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2D NMR Study of Solution Conformations and Complete ^{1}H and ^{13}C Chemical Shifts Assignments of Vitamin D Metabolites and Analogs

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Abstract: In addition to conventional 1D ^{1}H and ^{13}C NMR, including DEPT and NOE methods, 2D -shift-correlated NMR techniques (^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC) were used, providing an approach to the complete signal assignments for vitamin D analogs and model compounds. On the basis of NMR data, solution conformations of vitamin D and analogs were studied.

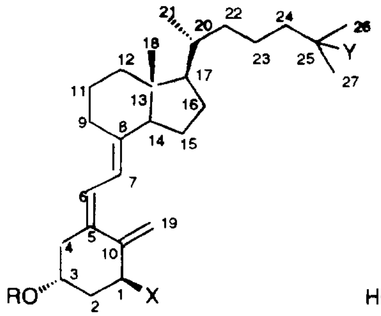
INTRODUCTION

1α,25-dihydroxyvitamin D_3 or its synthetic analogue 1α-hydroxy- vitamin D_3 play not only a major role in calcium homeostasis and bone mineralization,[1] but also suppress tumour growth, inhibit metastasis and prolong survival.[2,3] Preliminary clinical studies suggest that the oral administration of 1α-hydroxylated vitamin D compounds may be beneficial in myelofibrosis, myelodysplastic syndromes and non-Hodgkin's lymphomas.[3,4] The potential therapeutic value of these compounds is, however, limited by their strong effects on intestinal calcium uptake. These results prompted chemists to synthesize vitamin D analogues possessing differentiation-inducing activity and immuno-regulating action, but lacking potent hypercalcemic effect.[3,5] It is known that vitamin D analogs showing bone calcium mobilization and intestinal calcium absorption activities all possess an "cholesterolic" side-chain. In order to estimate or reduce the calcemic effects by retaining or increasing the activity on the cell regulatory processes, a range of side chain analogues of vitamin 1α-D_3 were synthesized, where possible sites for side-chain hydroxylation were blocked [3,6-8] or in which the cholesterol side-chain was substituted with that of pregnane.[9]

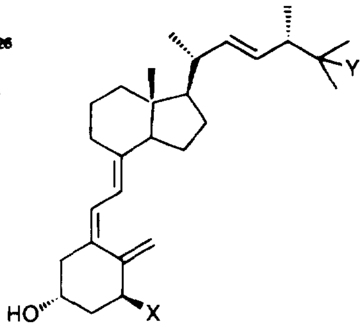
On the other hand, introduction of a OH-group in 2-position of the *A* -ring of 25-hydroxyvitamin D_3 produces an analog, whose activity is only 1/150 of that of 1α,25-dihydroxyvitamin D_3.[10]

These facts indicate the important role of the 1-α-OH group for vitamin D anticancer activity. In addition, it was established that the conformation of the *A* -ring is also significant for binding of vitamin D to its receptor protein.[11]

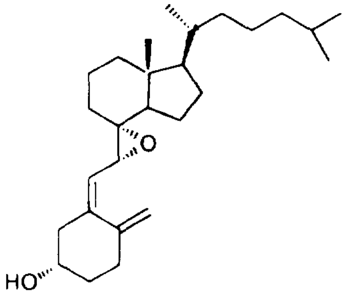
This paper deals with the study of the solution conformations of vitamin D and of its analogs **1-5, 6** (vitamin D_3 - *trans*), **7-13**, which can provide the basis for the syntheses of prospective anticancer agents.



1 R = $CH_3(CH_2)_2C(O)$, X =H, Y = H
2 R = H, X = H, Y = OH
3 R = H, X = OH, Y = H
5 R = H, X = H, Y = H (vitamin D_3-*cis*)
7 R = Ts, X = H, Y = H
8 R = t-Bu_2Si, X = H, Y = H



4 X = H, Y = H



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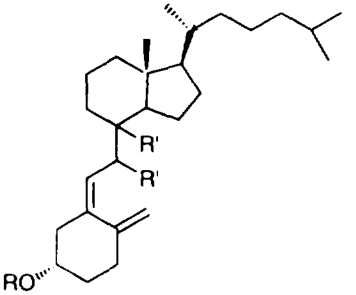

10 R = H, R' = OH
11 R = t-Bu_2Si, R' = OH
12 R = Ts, R' =OH
13 R = $CH_3(CH_2)_2C(O)$, R'+ R' = O

RESULTS and DISCUSSION

Suprisingly, we came up against the problem that ^1H and ^{13}C chemical shifts of vitamin D and analogs have not yet been assigned completely, which should preceded an examination of solution conformation of compounds **1-13.**

Complete ^1H and ^{13}C Chemical Shifts Assignments of vitamin D and its analogs. In existing publications (for instance, [11-16]) NMR ^1H spectra of vitamin D and its analogs were characterized as containing an undecipherable, broad, almost featureless "hump" between ~1 and 2.5 ppm. Most information was consequently derived from chemical shift data for angular methyl singlet (C-18) and doublets (C-21, 26, 27) or double bond signals (C-5, 6, 7, 8, 19) and C-3 multiplet.[12,21,22] The steroid hump, which usually contains over 60% of the ^1H resonances, was virtually inaccessible to interpretation.

Full ^{13}C NMR chemical shifts assignment, using the two-dimensional (2D) INADEQUATE technique, was reported only for vitamin D_3.[17]

Our strategy for assigning ^1H and ^{13}C signal of the compounds **1-13** is as follows: we selected compounds **1-4** as a models. Conventional 1D ^1H and ^{13}C NMR techniques, including ^{13}C NMR DEPT and ^1H NMR NOE difference experiments, the 2D ^1H-^1H COSY (with variants) and ^1H-^{13}C shift correlation (HMQC) were carried out and corresponding spectra were collected for each of these compounds (Table 1).

^{13}C Chemical Shift Assignments. Our results for compound **1** are in full agreement with those described previously.[17] The signals of C-2, 3, 4 were found to be shifted by ~3 ppm (C-2 and C-4 - to upfield, C-3 - to downfield) under the influence of the carbonyl group.

A hydroxyl group in the 1 α-position of an *A* -ring as in compound **3** causes not only changes of the chemical shifts of the nearest carbons C-2 (+7.65), C-3 (-2.28), C-10 (+2.54), but also influences those of the carbons of double bonds: C-5 (-2.34), C-6 (+2.71), C-7 (-0.56), C-8 (+1.29).

Substitution of H-25 by a hydroxyl group (compound **2**) causes changes in the chemical shifts of side-chain carbons: C-22 (+0.25), C-23 (-0.31), C-24 (+1.02), C-25 (+43.12), C-26 (+6.66), C-27 (+6.55).

The chemical shifts assignment of the carbons (C-1 - C-19) for vitamin D_2 **4** is the same as for vitamin D_3. The resonances of side chain carbons (C-20 - C-28) were assigned from their respective connectivities with the corresponding proton patterns, using HMQC.

DEPT experiment. Standard DEPT technique was used to differentiate the methylene and methine carbons: C-12 and C-20 (at 41.40 ppm), C-4 and C-13 (at 46.55 and 46.41 ppm, respectively) for **4**. Carbons C-1, C-2 (32.66 and 32.86 ppm) and C-16, C-25 (28.32 and 28.66) were differentiated for compound **1** . The same procedure was used for **2** [C-4, C-13 (46.57 and 46.49 ppm)] and **3** [C-20, C-22 (36.76 ppm) and C-4, C-13 (45.91 and 46.57 ppm)].

Table 1 ^{13}C and ^{1}H NMR chemical shifts of compounds **1-4** in $CDCl_3$

Carbon	**1**	**2**	**3**	**4**	Proton	**1**	**2**	**3**	**4**
1	32.66	32.56	71.48	32.55	1α	2.204	2.157	4.439	2.181
					1β	2.400	2.388	-	2.416
2	32.86	35.81	43.49	35.78	2α	1.965	1.901	2.003	1.940
					2β	1.727	1.650	1.898	1.685
3	72.12	69.84	67.52	69.83	3α	4.963	3.945	4.235	3.952
4	42.85	46.57	45.91	46.55	4α	2.567	2.572	2.587	2.578
5	135.02	135.74	133.38	135.69	4β	2.381	2.265	2.297	2.298
6	123.07	123.07	125.70	123.10	6	6.214	6.236	6.389	6.234
7	118.10	118.15	117.58	118.13	7	6.032	6.034	6.005	6.036
8	143.06	142.89	144.04	142.90	9α	1.645	1.665	1.669	1.685
9	29.68	29.65	29.74	29.65	9β	2.811	2.829	2.810	2.829
10	145.37	145.73	148.26	145.70	11α	1.645	1.665	1.654	1.670
11	24.21	22.89	24.26	24.21	11β	1.531	1.481	1.509	1.537
12	41.19	45.04	41.13	41.04	12α	1.287	1.280	1.280	1.331
13	46.56	46.49	46.57	46.41	12β	2.023	1.993	1.993	1.974
14	57.01	56.96	57.01	57.06	14	1.975	1.973	1.968	1.994
					15α	1.469	1.271	1.325	1.444
15	22.87	21.45	22.93	22.87	15β	1.469	1.440	1.444	1.537
16	28.32	28.32	28.27	28.43	16α	1.870	1.855	1.858	1.734
					16β	1.520	1.230	1.210	1.287
17	57.24	57.15	57.24	57.08	17	1.263	1.255	1.270	1.326
18	12.62	12.64	12.64	12.91	18	0.543	0.544	0.547	0.558
19	113.27	113.07	112.42	113.06	19(*Z*)	4.839	4.821	5.010	4.819
					19(*E*)	5.060	5.050	5.333	5.047
20	36.78	36.76	36.76	41.04	20	0.991	1.019	0.986	2.014
21	19.21	19.46	19.49	18.23	21	0.922	0.937	0.923	0.916
22	36.78	37.03	36.76	136.25	22	1.349	1.373	1.320[a]	b
								1.360[a]	
23	24.51	24.21	24.50	132.58	23	1.340	1.481	1.225[a]	b
						1.113		1.110[a]	
24	40.15	41.17	40.14	43.45	24	1.120	1.413[a]	1.100	1.861
							1.353[a]		
25	28.66	71.76	28.66	34.74	25	1.254	-	1.499	1.478
26	23.20	29.85	23.20	20.29	26	0.862	1.216	0.863	0.824
27	23.46	29.99	23.46	20.60	27	0.878	1.216	0.879	0.840
28	-	-	-	21 75		-	-	-	1.020

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[a] α or β protons. [b] 5.195 m

^{1}H - ^{13}C Chemical Shift Correlation (HMQC). From the assigned ^{13}C spectrum it was possible to find a proton or a pair of protons bound to a carbon, using HMQC. Despite the extensive overlapping of ^{1}H resonance signals appearing in the region of 1.2-2.5 ppm, the chemical shifts of each proton could be read of by correlating the carbon-proton resonances from the contour plots of the diagrams (Fig. 1).

The ^{1}H-^{13}C spectra provided a facile and straightforward identification of the protons in **1**: the chemical shifts of 4β-H, 1α-H and 9β-H are in agreement with previous data.[11,13,14] The signal of the 4a-H overlaps with that of 1β-H, and 2β-H was found to overlap with 12-H and 14-H protons. The peaks, corresponding to the protons of *C,D* -rings overlap each other (Fig. 1) and the pattern of 20-H appears as a broad triplet (0.991 ppm).

The chemical proton shifts of other derivatives **2-4** were assigned unequivocally from the corresponding ^{1}H-^{13}C spectra (Table 1).

^{1}H- ^{1}H Chemical Shift Correlation (COSY).[18] The chemical shifts of the *A* -ring protons may all be determined directly from the ^{1}H-^{13}C spectrum. The chemical shifts of 4α-H and 1β-H are known for vitamin D_3 from a previous study.[11,14] The chemical shift of 1β-H provides the starting point for tracing the coupling partners. Spin-coupling network is observed for four protons - 1α-H, 1β-H, 2α-H and 2β-H (Fid.3), that indicates positions of these protons unambiquously. The 11β-H signal is easily located by its coupling to 9β-H, whose chemical shift is known[11,13,14] and confirmed by our NOE difference experiment (see corresponding section). The 9α-H is also identified by its coupling to the 11α-H and 12β-H - by its coupling to 12α-H and 11β-H. Assignments for α and β protons of C-15 and C-16 are not so obvious.

1D NOE Difference experiment. Our assignment of the signals of exocyclic methylene protons [19(*E*)-H and 19(*Z*)-H] is based on the results of 1D NMR NOE difference experiments performed on **1-4** (Fig.2) and is confirmed by NOE experiments on vitamin D_2,D_3[19] and 11-α-(3-carboxyalkoxy)-25-hydroxyvitamin D_3 derivatives,[20] but is opposite to the conventional assignment for other vitamin D_3 and D_2 compounds.[11,13,14, 21, 22, 23]

The NOE enhancement was also observed between the 7-H/15α-H, 6-H/9β-H and 6-H/4-H (Fig. 2).

15β-H was identified from COSY spectra by coupling to 15α-H; 16α-H and 16β-H - by their coupling to the corresponding 15α-H and 15β-H and to each other (Fig. 3)

It should be noted that the attempts to apply NOESY and ROESY techniques to the molecules of vitamin D type were unsuccessful, due to poor detectability of the small NOE's or ROE's effect in rapidly tumbling small molecules.[24]

Conformation of the Vitamin D Compounds derived from NMR Data. The vitamin D structure can be viewed as a *C,D* -ring system, to which are connected two flexible entities, the upper side chain and the lower part consisting of the *A* -ring and the connecting diene (so-called seco-*B* -ring).

In our opinion to estimate the conformations of *A* - and *C,D* -rings the spectra of *A* -ring region and *C,D* -ring pattern should be compared with the spectra of model systems with known stereochemistry. This approach was recommended as an effective method for describing conformational and geometrical properties of the complex molecules, for example, steroids.[25,26]

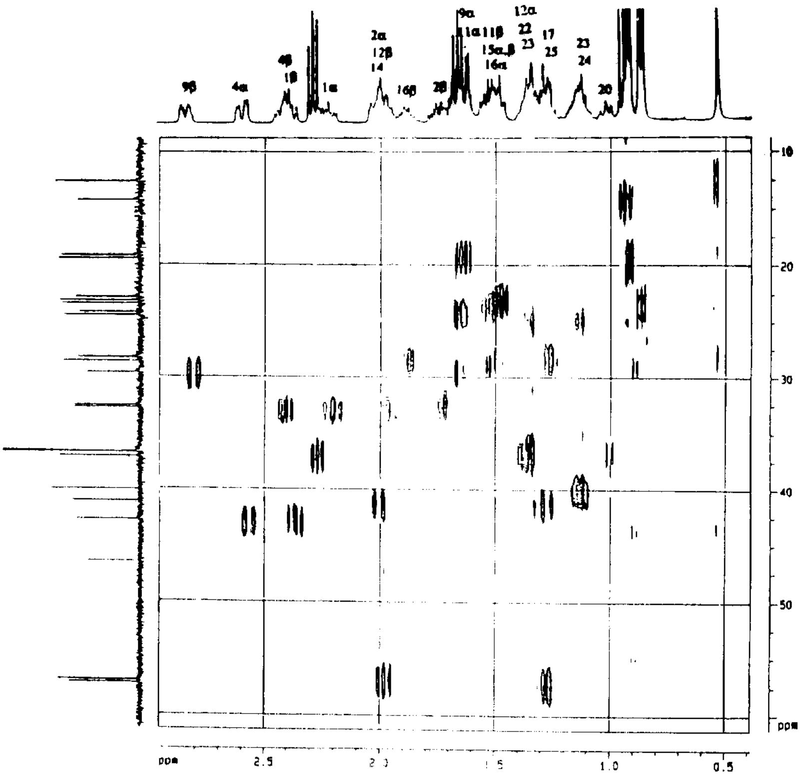


Figure 1. 400 MHz spectrum of 1.

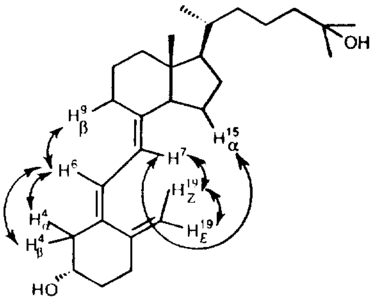


Figure 2. NOE enhancements of compound **2**. The arrows show the NOE relationships confirmed by 1D NOE experiments in $CDCl_3$ at 300 K.

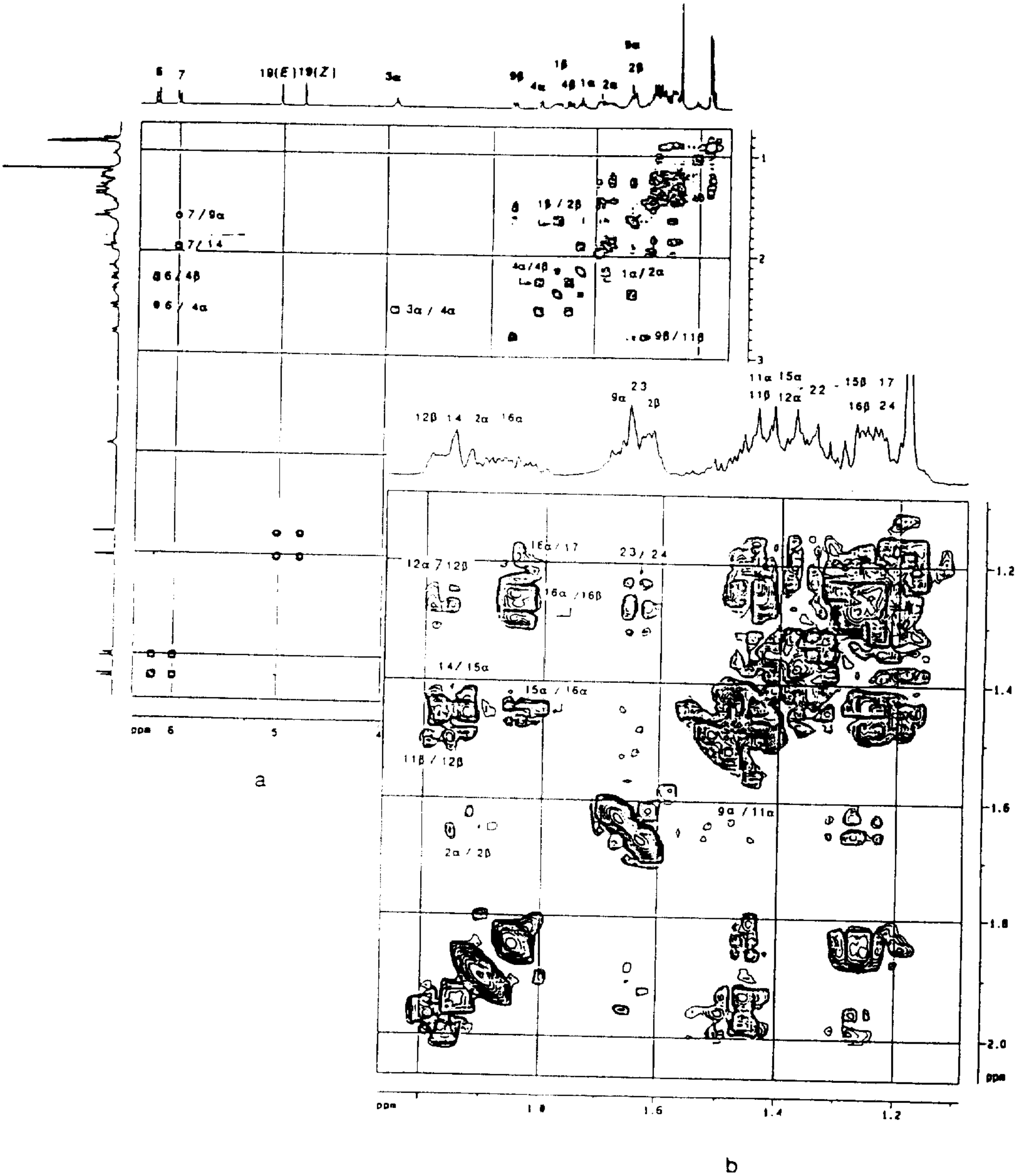


Figure 3. [a] The contour map from the DQF-COSY experiment for **2**.
[b] Selected part of map[a] which comprises the cross-peacks between the *C,D* -ring hydrogens.

(a) Conformation of A-ring system. NMR studies[27,28] of vitamin D_3 and D_2 structures in solution have established a dynamic equilibrium between nearly equimolar populations of two chair conformations with equatorial (*eq.*) and axial (*ax.*) position of 3-β OR- group.[29] These two forms were also found in the crystals of vitamin D in a 1:1 ratio.[30,31]

As a model for *A* -ring we synthesized two cyclohexanol derivatives (**14** and **15**) (Scheme).

The chair conformation of analogous compounds are known[32,33] , but for **14** and **15** was confirmed by our computer molecular modeling experiments, using MM2 force field calculations.[34]

The *A*- ring proton coupling constants for compounds **14** and **15** are the same as for **1-4** (Table 2).[35]

In addition, it should be noted that the coupling constants of *A* -ring in vitamin D analogs do not vary upon solvent change ($CDCl_3$, C_6D_{12}, THF-d_8, CD_3OD).[36]

From the data (Table 2 and 3) the following deductions were made for vitamin D derivatives **1-13**: (i) *A* -ring conformation is unaffected by the nature of the side chain; (ii) acylation, tosylation or silylation change the *eq.* : *ax.* ratio in favor of *ax.* -conformer, in range: silyl ether> tosylate> butyrate; (iii) a conformational equilibrium is shifted to *ax.* -conformer by conversion of *cis* -isomer to *trans*-, but the introduction of hydroxyl to the 1a-position shifts the *eq.* : *ax.* ratio to slightly *eq.*-conformer (~55%); (iv) hydroxylation of 7,8-double bond causes a maximum change in conformational equilibrium to *ax.* -conformer .

It is interesting to note that the *eq.* : *ax.* ratio for 3-tert-butyldimethylsiloxyphosphine oxide **15** (M.w. = 451) is approximately the same as of tert-butyldimethylsiloxyvitamin D_3 (M.w. = 499), it seems that the diphenylphosphine oxide group plays a role of only a "heavy" bulky substituent whose weight is comparable to *C,D* -ring. Probably, the presence a heavy, bulky group in the 7-position ensures preservation of conformation of the diene moitety as a *s-trans* for molecules **1-15.**

(b) Conformation of C,D -rings system. A compound **16** (de-*A,B* -8-cholestanone) and its hydroxylated derivatives **17** and **18** (Scheme) were selected as a suitable models.

Although ketone **16** was well known,[37] full assignment of the ^1H and ^{13}C NMR spectra has to our knowledge not been reported.

This assignment was carried out using the same strategy as for vitamin D compounds (see corresponding section): a combination of conventional 1D ^1H and ^{13}C NMR with 2D ^1H-^1H COSY, 2D COSY long range and ^1H-^{13}C HMQC techniques (Table 4).

Introduction of OH- or bulky $OSiMe_3$-groups in the 25-position of hydroindan-4-one **16** does not produce large shifts in the positions of protons (Table 4).

It is known on the basis of X-ray[25,38] and molecular mechanic calculations[25,39,40] that the hydroindan-4-ones have a six-membered ring in a chair form and a *trans*- or *cis* - fused five-membered ring (Fig. 4).

Comparison of the chemical shifts for protons of vitamin D compounds **1-4** and hydroindan-4-ones **16-18** shows essential distinction in the main 1.0-2.0 ppm region (Tables 1 and 4). Because of this there is no reason to assertain that the conformation of *C,D* -rings in vitamin D compounds is a same that for model hydroindanones **16-18.**

The preferred conformation of *C* -ring in solution is, most probably, a "boat"-form, according to our molecular dynamics experiment (Fig. 4) and X-ray data for solid state.[41]

Table 2 The *J* values of compounds **1-4**, **14** (in Hz)

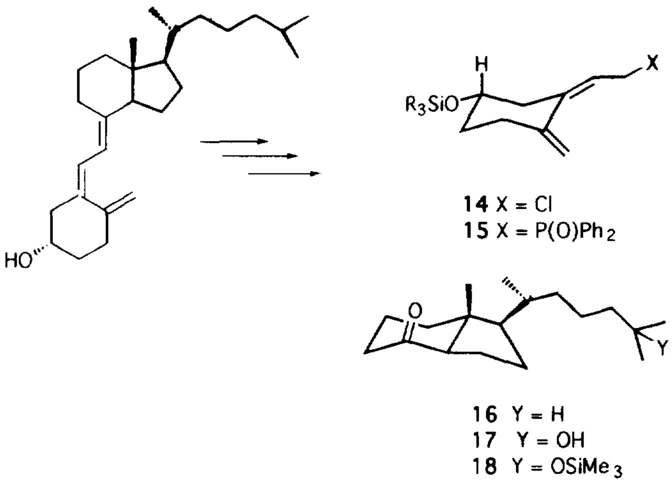
Proton	**1**	**2**	**3**	**4**	**14**
1α	4.5; 9.2; 13.7	4.8; 8.7; 13.6	-	4.9; 8.6; 13.7	1.3; 4.3; 9.6; 13.7
1β	4.6; 9.1; 13.5	4.8; 7.8; 13.4	3.7; 3.7; 7.4	5.3; 8.6; 13.5	a
2α	a	a	a	a	1.7; 4.5; 8.0; 13.7
2β	4.5; 8.8;	a	a	a	4.5; 8.6; 9.5; 13.8
3α	4.0; 4.0; 8.0; 8.0	3.7; 3.7; 7.4; 7.4	bm	3.7; 3.7; 7.4; 7.4	4.0; 4.0; 8.1; 8.1
4α	3.9; 13.3	3.6; 13.1	3.5; 13.4	3.6; 13.1	4.3; 13.0
4β	8.0; 12.6	7.5; 13.1	6.6; 13.3	7.5; 13.1	8.4; 13

[a] A signal of corresponding proton is overlapped with others protons

Table 3 The $J_{3\text{-H},4\text{-H}}$ values (in Hz) and α : β ratio of *A* -ring conformers for vitamin D compounds

Compounds	$J_{3\alpha\text{-H},4\alpha\text{-H}}$	$J_{3\alpha\text{-H},4\beta\text{-H}}$	α : β	$\delta_{3\text{-H}}$ (ppm)
Vitamin D_3-*cis* **5**	3.7	7.4	45:55	3.954
2 and **4**	3.7	7.4	45:55	a
Vitamin D_3-*trans* **6**	4.1	8.7	30:70	3.889
Vitamin D_3 butyrate **1**	4.0	8.0	38:62	a
Vitamin D_3 tosylate **7**	3.9	7.9	39:61	4.667
Vitamin D_3 *tert* -BuMe$_2$SiO **8**	4.0	9.1	24:76	3.828
Phoshine oxide **15**	4.6	9.1	24:76	3.528
5,6- Epoxy- 5,6-dihydro-D_3 **9**	4.3	8.8	28:72	3.884
7,8-(OH)$_2$-7,8-dihydro-D_3 **10**	4.0	9.7	16:84	3.804
7,8-(OH)$_2$-7,8-dihydro-(*t*-BuMe$_2$SiO)-D_3 **11**	4.2	9.9	14:86	3.692
7,8-(OH)$_2$-7,8-dihydro-D_3 tosylate **12**	4.4	10.1	11:89	4.542
7,8- Epoxy-7,8-dihydro-D_3 butyrate **13**	3.5	7.0	50:50	5.004
1α-Hydroxy-vitamin D_3 **3**	-	6.6	55:45	a

[a] See Table 1.

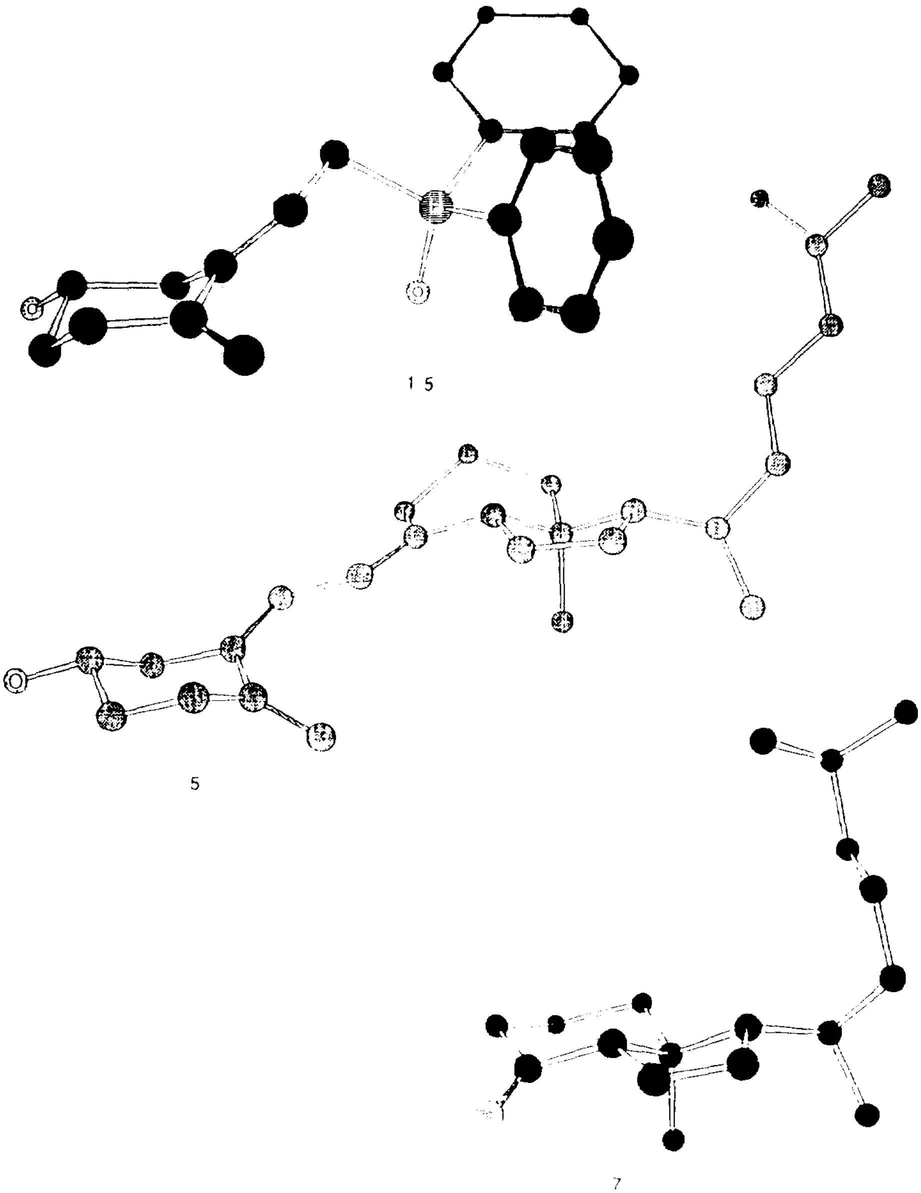


Scheme

Table 4 NMR chemical shifts of compounds **16-18** in $CDCl_3$

	16		17	18
Carbon[a]	^{13}C		^{1}H	
8	212.14	-	-	-
9	41.60	2.146α	2.253	2.257
		2.192β		
11	24.41	1.941α	2.004α	2.033α
		1.835β	1.926β	1.908β
12	39.62	1.520α	1.587α	1.579α
		2.068β	2.130β	2.125β
13	50.56	-	-	-
14	62.62	2.392	2.457	2.447
15	19.70	1.446α	1.525α	1.525α
		1.665β	1.721β	1.716β
16	28.14	1.820α	1.908α	1.834α
		1.251β	1.287β	1.265β
17	57.34	1.363	1.311	1.426
18	13.10	0.578	0.646	0.636
20	36.14	1.347	1.259	1.277
21	19.36	0.893	0.974	1.196
22	36.61	1.290	b	b
23	24.71	1.286[c]	b	b
		1.085[c]		
24	40.05	1.060	b	b
25	28.61	1.453	-	-
26	23.16	0.802	1.227	0.955
27	23.42	0.818	1.227	0.955

[a] Steroid numbers. [b] Overlapped multiplets. [c] α or β protons.



15

5

7

Figure 4. Ball-and-stick representation of the MM2-optimized structures of 15, 5 and 7

Since the position of *C* -ring protons practically does not change in spectra of **1-4** and small changes occur only with *D* -ring protons, apparently, *cis* - or *trans* - fused cyclopentane-*D* -ring performs total pseudorotation, it being known that all such conformers have approximately equal energy.[25,39] These changes in conformation of *D*-ring are reflected in NMR time scale as slight shifts of *D* -ring protons.

CONCLUSION

On the basis of NMR data and molecular modeling experiments it was established that the changes in side-chain of vitamin D do not influence the conformation of *A* -ring, which exists in solution in an eq., *ax.*-equilibrium chair form. This equilibrium depends only on the substituents in *A* - and *seco -B* -rings, but not on the changes in side-chain. The substitution of *C,D* -ring by bulky-group with the same molecular weight causes the same *eq.* : *ax.* ratio of conformers as for vitamin D compounds with the conservation of chair conformation of *A* -ring.

Since the necessary prerequisite for the antitumor activity of vitamin D analogs is 1-a and 3-b hydroxylated *A* -ring and the *seco -B* ring, *C,D* -ring and usual "cholesterol" side-chain can be substituted by different alkyl, alkenyl, polyalkenyl and aryl groups.

EXPERIMENTAL

The vitamin D_3 butyrate **1**, vitamin D_3 **5** and vitamin D_2 **4** are available commercial products and were used without further purification. Compounds **2**[12,42], **3**[12,43], **6**[43],**7**[44], **8**[43], **9**[45], **10**[46], **11**[46], **12**[44], **13**[45], **14**[42], **15**[42], **16**[42], **17**[47] and **18**[47] were synthesized according to known procedures.

NMR Experiments. Compounds **1-18** (ca.150 mg) were dissolved in 0.7 ml of $CDCl_3$ in a 5 mm NMR tubes. ^1H and ^{13}C spectra were recorded on a Bruker AMX-400 spectrometer at 400.13 and 100.61 MHz, respectively, and at 300 K. ^1H and ^{13}C chemical shifts (d) are given from internal TMS.

COSY. Proton-proton chemical shift correlation double-quantum-filtered COSY (DQF-COSY) and long-range COSY were employed using standard Bruker software (cosydf and cosylr) with a spectral width of 1.2 kHz or 2.6 kHz in 1K data points using 8 transients for each of 256 t_1 increments. Double Fourier transformation after zero filling to 512 in F_1 dimension was performed. In the long-range COSY experiment the last "read" pulse was set at 50°C (for sensitivity improvement) and the additional delay was 0.3 s.

HMQC. A two-dimensional heteronuclear correlation ^1H-^{13}C inverse detection experiment was performed using standard Bruker software (invbtp) and was acquired with a BIRD pulse. A total of 64 increments were used with a spectral width of 16 kHz in 1 K data points with 4-8 scans for each increment. The F_1 (^1H) dimension covered a range of 6 ppm. Double Fourier transformation after zero filling yielded a data matrix of 2 K X 128. Sine-bell windows shifted by $\pi/2$ were applied for both dimensions, prior to Fourier transformation.

NOE - experiments. NOE difference spectra were obtained using standard steady-state difference experiments. The irradiation time was 1.5-2 s with sufficient power (55-57 dB). The resulting FIDs were Fourier transformed using an exponential multiplication with 1 Hz line broadening and subtracted. The solutions were degassed directly in the NMR tubes just before sealing. The phase-sensitive NOESY and

ROESY spectra were performed by standard Bruker software (noesytp and roesytp). The spectra were acquired with spectral width of 2.6 kHz in 1 K data points using 16 scans for each of 512 t_1 increments. Mixing time (for NOESY) was 50, 100, 200 ms. Spin-lock time (for ROESY) was 100 and 200 ms. Double Fourier transformation resulted in real matrix of 1 K x 1 K after zero filling. A sine window function was multiplied in both dimensions prior to Fourier transformation.

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