:** HPLC, R_v 57 ml; uv λ_{max} 242.5 nm, 250.5, 260.5 ($A_{242}/A_{250}/A_{260}$ = 0.87 : 1 : 0.66); NMR (Table 1); MS *m/e* (rel intensity), 418 (M^+, 100), 400 (48), 289 (36), 245 (39); exact mass calcd for $C_{27}H_{46}O_3$ 418.3447, found 418.3433.

BIOLOGICAL TESTS

Binding to porcine intestinal nuclear receptor. This test was carried out according to procedures previously described (*62*) using porcine nuclear extract prepared according to Dame *et al.* (*63, 64*). The receptor was incubated overnight with 2 nM tritiated $1,25\text{-}(OH)_2D_3$ by itself or in the presence of the indicated concentrations of analog or nonradioactive $1,25\text{-}(OH)_2D_3$. The samples were then treated with hydroxyl apatite and centrifuged, and the pellet was washed three times as previously described (*62–64*). The radioactivity in the pellet was then extracted and counted by liquid scintillation counting. From these data, the displacement curves shown in Figs. 6A and 6B were constructed. The degree of displacement of the tritiated $1,25\text{-}(OH)_2D_3$ is taken as a measure of the ability of the analog to bind to the receptor binding site.

Preparation of doses. An extinction coefficient of 31,000 at 250 nm was used to calculate concentration of the compounds. This is based on the extinction coefficient determined for the corresponding compounds without the 25-hydroxyl group (*37*). This extinction coefficient is similar to the extinction coefficient found for 1α-OH-19-nor-vitamin D compounds. The absorption spectra were taken in absolute ethanol from which the dosing solutions were prepared. For *in vivo* dosing, the preparations were dissolved in propane-1,2-diol such that the final dosing solution contained 95% propane-1,2-diol and 5% ethanol. Each dose was given intraperitoneally in 0.1 ml every day for 7 days. For the displacement studies from the receptor, concentrations were prepared in absolute ethanol and added to the receptor solution in 0.05 ml of ethanol. As for the differentiation experiments, the compounds were added in 0.05 ml of ethanol to the culture fluid. In all cases the amount of ethanol did not exceed 1% of the culture medium.

HL-60 differentiation measurements. HL-60 cells, originally obtained from ATTC, were plated at 10^5 cells per plate, incubated in Eagle's modified medium as described previously (*17*). The compounds tested were added in the indicated concentrations in 0.05 ml of ethanol so that the ethanol concentration never exceeded 1%. The incubation was carried out for 4 days and at the end of 4 days, superoxide production was measured by nitro blue tetrazolium (NBT) reduction. The cells showing NBT reduction were then counted. Percentage differentiation represents percentage cells providing NBT reduction appearance. This method is described in detail elsewhere (*17*).

Measurement of intestinal calcium transport and bone calcium mobilization. Male, weanling rats obtained from the Sprague–Dawley Co. (Madison, WI) were placed on a normal calcium, normal phosphorus, vitamin D-deficient diet for a period of 3 weeks and then shifted to a low calcium (0.02%), normal phosphorus diet for the remainder of the test (*65*). The indicated compound was injected intraperitoneally each day for 7 days. Twenty-four hours after the last dose, the animals were killed for measurement of intestinal calcium transport by the everted sac technique (*62, 66*) and serum calcium analysis using the Calcette automatic calcium titrator (Precision Systems, Inc., Natick, MA). Intestinal calcium transport is expressed as serosal : mucosal ratio of calcium in the sac to the calcium in the final incubation medium or *S/M*. Bone calcium mobilization represents the

rise in serum calcium of the rats maintained on a very low calcium diet. In that measurement, the rise in serum calcium must arise from bone and hence is a determination of bone calcium mobilization.

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