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Conformational Analysis of 1α,25-Dihydroxyvitamin D_3 by Nuclear Magnetic Resonance

TADASHI EGUCHI AND NOBUO IKEKAWA

Department of Fundamental Science, Iwaki Meisei University, Iwaki, Fukushima 970, Japan

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The conformational equilibrium of 1α,25-dihydroxyvitamin D_3 (**3**) was studied by variable temperature ^1H and ^{13}C NMR spectroscopy. At temperatures below −100°C, two conformations in a ratio of 55 : 45 were observed by splitting of the signals for H-3α, H-4α, and H-6. A free enthalpy of interconversion was estimated to be $\Delta G^{\ddagger}_{193°K}$, 39.6 kJ/mol from the ^{13}C NMR spectrum at −80°C. From an analysis of the A-ring coupling constants in various solvents, we propose that the A-ring of **3** undergoes chair–chair interconversion in hydrophobic media and chair–half-chair interconversion in hydrophilic media.

INTRODUCTION

It is well known that vitamin D_3 (**1**) is hydroxylated to 25-hydroxyvitamin D_3 (**2**) in the liver and subsequently to 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$, (**3**)] in the kidney (*1*). The renal metabolite 1,25-$(OH)_2D_3$ is capable of producing all the known physiological responses attributable to vitamin D_3, such as calcium absorption in intestine, bone calcium mobilization, and calcification of bone (*1, 2*). The mode of action of 1,25-$(OH)_2D_3$ is believed to be mediated by an intracellular receptor as is the case for other classical steroid hormones (*3*). An active form of vitamin D_3, 1,25-$(OH)_2D_3$, binds an intracellular receptor protein present in the target organs with high affinity and specificity, and formation of this hormone–protein complex is thought to be the first step for vitamin D actions. Topological features of vitamin D metabolites as receptor ligands are very important. Therefore, actual conformations of vitamin D metabolites have been a subject of considerable interest. Recently the primary structure of the human vitamin D receptor protein was determined (*4*). However, topological features of the binding site(s) have yet to be clarified. To obtain insights into this important hormone action, further in-depth knowledge of the actual conformation of the active hormone is necessary (Scheme 1).

In the crystalline form, vitamin D_3 exists as a 1 : 1 mixture of two different chair forms of the A-ring (*5*). The conformation of 25-hydroxyvitamin D_3 in the crystalline state appears to exist as only one of those conformations with the 3β-hydroxyl group equatorial (*6*). However, no X-ray analysis of vitamin D_3 derivatives possessing a biologically important 1α-hydroxyl group has been reported.

In solution, vitamin D and its derivatives, including 1,25-$(OH)_2D_3$, have been discussed by means of NMR spectroscopy. These studies have shown that the A-

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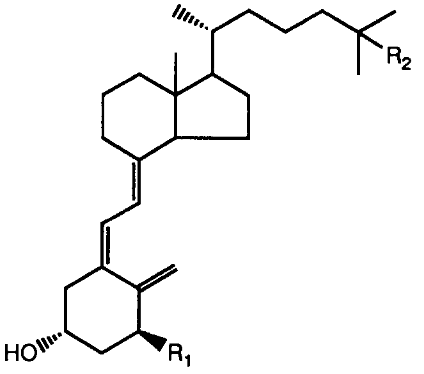

(1) Vitamin D_3 (R_1=H, R_2=H)
(2) 25-Hydroxyvitamin D_3 (R_1=H, R_2=OH)
(3) 1α,25-Dihydroxyvitamin D_3 (R_1=OH, R_2=OH)

SCHEME 1

ring of these compounds exists in two chair conformations, which are under dynamic equilibrium in solution (*7–18*). However, two conformations were not directly observed in solution and the equilibrium was only discussed on their averaged proton–proton vicinal coupling constants. As far as 1,25-$(OH)_2D_3$ is concerned, a few reports suggested that two chair conformations exist in an almost equal ratio based on the vicinal coupling constant of H-3α-H-4 (*10, 18*). To obtain further information on the actual conformation of this class of important hormones we undertook a study on solution conformation of 1,25-$(OH)_2D_3$, taking advantage of variable temperature ^1H and ^{13}C NMR spectroscopy. Here we describe a conformational equilibrium of the A-ring of 1,25-$(OH)_2D_3$, and solvent effects for conformation are also discussed.

RESULTS AND DISCUSSION

NMR spectroscopy is most appropriate for the conformational analysis, especially cyclohexane derivatives like the A-ring of 1,25-$(OH)_2D_3$. Various parameters can be used for the investigation of conformational equilibrium such as the areas of the separated signals, the chemical shifts, the coupling constants, and the bandwidth of the signals (*19*). The variable temperature NMR technique is one of the most frequently used technique for analysis of conformational interconversion. First, we investigated the ^1H NMR spectra of 1,25-$(OH)_2D_3$ at a variable low temperature, which are shown in Fig. 1. While the resolution was not high due to high viscosity of the solvent under these conditions, each signal at H-3α, H-4α, and H-6 was well split into pairs below −100°C. These experiments clearly indicated that 1,25-$(OH)_2D_3$ exists in two major conformations under the conditions,

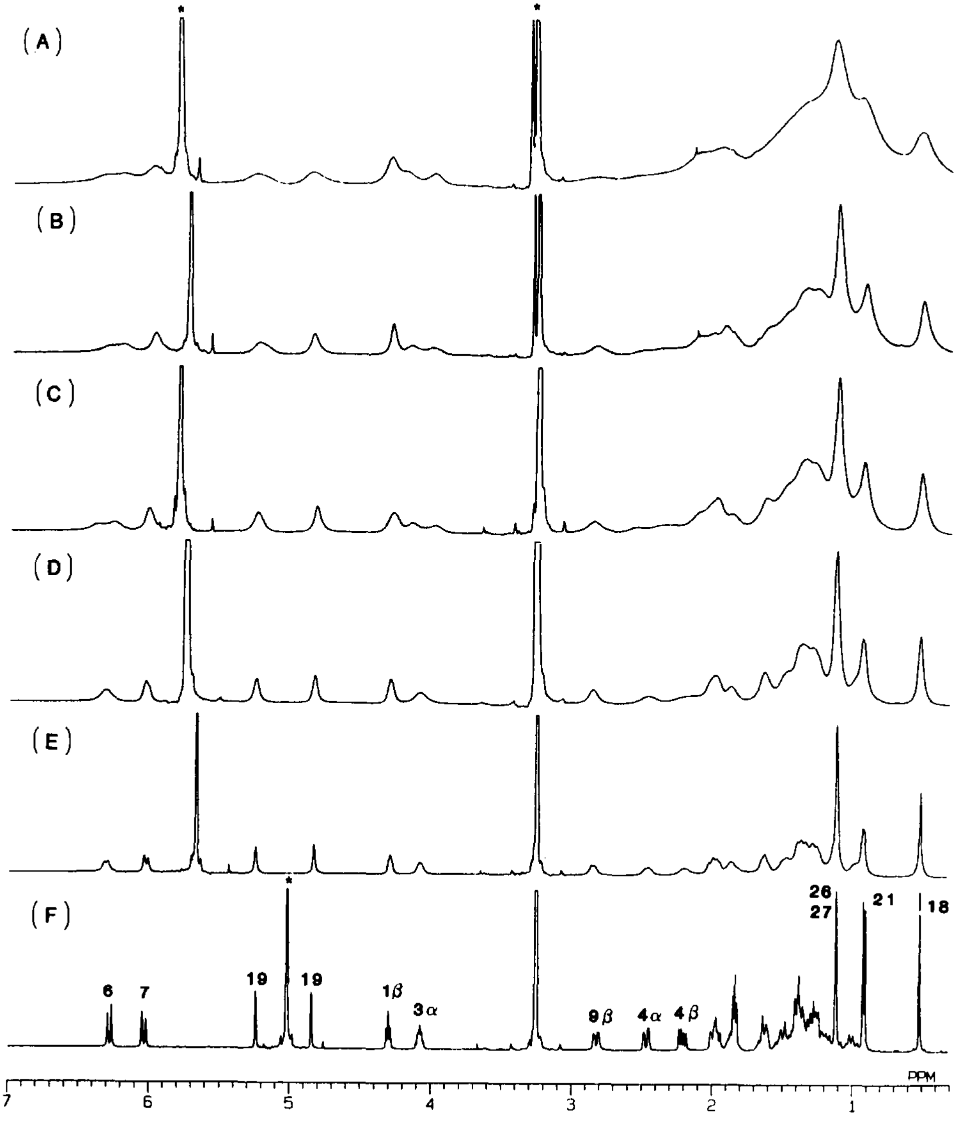

FIG. 1. Variable temperature ^1H NMR (400 MHz) spectra of 1,25-$(OH)_2D_3$: (A) at −110°C in CD_3OD–$CFCl_3$ (1 : 1), (B) at −100°C in CD_3OD–$CFCl_3$, (C) at −100°C in CD_3OD, (D) at −90°C in CD_3OD, (E) at −80°C in CD_3OD, and (F) at 0°C in CD_3OD. The asterisks denote the solvent signals.

and the relative equilibrium ratio was estimated to be approximately 55 : 45 from these spectra. One of the split signals of the H-3 observed at about 4.2 ppm at −100°C must be assigned to an equatorially oriented proton and the other at about 4.0 ppm to an axially oriented proton on the basis of the shielding effect of a cyclohexane ring (*19*). This ratio is in good agreement with the calculated ratio from the coupling constant between H-3α and H-4β (*10, 18*). Unfortunately, other kinetic parameters could not be determined from the spectra, because a lower temperature NMR spectrum was not informative due to low resolution. Consequently, the exact chemical shifts of protons on the A-ring due to two frozen conformations were not accurately obtained and it was also difficult to analyze the separated signal shapes due to homonuclear coupling. But, at any rate, this is the first, to our knowledge, to directly observe two conformations of the vitamin D derivatives in solution.

It should be noted that, as to the protons on the A-ring, the signals of H-3α and H-4α were observed in paired signals, but the signal of H-1β could not be observed as two signals under the same conditions. Since the chemical shift differences between equatorially and axially oriented oxymethine protons in chair-formed cyclohexane derivatives are generally relatively large ((*20, 21*) see also Fig. 3), H-1 nucleus would have given rise to large chemical shift differences in two conformations such as those of the previously proposed complete chair–chair interconversion (*10, 18*). The fact was reverse; therefore, the present data may imply that two A-ring conformations of 1,25-(OH)$_2$D$_3$ are not in the complete chair–chair interconversion and that either one or both conformations might be deformed chair conformation under these conditions.

^{13}C NMR analysis is suitable for the study of the conformational equilibrium because of superior signal separation and of elimination from complication due to homonuclear coupling (*22*). We therefore investigated this equilibrium by means of ^{13}C NMR spectroscopy under variable temperature. As can be seen in Fig. 2, the signals, especially those due to the A-ring carbons, changed from sharp lines at 20°C into the broad signals below −80°C. Signal splitting was not detected even at −100°C. Thus, as in the case of ^{1}H NMR spectra, the exact chemical shifts of the two possible conformations could not be determined. However, since the coalescence temperatures of the signals of the C-1 and C-4 carbons were estimated to be near −80°C from the spectra, an approximate value for the equilibrium rate constant K_r was estimated to be about 80 s^{-1} from the half-maximum intensity linewidth (*23*) from the signals of the C-1 and C-4 at −80°C. A free enthalpy of the interconversion barrier at this temperature was calculated to be about, $\Delta G^{\ddagger}_{193^{\circ}\mathrm{K}}$, 39.6 kJ/mol by the Eyring equation (*22*).

To determine the actual two conformations of 1,25-(OH)$_2$D$_3$, we analyzed the averaged coupling constants on ^{1}H NMR compared with model compounds as well as the previous studies (*7–18*). Previous work used the coupling constants of 3,3,4,4,5,5-hexadeuteriocyclohexanol as the model for the coupling constants of H-3α of vitamin D derivatives. Because the coupling constants in various solvents were not obtained, we chose *cis*- and *trans*-4-*tert*-butylcyclohexanol as the model compounds for the coupling constants of H-3α. As the suitable model compounds of the coupling constants of H-1β, *cis*- and *trans*-5-*tert*-butyl-2-methylenecyclohexanol were synthesized. The chemical shifts and the coupling constants of the model compounds are shown in Fig. 3, and the chemical shifts and the coupling constants of 1,25-(OH)$_2$D$_3$ are listed in Table 1. We were aware, at this point, of solvent dependency of the proton–proton coupling constants on the A-ring, especially the H-1β signal in ^{1}H NMR spectra. In CD$_3$OD, the H-1 signal of 1,25-(OH)$_2$D$_3$ was observed as a triplet at 4.29 ppm (J = 6.0 Hz), but as a double

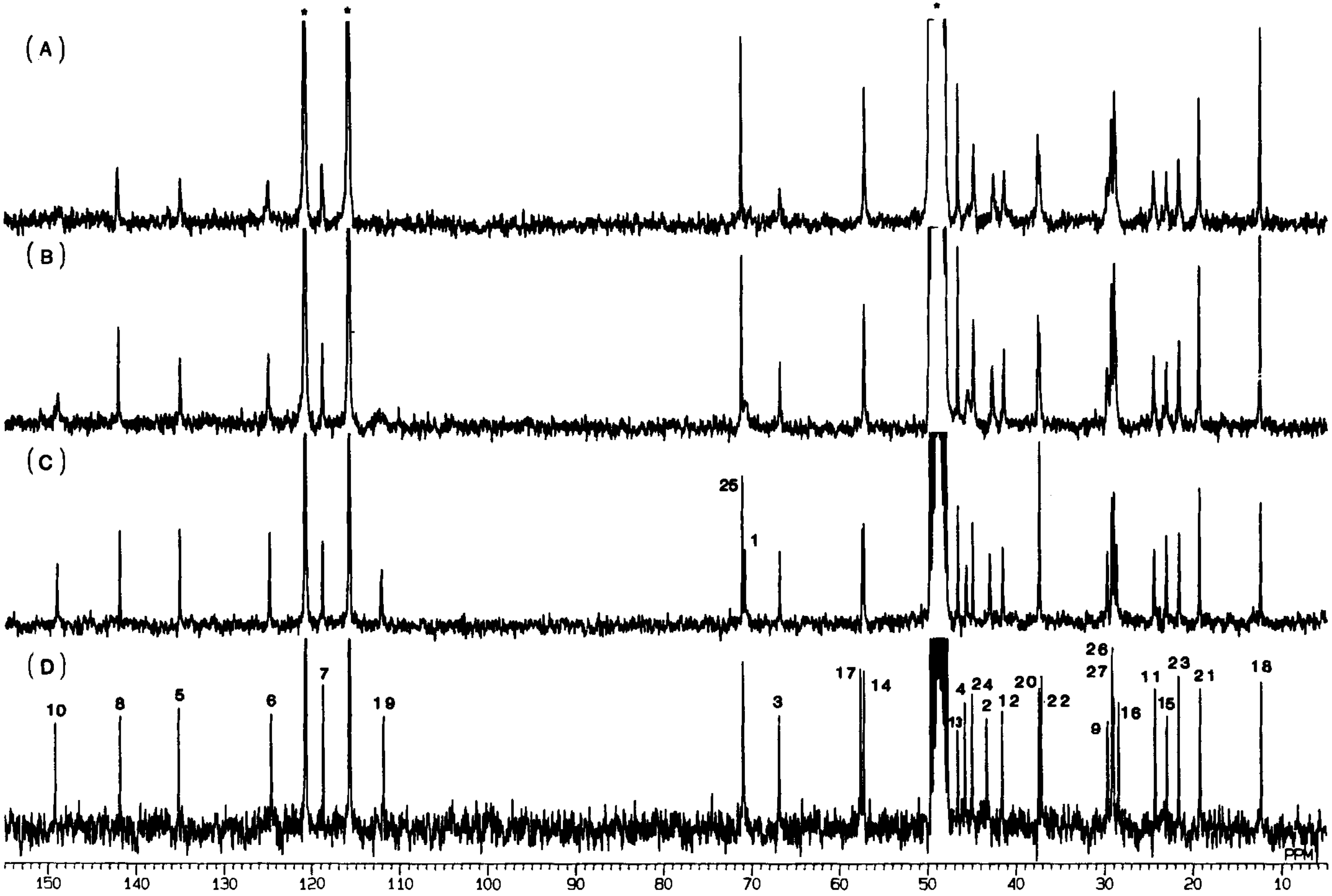

FIG. 2. Variable temperature ^{13}C NMR spectra (67.5 MHz) of 1,25-$(OH)_2D_3$ in CD_3OD–$CFCl_3$ (1 : 1): (A) at −100°C, (B) at −80°C, (C) at −40°C, and (D) at 20°C. Assignments of the signals were unambiguously carried out based on C–H COSY and C–H long range COSY spectra. The asterisks denote the solvent signals.

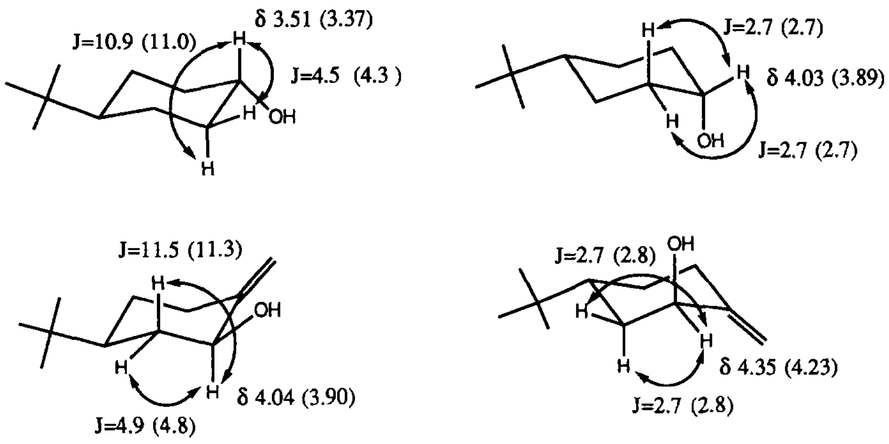

FIG. 3. Observed chemical shifts and coupling constants of the model compounds in $CDCl_3$ and CD_3OD. Coupling constants are given in hertz and data recorded in CD_3OD are shown in parentheses.

doublet at 4.43 ppm (J = 4.0 and 7.5 Hz) in $CDCl_3$ (*24*). The partial spectra of 1,25-$(OH)_2D_3$ in CD_3OD, $CDCl_3$, and mixed solvents are shown in Fig. 4.

Averaged values of $J_{\text{axial,axial}}$-$J_{\text{equatorial,equatorial}}$ and $J_{\text{axial,equatorial}}$-$J_{\text{equatorial,axial}}$ of the model compounds based on the equilibrium ratio (55 : 45) appear to agree quite satisfactorily with the vicinal coupling constants of H-3α-H-4α and H-3α-H-4β in all solvents, respectively. In the case of the H-1 signal, calculated values according to the equilibrium ratio are reproducible with those of the experimental values in $CDCl_3$, but, in CD_3OD, averaged values are largely out of the experimental coupling constants. The larger coupling constant of H-1 (i.e., $J_{\text{H-1}\beta,\text{H-2}\alpha}$) tends to

TABLE 1

Chemical Shifts and Coupling Constants of 1,25-$(OH)_2D_3$ in CD_3OD and $CDCl_3$[a]

	In CD_3OD	In $CDCl_3$
18-H	0.52 (s)	0.54 (s)
21-H	0.91 (d, J = 6.3)	0.94 (d, J = 6.2)
26- and 27-H	1.11 (s)	1.20 (s)
4β-H	2.20 (dd, J = 13.5, 6.8)	2.32 (dd, J = 13.6, 6.7)
4α-H	2.46 (dd, J = 13.5, 3.5)	2.59 (dd, J = 13.5, 3.4)
9β-H	2.82 (dd, J = 11.9, 3.6)	2.83 (dd, J = 11.8, 3.7)
3α-H	4.07 (m)	4.23 (tt, J = 6.5, 3,5)
1β-H	4.29 (t, J = 6.0)	4.43 (dd, J = 7.5, 4.0)
19*E*-H	4.84 (dd, J = 2.3, 0.9)	5.00 (br s)
19*Z*-H	5.23 (dd, J = 1.2, 2.4)	5.33 (br t, J = 1.4)
7-H	6.03 (d, J = 11.3)	6.02 (d, J = 11.3)
6-H	6.27 (d, J = 11.3)	6.38 (d, J = 11.3)

[a] Chemical shifts are in δ units. Multiplicities and coupling constants (in hertz) are given in parentheses.

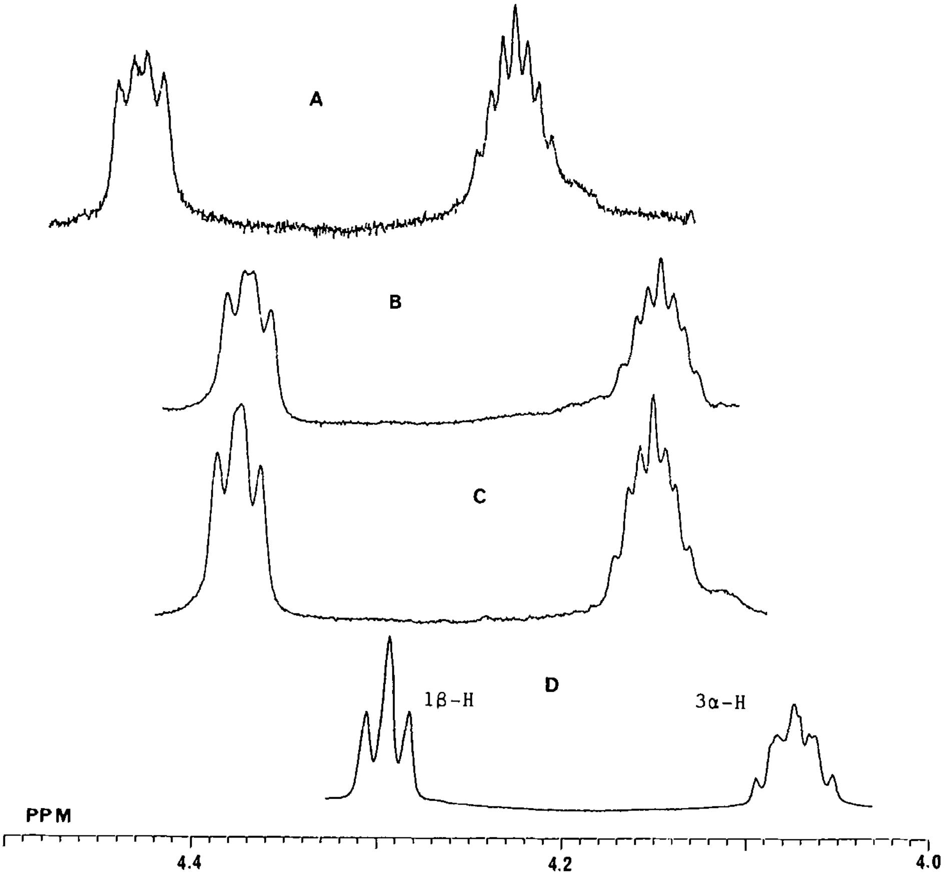

FIG. 4. Segments of ^{1}H NMR spectra (500 MHz) of 1,25-$(OH)_2D_3$ in various solvents: (A) in $CDCl_3$, (B) in $CDCl_3$–CD_3OD (5 : 5), (C) in $CDCl_3$–CD_3OD (3 : 7), and (D) in CD_3OD.

decrease and the smaller one (i.e., $J_{H\text{-}1\beta,H\text{-}2\beta}$) to increase depending upon the hydrophilic nature of the solvent. These results suggest that, in a nonpolar solvent, the A-ring of 1,25-$(OH)_2D_3$ exists in two chair conformation, whereas, in hydrophilic media, such a complete chair–chair interconversion becomes less favorable. In methanol the coupling constant of H-2-H-3 was also affected by deformation of the A-ring.

Thus, we propose that the A-ring of 1,25-$(OH)_2D_3$ exists in equilibrium between two chair conformations in chloroform and that, in methanol, the A-ring of 1,25-$(OH)_2D_3$ is under equilibrium between a chair conformation and a half-chair conformation. As shown in Fig. 5, in CD_3OD, one conformer consists of a chair conformation possessing the equatorial orientation of the 3β-hydroxyl group and the axial-oriented 1α-hydroxyl group. The other has the axial 3β-hydroxyl group, the pseudo-equatorial 1α-hydroxyl group, and the flattered nature of conjugated double bond moiety. These arguments were reinforced by calculations of the vicinal coupling constants of the proposed conformations using the Karplus-Al-

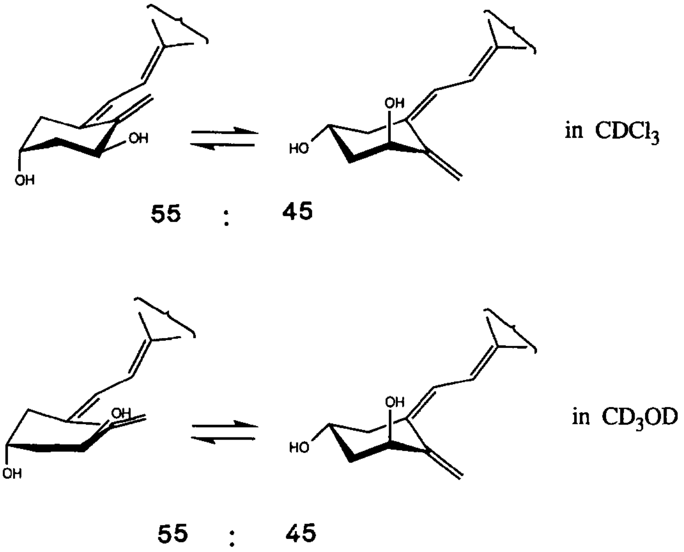

FIG. 5. Proposed conformations under dynamic equilibrium of 1,25-$(OH)_2D_3$ in $CDCl_3$ and CD_3OD.

tona correlations (*25*). The deformed cyclohexane conformation may be attributed to an unfavorable interaction between the exomethylene group and the 1α-hydroxyl group, and a coplanarity of the conjugated double bond may play an important role. Since such an interaction between the exomethylene and the 1α-hydroxyl group may be increased by hydrogen bonding of the 1α-hydroxyl group with hydroxylic media, in methanol such a repulsion might be superior to the energy due to the deformation of the cyclohexane ring, but in chloroform the reverse may be the case. Since the proposed equilibrium may cause close shielding environments of the H-1 nuclei between two conformations, it seems likely that the H-1 signal was not split by ^1H NMR spectroscopy at low temperatures.

To confirm the supposed conformations under equilibrium, MM2 calculations together with a π system treatment were carried out taking into account the possible conformations on the model compounds of the A-ring of 1,25-$(OH)_2D_3$. As shown in Fig. 6, two minima were obtained within a reasonable energy range. The calculations are in good agreement with the experimental results, thereby concluding that the A-ring of 1,25-$(OH)_2D_3$ exists in two chair conformations in the hydrophobic media. But, the calculations were not reproducible for the conformations in hydrophilic media, because the MM2 calculation did not consider the interaction with the solvent.

Studies on structure–activity relationships for vitamin D and related derivatives discussed previously that the 1α-hydroxyl group was essential for the biological activities (*26*). It was also claimed that the equatorial orientation of the 1α-hydroxyl group was important for optimization of biological activities on the basis of studies on relationships between conformations and biological activities of dihydrotachysterols (*8, 9*). However, the present studies, using natural hormone 1,25-$(OH)_2D_3$, suggest that the equatorial orientation of the 1α-hydroxyl group is less favorable and prefers the pseudo-equatorial direction at the ground state under

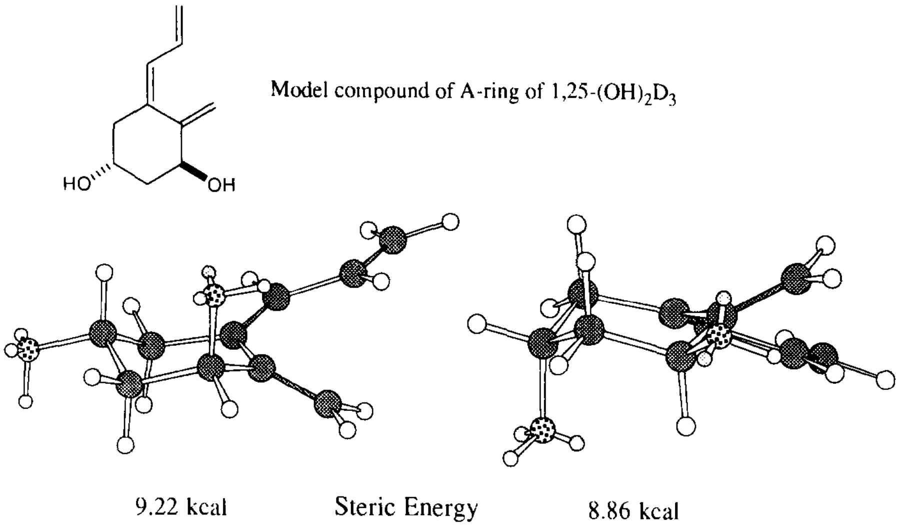

FIG. 6. The results of MM2 calculation of the model compound for the A-ring of 1,25-$(OH)_2D_3$.

hydrophilic conditions. We recently discussed that a zigzag conformation of the side chain in 1,25-$(OH)_2D_3$ was important for biological activities (*27*). But, the concept of conformational optimization of biological activities, especially elucidating an actual binding conformation of 1,25-$(OH)_2D_3$ to the receptor, is clearly one that should be further examined.

In conclusion, we were successful in directly observing the two major conformations of 1,25-$(OH)_2D_3$ with the low temperature ^1H NMR spectroscopy and the equilibrium ratio of two conformations at $-100°C$ was determined as approximately 55 : 45. Kinetic parameters of the equilibrium at $-80°C$ were approximated from a ^{13}C NMR spectrum. The analysis of proton coupling constants allowed us to propose that two conformations of the A-ring of 1,25-$(OH)_2D_3$ underwent chair–chair and chair–half-chair interconversion in hydrophobic and hydrophilic media, respectively.

EXPERIMENTAL

Crystalline 1,25-$(OH)_2D_3$ was obtained from Duphar B. V. (Amsterdam, Holland). The samples of *cis*- and *trans*-5-*tert*-butyl-2-methylenecyclohexanol were synthesized in the present study. A mixture of *cis*- and *trans*-4-*tert*-butylcyclohexanol were commercially available from Tokyo Kasei Co., Ltd. (Tokyo, Japan) and their ^1H NMR spectra were measured as a mixture. Variable temperature ^1H and ^{13}C NMR spectra were recorded on JEOL GSX-400 (400 MHz) and JEOL GX-270 (67.5 MHz) spectrometers, respectively. The other ^1H NMR spectra were taken on JEOL GX-270 (270 MHz) and/or GSX-500 (500 MHz) spectrometers. All chemical shifts were measured relative to internal tetramethylsilane. The melting point was determined on a hot stage with a microscope and is uncorrected.

Column chromatography was carried out on silica gel 60 (E. Merck, 70–230 mesh).

trans-5-tert-Butyl-2-methylenecyclohexanol. To a solution of 4-tert-butylmethylenecyclohexane (3.7 g, 0.243 mmol) were added SeO_2 (134 mg, 5 mol%) and a solution of tert-butylhydroperoxide in 2,2,4-trimethylpentane (8.1 ml, 1.0 eq). The mixture was stirred overnight at room temperature and a saturated solution of Na_2SO_3 solution was added. After 1 h of stirring, the organic phase was separated and an aqueous layer was extracted with ether. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated to give a pale yellow oil. This oil was purified by column chromatography on silica gel (eluted with hexane–ether, 5 : 1) to give the title product as an oil (3.5 g, 86%). This was kept in a refrigerator to crystallize into colorless needles; mp 45–48°C; ^1H NMR ($CDCl_3$) δ; 0.85 (9H, s, C–CH_3), 4.35 (1H, br t, $J = 2.7$ Hz), 4.73 (1H, t, $J = 2.1$ Hz, exomethylene), 4.82 (1H, t, $J = 1.9$ Hz, exomethylene). *Anal.* Calcd for $C_{11}H_{20}O$: C, 78.51; H, 11.98. Found: C, 78.29; H, 11.86.

cis-5-tert-Butyl-2-methylenecyclohexanol. Pyridinium chlorochromate (2.6 g, 11.8 mmol) was added to a mixture of *trans*-5-tert-butyl-2-methylenecyclohexanol (1.0 g, 5.95 mmol) and powdered molecular sieves 3A (5.0 g) in dry CH_2Cl_2 (30 ml). After 1 h of stirring, the mixture was diluted with a 5 times volume of ether and passed through a column of Florisil (25 g). After removal of the solvent, the oily residue was dissolved in THF (10 ml) and cooled to 0°C. $LiAlH_4$ (226 mg, 5.95 mmol) was added. The mixture was stirred for 1 h at the same temperature. Water was carefully added to destroy the excess reagent and the mixture was extracted with ether. The organic phase was washed with an aqueous 10% solution of tartaric acid, saturated aqueous $NaHCO_3$ solution, and brine, successively, dried over Na_2SO_4, filtered, and evaporated. The oily residue was purified by column chromatography eluting with hexane–ether (5 : 1) as eluant to give the title product (223 mg, 22%) as a colorless oil. ^1H NMR ($CDCl_3$) δ; 0.86 (9H, s, C–CH_3), 4.04 (1H, br dd, $J = 10.5$ and 4.2 Hz), 4.75 (1H, q, $J = 1.7$ Hz, exomethylene), 4.90 (1H, q, $J = 1.5$ Hz, exomethylene). *Anal.* Calcd for $C_{11}H_{20}O$: C, 78.51; H, 11.98. Found: C, 78.23; H, 11.79. Further elution of hexane–ether (5 : 1) gave 4-tert-butyl-1-cyclohexenylmethanol (479 mg, 48%) as a slightly yellow oil. ^1H NMR ($CDCl_3$) δ; 0.85 (9H, s, C–CH_3), 4.00 (2H, br s, –CH_2OH), 5.65 (1H, m, olefinic).

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REFERENCES

1. DeLuca, H. F., and Schnoes, H. K. (1985) *Annu. Rev. Biochem.* **52,** 411.
2. Norman, A. W., and Wong, R. G. (1972) *J. Nutr.* **102,** 1709.
3. O'Malley, B. W., McGuire, W. L., Kohler, P. O., and Korenman, S. G. (1969) *Recent Prog. Horm. Res.* **25,** 105.

4. Baker, A. W., McDonnel, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., and O'Malley, B. W. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3294.
5. Trinh, T., DeLuca, H. F., and Dahl, L. F. (1976) *J. Org. Chem.* **41,** 3476.
6. Trinh, T., Ryan, R. C., Simon, G. L., Calabrtese, J. C., Dahl, L. F., and DeLuca, H. F. (1977) *J. Chem. Soc. Perkin* 2, 393.
7. La Mar, G. N., and Budd, D. L. (1974) *J. Amer. Chem. Soc.* **96,** 7317.
8. Wing, R. M., Okamura, W. H., Pirio, M. R., Sine, S. M., and Norman, A. W. (1974) *Science* **186,** 939.
9. Okamura, W. H., Norman, A. W., and Wing, R. M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4194.
10. Wing, R. M., Okamura, W. H., Rego, A., Pirio, M. R., and Norman, A. W. (1975) *J. Amer. Chem. Soc.* **97,** 4980.
11. Sheves, M., Berman, E., Freeman, D., and Mazur, Y. (1975) *J. Chem. Soc. Chem. Commun.*, 643.
12. Sheves, M., and Mazur, Y. (1977) *J. Chem. Soc. Chem. Commun.*, 21.
13. Okamura, W. H., Hammond, M. L., Rego, A., Norman, A. W., and Wing, R. M. (1977) *J. Org. Chem.* **42,** 2284.
14. Berman, E., Luz, Z., Mazur, Y., and Sheves, M. (1977) *J. Org. Chem.* **42,** 3325.
15. Sheves, M., Fridman, N., and Mazur, Y. (1977) *J. Org. Chem.* **42,** 3597.
16. Paoren, H. E., Schnoes, H. K., and DeLuca, H. F. (1977) *J. Chem. Soc. Chem. Commun.*, 890.
17. Mourino, A., Lewicka-Piekut, S., Norman, A. W., and Okamura, W. H. (1980) *J. Org. Chem.* **45,** 4015.
18. Helmer, B., Schnoes, H. K., and DeLuca, H. F. (1985) *Arch. Biochem. Biophys.* **241,** 608.
19. Franklin, N. C., and Feltkamp, H. (1969) *Angew. Chem. Int. Ed. Engl.* **4,** 774.
20. Klein, J., and Levene, R. (1969) *Tetrahedron Lett.*, 4833.
21. Chamberlain, P., and Whitham, G. H. (1970) *J. Chem. Soc. B*, 1382.
22. Breitmaier, E., and Voelter, W. (1987) Carbon-13 NMR Spectroscopy, pp. 127, VCH Publishing, New York.
23. Kessler, H. (1970) *Angew. Chem. Int. Ed. Engl.* **9,** 219.
24. Helmer *et al.* also observed the same solvent effect (see Ref. (*18*)), but they have not discussed this phenomenon.
25. Haasnoot, C. A. G., de Leeuw, F. A. M., and Altona, C. (1980) *Tetrahedron* **36,** 2783.
26. Ikekawa, N. (1987) *Med. Res. Rev.* **7,** 333.
27. Eguchi, T., Yoshida, M., and Ikekawa, N. *Bioorg. Chem.* **17,** 294–307.