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Studies on Vitamin D and Its Analogs. VII. Solution Conformations of Vitamin D_3 and 1α,25-Dihydroxyvitamin D_3 by High-Resolution Proton Magnetic Resonance Spectroscopy[1]

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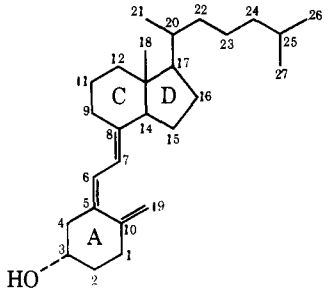
Abstract: The conformations of the A and seco-B rings of vitamin D_3 have been studied by two ^1H NMR methods: correlation of the observed coupling constants with the Karplus equation; and computer analysis of the 300-MHz tris(dipivalomethanato)europium(III) [Eu(dpm)$_3$] shifted spectra. Both methods show that the A ring of vitamin D_3 exists as an approximate equimolar mixture of rapidly equilibrating chair conformers. The torsion angle about the C_6–C_7 bond is essentially the same in solution as previously determined by X-ray diffraction studies. Comparison of the spectra of side chain modified analogs (20,21,22,23,24,25,26,27-octanorvitamin D_3 and vitamin D_2) with that of vitamin D_3 establish that A and seco-B ring conformations are independent of the nature of the side chain. Analysis of the ^1H NMR spectra obtained for 1α,25-dihydroxyvitamin D_3 (the natural hormone) and 1α-hydroxyvitamin D_3 shows that the A-ring conformational populations are identical with one another and similar to that observed for D_3. Observed coupling constants of D_3 in the presence of 0.55 molar equivalents of La(dpm)$_3$ (a diamagnetic analog of the europium shift reagent used in these studies) are smaller than those observed for D_3. This implies that the shift reagent detectably affects conformational populations. An estimate of the relative association constants for axial vs. equatorial hydroxyl groups (K_a/K_e = 1.29) was determined by a competitive titration of *cis*- and *trans*-4-*tert*-butylcyclohexanol with Eu(dpm)$_3$ and is in agreement with the observed perturbation of the chair–chair equilibrium of D_3 by La(dpm)$_3$.

Vitamin D_3 (**1**, D_3) is metabolized in the liver[4] to 25-hydroxyvitamin D_3 (**2**, 25-OH-D_3), which is then further hydroxylated in the kidney[5] to give 1α,25-dihydroxyvitamin D_3 (**3**, 1α,25-(OH)$_2$-D_3). The renal metabolite [1α,25-(OH)$_2$-D_3] has been shown to produce all of the known physiological responses attributable to vitamin D_3 including stimulation of intestinal calcium transport and bone calcium mobilization.[6] From all indications, 1α,25-(OH)$_2$-D_3 appears to be the active form of vitamin D_3[6] and behaves in a manner characteristic of classical steroid hormones.[6,7]



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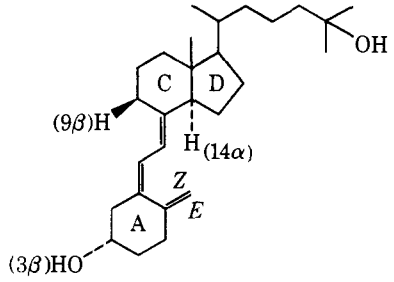

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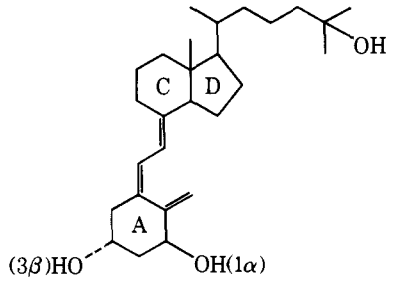

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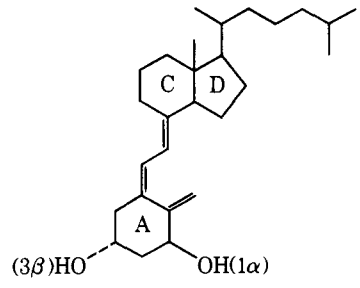
The high biological activity of these metabolites has prompted a number of chemical syntheses of $1\alpha,25\text{-}(OH)_2\text{-}D_3$[8] and of a biologically active analog 1α-hydroxyvitamin D_3 (**4**, $1\alpha\text{-OH-}D_3$).[9] It has been noted that the 1α-hydroxyl group is particularly critical for biological activity.[10] Observation of high biological activity for 3-deoxy-1α-hydroxyvitamin D_3 (**5**, 3-D-1α-OH-D_3), which we recently reported,[11] further emphasizes the crucial role played by the 1α-hydroxyl group.



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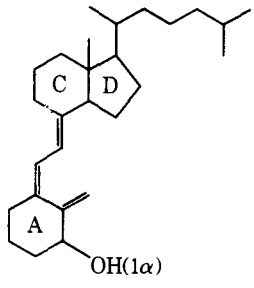

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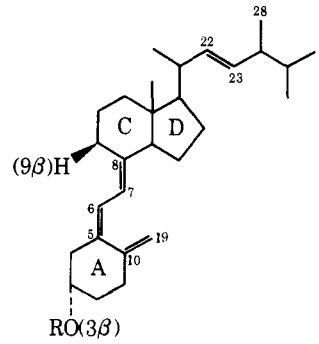
More recently, we have proposed that the 1α-hydroxyl group must occupy an equatorial orientation for optimization of the biological response.[10] Besides testing this structure-function model, ongoing synthetic and biological studies demanded a detailed examination of the structures of vitamin D_3, its metabolites, and analogs in solution. X-ray diffraction studies of two vitamin D analogs have been reported. Hodgkin et al.[12] published in 1963 the full details of the structure of a heavy atom ester derivative of vitamin D_2 (**6**, R = 4-iodo-3-nitrobenzoate), and more recently Knobler et al.[13] reported the structure of the diketal analog **7**.



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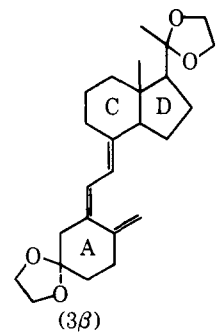

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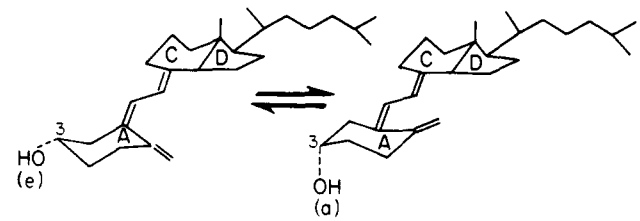
The essential difference between these two structures is that the 3β-oxygen is equatorial in **6**, whereas this same oxygen is axial in **7**. The A ring is very nearly chair shaped as in cyclohexane[14] with the $C_6\text{-}C_5\text{-}C_{10}\text{-}C_{19}$ torsion angle being ca. 58° for both structures. The seco-B ring is nearly planar; the $C_5\text{-}C_6\text{-}C_7\text{-}C_8$ torsion angle is 168° in both structures such that proton H_6 lies on the α side of the plane defined by $H_{9\beta}\text{-}C_9\text{-}C_8$. This paper reports the solution structures[15] of vitamin D_3 (**1**) and 1α,25-dihydroxyvitamin D_3 (**3**).

Results and Discussion

High-resolution 300-MHz ^1H NMR spectra for vitamin D_3 and four related molecules, including the metabolite $1\alpha,25\text{-}(OH)_2\text{-}D_3$ (**3**), are given in Figure 1. The assignments for vitamin D_3 (given in Figure 1 and Table I) were established on the basis of (a) an analysis of chemical shifts and coupling constants and, in the case of the A-ring, computer simulation[16] of its proton resonances (Figure 2) and (b) iterative refinement[17] of the shifts of the proton resonances induced by tris(dipivalomethanato)europium(III) [Eu(dpm)$_3$] (Figure 3).

It is immediately apparent from a first-order analysis of the couplings to the 3α proton, $|J| \sim 7.6$, 7.6, 3.7, and 3.7 Hz, that the A-ring must be dynamically partitioned between two chair forms in an approximate equimolar ratio as follows:[18,19]



The assignment of resonance frequencies to the A-ring protons by iterative application of the McConnell-Robertson equation[17,20] was initially based on an assumed 1:1 mixture of cyclohexane-like chair conformers and identical europium-substrate geometries for the two conformers (vide infra). When the proton assignments finally became clear (see Figure 2 for a computer simulation[16] of the seven spin aliphatic A-ring proton system and column 4, Table I), an optimal value of the ratio equatorial:axial 3β-OH of 43:57 was computed from the LIS data.[17,21]

A number of researchers[22] have noted that conformational analysis by LIS requires a knowledge of the relative association constants of the paramagnetic probe with the conformers. This is due to the fact that LIS calculations must by reason of mass balance give the ratio of conformers *bound to shift reagent*. Therefore it is clear that some measure of the extent of perturbation of the substrate's conformational equilibrium by shift reagent is required before

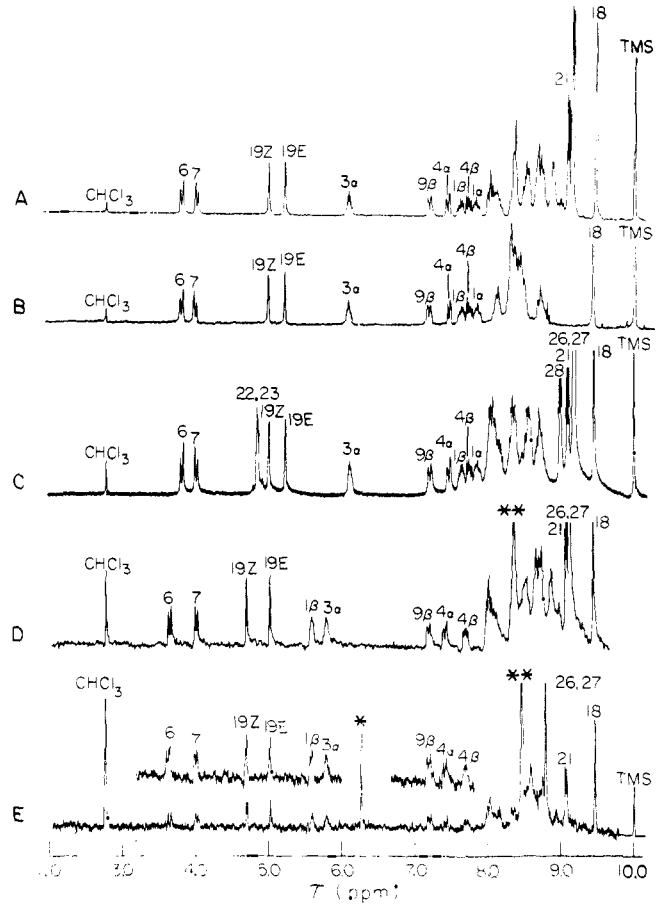


Figure 1. Proton NMR spectra at 300 MHz of (A) D_3, (B) octanor-D_3, (C) D_2, (D) 1α-OH-D_3, and (E) 1α,25-$(OH)_2$-D_3 in deuteriochloroform solvent. Me_4Si (TMS) and chloroform (2180 Hz apart) appear as internal standards. Impurities are marked with asterisks.

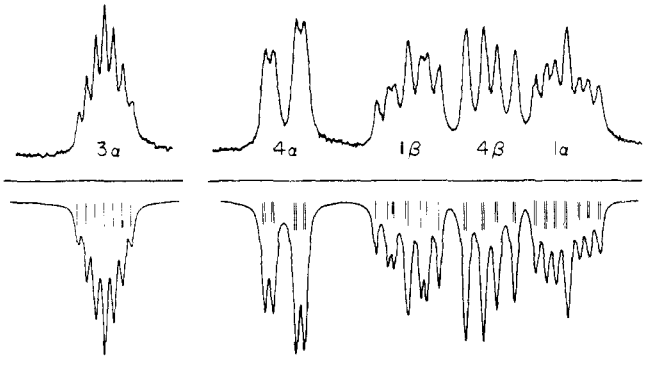


Figure 2. Frequency expansion of the A-ring resonance of D_3, with a LAOCN3 computer simulation given below in inverted form. See footnote *c* of Table I for a description of parameters.

LIS methods may be used to predict conformational equilibria of free substrate. Indeed, we note that the value of the *trans vicinal* coupling $J_{3\alpha,4\beta} = 7.6$ Hz implies the somewhat higher value[23] of 57 ± 4% for equatorial 3β-OH rather than the 43 ± 2% extracted from the fit of LIS.

Thus, one should see a decrease in $J_{3\alpha,4\beta}$ as one titrates vitamin D with shift reagent. This change, estimated from a titration with the diamagnetic reagent $La(dpm)_3$ (Figure 4), gave a limiting value of $J_{3\alpha,4\beta}$ of 6.6 ± 0.2 Hz for fully bound D_3 and hence an estimate of 45 ± 4% 3β-OH equatorial.

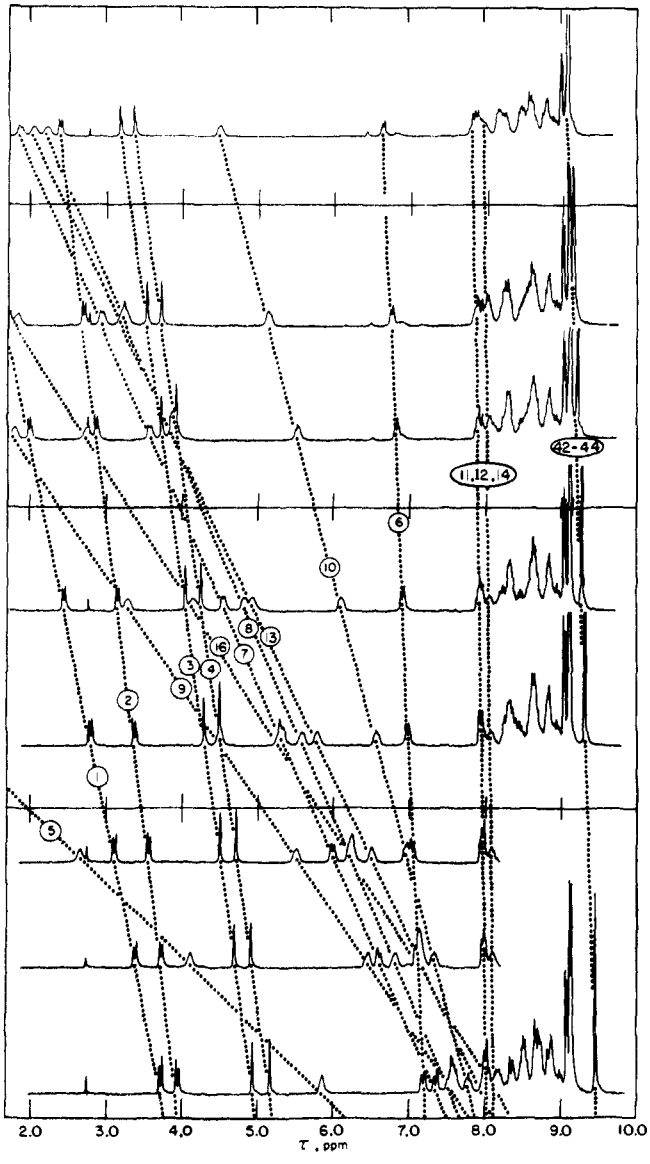


Figure 3. An $Eu(dpm)_3$ titration of vitamin D_3. The vertical scale represents increasing amounts of shift reagent, and the dotted lines denote those LIS which could be unambiguously followed. The numbers refer to resonances in order of increasing field and are further defined and correlated in Table I.

Writing the mass balance as

$$\begin{array}{ccc} \text{free e} & \xrightleftharpoons{K_{free}} & \text{free a} \\ K_e \Big\| & & \Big\| K_a \\ \text{bound e} & \xrightleftharpoons{K_{bound}} & \text{bound a} \end{array}$$

we note that $K_{bound} = (K_a/K_e)K_{free}$ so that we would estimate from our coupling constants that $(K_a/K_e) = 1.62$.

Since the predicted preference of $Eu(dpm)_3$ for an axial OH group seemed a bit unusual, we made a competitive measurement (see Experimental Section) of the binding of *cis*- and *trans*-4-*tert*-butylcyclohexanol to the same shift reagent, finding a similar although smaller ratio of 1.29 in favor of the cis form (i.e., axial OH group) of the rigid substrate molecule. In addition titration with up to a twofold excess of $Eu(fod)_3$ shows the limiting shifts of the geminal H's to be identical for *cis*- and *trans*-4-*tert*-butylcyclohexanol and thus supports our assumption of identical (lanthanide eclipsed with the 3α hydrogen of vitamin D_3) metal–substrate geometries made above.[24]

Table I. Summary of NMR Results for Vitamin D_3[a]

Line no.	Assignment[b]	Chemical shift (τ, ppm)	Fine structure[c]	Geometric shift	
				Obsd[d]	Calcd[e]
1	6	3.75	d (11.2)	213	200
2	7	3.97	d (11.2)	138	136
3	19*Z*	4.95	dt (2.5, 1.2)[f]	146	157
4	19*E*	5.18	d (2.5)[f]	150	161
5	3α	6.06	dddd (3.6, 3.8, 7.5, 7.6)	1094 (−55)	1037
6	9β[h]	7.18	d (12.0)	44	52
7	4α	7.44	dd (3.6, 13.2)	459 (−7)	448
8[g]	1β	7.61	ddd (5.0, 7.7, 13.9)	442 (5)	446
9	4β	7.72	dd (7.5, 13.2)	694 (−5)	686
10[g]	1α	7.83	ddd (4.5, 8.6, 13.9)	274 (5)	267
12	14 or 9α	8.02		14	34
13	2α	8.07		493 (−7)	489
14	9α or 14	8.12		16	39
15	OH	(Variable)	s	Very large	Not calcd
16	2β	8.30		665 (−5)	656
33–35	21 methyl	9.08	d (6.0)	4	Not calcd
36–41	26,27 methyl	9.13	d (6.5)	1	Not calcd
42–44	18 methyl	9.46	s	29	19

[a] Solution containing 25 mg of D_3 in 0.5 ml of $DCCl_3$. [b] The numbering scheme is defined in **1** and **2** (see L. F. Fieser and M. Fieser, "Steroids", Reinhold, New York, N.Y., 1959, Chapter 4). [c] LAOCN3 refined coupling constants (standard deviations of least significant figures in parentheses): $J_{1\alpha,1\beta} = -13.89$ (8); $J_{1\alpha,2\alpha} = 4.53$ (11); $J_{1\alpha,2\beta} = 8.56$ (11); $J_{1\beta,2\alpha} = 7.73$ (11); $J_{1\beta,2\beta} = 4.98$ (11); $J_{2\alpha,2\beta} = -13.2$; $J_{2\alpha,3\alpha} = 3.83$ (15); $J_{2\beta,3\alpha} = 7.62$ (12); $J_{3\alpha,4\alpha} = 3.62$ (6); $J_{3\alpha,4\beta} = 7.54$ (7); $J_{4\alpha,4\beta} = -13.18$ (5) Hz; long range couplings of 0.9 Hz were used for $J_{1\alpha,3\alpha} = J_{2\alpha,4\alpha} = J_{2\beta,4\beta}$. [d] Diamagnetic corrections are given in parentheses. [e] The 'PSEUDO' optimized structure gave Eu–O = 2.66 (4) A; Eu–O–C = 117 (2)°, Eu–O–C–$H_{3\alpha}$ torsion angle 7 (2)°, % axial 3β-OH conformer 57 (2) with a residual error of 1.24% based on A ring protons only and 2.66% for all protons. [f] From 60-MHz spectral data. [g] From 300-MHz spin decoupling studies, resonances 8 and 10 were found not to be coupled to resonance 5. [h] An alternative assignment has been given by G. N. LaMar, ref 21.

The resonances due to the 19 *Z* and 19 *E* protons could not be assigned wholly on the basis of our LIS analysis. The lower field resonance is assigned to proton 19 *Z* (τ 4.95) and the higher field one to 19 *E* (τ 5.18) for two reasons. First, as also discussed recently by La Mar,[21] only the lower field doublet is further split into triplets as expected of a proton (19 *Z*) located trans to the allylic protons.[25] Second, the 19 *Z* resonance should be at lower field because of the possibility of mutual deshielding by the proximal 7-proton.[26]

Further assignments, now for seco-B and C ring resonances, were confirmed on the basis of LIS values calculated using the X-ray value for the C_5–C_6–C_7–C_8 torsion angle and the LIS-A ring conformation established above. Finally the LIS for the 7, 9β, 18-methyl, 9α, and 14β protons (the latter two being uncertain assignments) were used to optimize the C_5–C_6–C_7–C_8 torsion angle. We are unable to detect any difference from the X-ray value of 168°. The uncertainty of our iterated LIS-computed torsion-angle, however, is large (±20°). We note that $J_{6,7}$ is 11.2 Hz, confirming the observation of Delaroff.[15b] This value is expected for the s-trans conformation about the 6-7 bond which, for a dihedral angle of about 180°, is usually in the range 10–12 Hz.[27]

Some other pertinent observations are as follows. (a) The A-ring conformation is unaffected by the nature of the side chain (see Figure 1A vs. 1B and 1C and Figure 1D vs. 1E). (b) The introduction of a hydroxyl at 1α in vitamin D_3 slightly shifts the conformational equilibrium to favor an axial 3β-OH ($J_{3\alpha,4\beta} = 6.5$ Hz) for 1α-OH-D_3. Thus the A ring consists of an equilibrium mixture containing ~56% equatorial 1α-OH conformer. (c) One must average computed LIS values over all conformers in solution in order to arrive at dependable assignments. However, conformational equilibria are not as easily derived from LIS analysis as from coupling constant analysis. This is due to the fact that *LIS calculations give a composite of conformer population and conformer–shift reagent adduct concentration, and it is not always possible to independently evaluate the shift reagent–substrate association constants.*

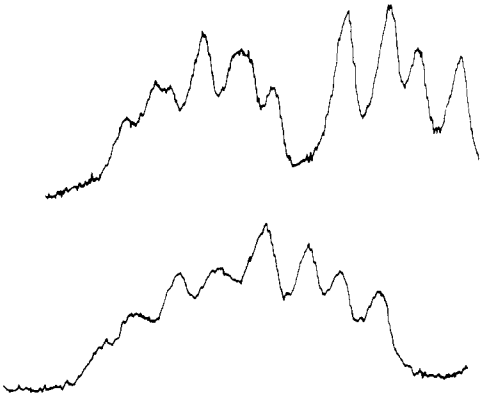


Figure 4. Frequency expansion of the 1β and 4β proton resonances of D_3. The upper trace shows the normal spectrum and the lower trace indicates the change due to the addition of 0.55 equiv of La(dpm)$_3$. Although the resonances overlap in the lower trace as a result of diamagnetic shifts, there are changes in coupling constants ($J_{3\alpha,4\beta}$ decreases from 7.6 to 7.0).

Experimental Section

Crystalline vitamins D_3 and D_2 (Aldrich) were used directly as purchased. Samples of 1α-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 were generously provided by Dr. Fürst (Basel) and Dr. Uskoković (Nutley) of Hoffmann-La Roche, Inc. A commercial (Aldrich) mixture of *cis*- and *trans*-4-*tert*-butylcyclohexanol was chromatographically separated (Woelm neutral alumina III, using low boiling petroleum ether–ether mixtures), and then each isomer was further purified by crystallization: cis, mp 81.5–82.5° (lit.[28] mp 82–83°); trans, mp 75.0–76.5° (lit.[28] mp 75–78°). Eu(dpm)$_3$ was used directly as obtained from Ventron, Inc., and La(dpm)$_3$ was a generous gift from Professor J. J. Uebel, University of New Hampshire.

All vitamin D-related ^{1}H NMR spectra were recorded on a Varian HR300 ^{1}H NMR instrument at a probe temperature of 24°. Deuteriochloroform was used as solvent in all cases, and small amounts of chloroform (τ 2.74) and tetramethylsilane (Me_4Si, τ 10.00) were used as internal standards. The *tert*-butylcyclohexanol study was carried out on a Varian A60D ^{1}H NMR spectrometer.

20,21,22,23,24,25,26,27-Octanorvitamin D_3 (Octanorvitamin D_3). Commercial 5-androsten-3β-ol-17-one was converted by successive Wolff-Kishner reduction, acetylation, bromination (1,3-dibromo-5,5-dimethylhydantoin), and dehydrobromination (*s*-collidine), as previously described,[29] to 3β-acetoxy-5,7-androstadiene, mp 115–116° (lit.[29a] mp 113–116°). Its ^{1}H NMR and uv spectra were in accord with the assigned structure.

The acetoxydiene (200 mg/200 ml of anhydrous ether), under a nitrogen atmosphere, was irradiated (Hanovia 450-W medium-pressure mercury arc) for 6.5 min through a Corex filter in an ice-cooled vessel (water-cooled quartz inner well in a Pyrex vessel). The solvent was removed under vacuum below room temperature, and the residue was chromatographed (Woelm alumina III, petroleum ether–ether mixtures) to afford the pure previtamin acetate. The neat previtamin was heated at 70° for 3 hr and then, after cooling, it was dissolved in a small volume of 5% potassium hydroxide–methanol solution. After allowing the saponification mixture to stand overnight in the refrigerator, the mixture was worked up in the usual way. The octanorvitamin D_3 was separated from the previtamin alcohol by chromatography. Vacuum drying afforded the octanorvitamin as a colorless oil (as previously described),[30] which was used in the NMR study (Figure 1B).

Shift Reagent Titration. Titration of vitamin D_3 was carried out by adding ca. 3–5 mg increments of solid Eu(dpm)$_3$ to 25 mg of vitamin D_3 in 0.5 ml of $CDCl_3$ until a near equimolar mixture of D_3 and shift reagent was obtained. ^{1}H NMR spectra were recorded immediately after each incremental addition of Eu(dpm)$_3$ (Figure 3).

Diamagnetic Correction. In order to examine whether the Eu(dpm)$_3$ magnetic probe influences the conformational equilibrium of the A ring, 0.55 mol equiv of La(dpm)$_3$, a diamagnetic analog of Eu(dpm)$_3$, was introduced to a 25 mg/0.5 ml solution of vitamin D_3 in ^{1}H NMR sample tube. A portion of the 300-MHz ^{1}H NMR spectrum obtained is shown in Figure 4. A small but detectable difference in some of the observable coupling constants could be detected. In particular, the $J_{3\alpha,4\beta}$ value was diminished to 7.0 Hz. There were also slight diamagnetic shifts noted for the observable A ring resonances, and these are listed in Table I.

Competitive Measure of K_a/K_e of 4-*tert*-Butylcyclohexanol.[31] A 1:1 mixture of pure *cis*- and pure *trans*-4-*tert*-butylcyclohexanol (40 mg total in 0.5 ml of $CDCl_3$) was prepared in a ^{1}H NMR tube. The induced shifts (Δ_{cis} or Δ_{trans}) of the H-1 protons of each were simultaneously followed as a function of Eu(dpm)$_3$ addition.

It was found that the H-1 proton resonances of each isomer diverged linearly up to 0.4 ± 0.1 equiv of Eu(dpm)$_3$ or Eu(fod)$_3$. They then converged to the same net LIS shift value in the limit of the titration (2:1 Eu(fod)$_3$/4-*tert*-butylcyclohexanol) so that $^B\Delta_c/^B\Delta_t = {}^B\Delta_a/{}^B\Delta_e$ is equal to one. The same end point ratio is indicated for titration with Eu(dpm)$_3$; however, we could not add much of an excess because of solubility limitations.[32]

Analysis of the titration curve using

$$\left(\frac{K_c}{K_t}\frac{^B\Delta_c}{^B\Delta_t}\right)\frac{1}{\Delta_c} + \left(\frac{1}{^B\Delta_t} - \frac{K_c}{K_t}\frac{1}{^B\Delta_t}\right) = \frac{1}{\Delta_t}$$

gave the relative basicity for the axial vs. equatorial hydroxyl group with the Lewis acid Eu(dpm)$_3$ as $K_c/K_t = K_a/K_e = 1.29$ at 24°.[33] This result is consistent with the ratio 1.8 ± 0.5 which can be computed from the results of Shapiro[34] et al. The a, e, c, and t subscripts refer to the hydroxyl in axial (cis) or equatorial (trans) orientations, K is the binding constant for Eu(dpm)$_3$ and the appropriately subscripted hydroxyl group, $^B\Delta$ refers to the titration endpoint shifts, and Δ refers to the observed shifts.

Computational Procedures. The program PSEUDO, described earlier,[17] has been modified to allow refinement of conformational populations as well as optimization of internal torsion angles.

Refinement of conformational populations has been accomplished by refining a parameter λ_i which is defined so that $\lambda_1 = (1 - \Sigma^n_{i=2}\lambda_i)$. At present, the coordinates for each conformer must be tabulated and submitted to PSEUDO so that observation 1 for the second conformer is the $(N + 1)$th observation in the overall list of N unique observations. Observed LIS are set to zero for all but the first conformation so that the data are properly weighted.

Refinement of torsion angles is accomplished by defining a transformation (B) to a coordinate system based on the desired bond j-k and an adjacent atom i. The torsional parameter is then defined in the local bond coordinates as

$$T = \begin{vmatrix} \cos\tau & -\sin\tau & 0 \\ \sin\tau & \cos\tau & 0 \\ 0 & 0 & 1 \end{vmatrix}$$

so that the overall coordinate transformation in PSEUDO becomes

$$\begin{vmatrix} L_x \\ L_y \\ L_z \end{vmatrix} = X_G + \phi\cdot\psi\cdot B^T\cdot T\cdot B\cdot \begin{vmatrix} D_x \\ D_y \\ D_z \end{vmatrix}$$

in which refinement of the internal angle is accomplished by B^TTB.

Thus T and λ_i are new parameters which have been added to the program. The required derivatives are computed as before and summed as required to give the appropriate normal equation matrix.

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Toxins Causing Noninflammatory Paralytic Neuronopathy. Isolation and Structure Elucidation

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Abstract: Four constituents of the neurotoxic principal of *Karwinskia humboldtiana* (Rhamnaceae) have been isolated and identified as 7-[3′,4′-dihydro-7′,9′-dimethoxy-1′,3′-dimethyl-10′-hydroxy-1′*H*-naphtho[2′,3′-*c*′]pyran-5′-yl]-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2*H*)-anthracenone (**1**), 3,4-dihydro-3,3′-dimethyl-1′,3,8,8′,9-pentahydroxy(7,10′-bianthracene)-1,9′(2*H*,10′*H*)-dione (**2**), 7-(2′-acetyl-6′,8′-dimethoxy-3′-methyl-1′-hydroxynaphth-4′-yl)-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2*H*)-anthracenone (**3**), and 3,3′-dimethyl-3,3′,8,8′,9,9′-hexahydroxy-3,3′,4,4′-tetrahydro(7,10′-bianthracene)-1,1′(2*H*,2′*H*)-dione (**4**). In addition, occurrence of the C-7′ desmethoxy analog of **1** was indicated indirectly and chrysophanol (**5**), 3,4-dihydro-7,9-dimethoxy-1,3-dimethyl-1*H*-naphtho[2,3-*c*]-5,10-pyrandione (7-methoxyelutherin, **6**), 3,4-dihydro-7,9-dimethoxy-1,3-dimethyl-6-hydroxy-1*H*-naphtho[2,3-*c*]pyran-5,10-dione (**7**), and 2-aceto-6,8-dimethoxy-3-methyl-1-naphthol (tarachrysone monomethyl ether, **8**) were isolated from a nontoxic hexane extract of *K. humboldtiana* seeds. Proton magnetic resonance spin decoupling experiments using **6** established that the C-1 and C-3 methyl groups in this compound are cis diequatorial; by analogy it is assumed that the stereochemistries of the dihydrodimethylpyran rings of **1** and **7** are similar.

Fractionation of the neurotoxic principle of *Karwinskia humboldtiana*, Zucc. (Rhamnaceae) has led to the isolation of four major constituents to which structures **1–4** (Figure 1) have been assigned. Compounds **1–4**, comprise respectively 0.75, 0.59, 0.39, and 0.46% of the weight of air-dried mature fruit and seeds and account for the observed neurotoxic properties.[2]

Clavigero,[3] almost 2 centuries ago, first reported the toxic effects which result from ingestion of the seeds[4,5] of *K. humboldtiana*, a plant indigenous to desert areas of southern Texas and northern and central Mexico. Clinically the toxicity is characterized by a progressive and symmetrical noninflammatory paralytic neuronopathy starting in the lower limbs and ending with respiratory and bulbar paralysis.[6]

Isolation of Toxins. The yellow, amorphous crude toxin,[4] obtained by chloroform extraction of the toxic seeds, was fractionated by chromatography on silica gel; compound **2** was eluted using benzene–acetone (50:1) with elution of the other, more polar constituents requiring increased acetone concentrations, i.e., **1** (20:1), **3** (10:1), and **4** (5:1). Pure samples of each of the toxins were obtained only after repeated rechromatography of these initial fractions. Fractionation of hexane soluble material from *K. humboldtiana*