Fluorinated Vitamin D Analogs to Probe the Conformation of Vitamin D in Its Receptor Complex: ^{19}F-NMR Studies and Biological Activity

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To investigate vitamin D conformation, specifically the A-ring and seco-B ring parts, in its complex with the vitamin D receptor (VDR), we have previously suggested the use of ^{19}F-NMR spectroscopy and have synthesized three fluorovitamin D derivatives to be used for the study, 4,4-difluoro-1α,25-dihydroxyvitamin D_3 [4,4-F_2-1,25-$(OH)_2D_3$, 2a], and (10*Z*)- and (10*E*)-19-fluoro-1α,25-dihydroxyvitamin D_3 [19-F-1,25-$(OH)_2D_3$, 3a, 4a]. In this paper, we examined the ^{19}F-NMR spectra of these and related fluorovitamin D compounds in detail. In the low temperature ^{19}F-NMR spectra, we observed two well-separated rigid conformations of 2a (51 : 49) and 4a (84 : 16), while only one conformation was detected with 3a. The two observed conformers of 2a and 4a were respectively assigned to the known α- and β-conformers formed by the flipping of the A-ring where the C(19) exocyclic methylene points to the α- and β-faces. The single conformation observed for 3a was assigned to the α-conformer. We detected no other conformation in the ^{19}F-NMR of all vitamin D compounds examined. The effect of solvents on the ^{19}F chemical shifts of fluorovitamin D compounds was found to be small (less than 6.3 ppm). This was much smaller than the difference between the two A-ring conformers (13—30 ppm). Using the dynamic ^{1}H-NMR studies of fluorovitamin D compounds, we determined the free energy of activation for the interconversion between the two conformations of 2a (9.9 kcal/mol) and 4a (10.8, 11.5 kcal/mol). Introduction of the 1α-hydroxyl group raised the activation energy about 1 kcal/mol. The affinity for VDR was evaluated, and the relative potency of 2a, 3a and 4a was found to be 1%, 8% and 9%, respectively, of that of 1,25-$(OH)_2D_3$ (1). Though the affinity for VDR was considerably reduced in these compounds, the ability to activate gene transcription was similar and remained in the range 30—50% of the effect of 1. This biological information in combination with the NMR properties indicates that 2a and 4a are promising probes for studying the VDR-bound A-ring conformation of vitamin D.

Key words fluorinated vitamin D; conformation analysis; vitamin D receptor; ^{19}F-NMR

The active vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$, **1**] (Fig. 1) is a multi-functional hormone. In addition to its central action in regulating calcium and phosphorus homeostasis, **1** plays a role in controlling growth and differentiation of cells and in immuno-modulation.[1] 1,25-$(OH)_2D_3$ has been widely used as a potential therapeutic agent in the treatment of bone metabolic diseases and the skin disorder psoriasis. However, the use of **1** is limited because a high dose causes critical hypercalcemia. Vitamin D analogs which have a selective activity profile, such as a high cell differentiating potential with little calcemic activity, have been developed by structural modification of the parent 1,25-$(OH)_2D_3$.[2]

1,25-$(OH)_2D_3$ exerts most of its effects *via* the vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily.[3] Binding of **1** to VDR induces a conformational change in the ligand-binding domain (LBD). VDR binds as a heterodimer with the retinoid X receptor (RXR)[4] to vitamin D response elements (VDREs), which are located in the promoter regions of 1,25-$(OH)_2D_3$ target genes. It is now clear that binding of a coactivator to this heterodimer is critical for the transactivation of target genes.

To understand, on a molecular basis, the mode of action of the native hormone at the transcription level, it is essential to determine in detail the three-dimensional structure of liganded VDR and its dynamic properties in solution. 1,25-$(OH)_2D_3$ can be conveniently divided into four parts with unique structural characteristics, the A-ring, the seco-B-ring, the C/D-ring, and the side chain. By our initial study on the conformation-function relationship of vitamin D employing conformationally restricted side chain analogs as tools, we have proposed a concept of the active space region of the side chain.[5—8] Our interest is now extended to the A-ring and the seco-B-ring triene parts: the geometry of the A-ring is important for vitamin D to bind to the VDR, and the conjugated triene has unique structural and electronic features compared with other steroid hormones. The A-ring is known to exist as a roughly equal population of two conformations in solution, which correspond to the two chair forms of the α- (1α-OH in axial and 3β-OH in equatorial orientation) and

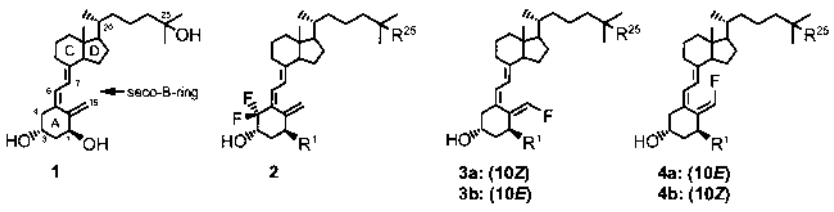

Fig. 1. The Structure of the Active Vitamin D_3 and Its Fluorovitamin D Analogs

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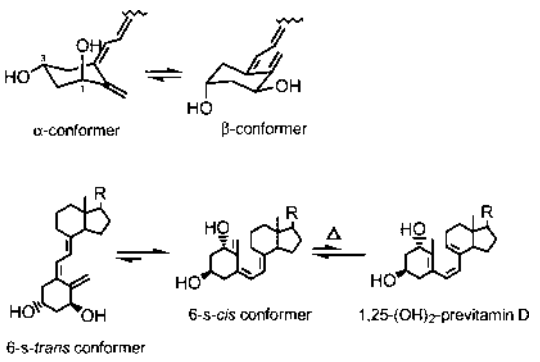

Fig. 2. Dynamic Behavior of the A-Ring and Seco-B-Ring of 1α,25-Dihydroxyvitamin D_3

β-conformers (1α-OH in equatorial and 3β-OH in axial position), by ^{1}H-NMR studies (Fig. 2).[9] These two conformers have also been found in the crystal structures of vitamin D: D_2 (both α- and β-forms),[10] D_3 (both α- and β-forms),[11] 25-hydroxyvitamin D_3 (α-form),[12] and 1,25-$(OH)_2D_3$ (β-form).[13] At physiological temperature, the C(6)–C(7) bond in 1,25-$(OH)_2D_3$ can rotate to yield two conformations, the 6-s-*trans* and 6-s-*cis* conformers, through which slow chemical isomerism of a [1,7]-sigmatropic hydrogen shift occurs to give 1,25-dihydroxyprevitamin D_3 (Fig. 2).[9] However, the 6-s-*cis* conformer has not been observed spectroscopically. There are reports suggesting that 1,25-$(OH)_2D_3$ can also generate biological responses *via* nongenomic pathways by binding to a membrane vitamin D receptor (m-VDR).[14] In this process, 1,25-$(OH)_2D_3$ is suggested to bind to the m-VDR in the 6-s-*cis* conformation.[15]

Since the mid 1990s, X-ray crystal structures of many nuclear receptor-LBDs complexed with the cognate ligand, such as RAR (retinoic acid receptor),[16,17] TR (thyroid hormone receptor),[18,19] ER (estrogen receptor),[20—23] PR (progesterone receptor),[24] and peroxisome proliferator-activated receptor (PPAR),[25,26] have been reported. However, the crystal structure of the wild-type VDR has not been solved so far. Recently, the crystal structure of a deletion mutant of VDR-LBD (human VDR 118—425 Δ165—215) complexed with the natural ligand (**1**) has been reported.[27] In this crystal structure, the A-ring of the natural ligand (**1**) adopts the β-conformer, which coincides with the crystal structure of 1,25-$(OH)_2D_3$. The conjugated triene is in the 6-s-*trans* conformation, its torsion angle being 30°. Four laboratories including ours proposed a three-dimensional model of VDR-LBD complexed with 1,25-$(OH)_2D_3$ constructed by homology modeling techniques.[28—31] Among the four, only our model which was constructed using human RARγ as a template and substantiated by mutational analysis accurately predicted the orientation of the ligand in the ligand binding pocket (LBP) and its hydrogen bond partners in the LBP.

To better understand the structure and dynamics of the A-ring and seco-B-ring of vitamin D complexed with VDR, we proposed the use of ^{19}F-NMR spectroscopy. The fluorine nucleus has properties that make its use in these studies attractive.[32] Substitution of hydrogen for fluorine is not expected to have a large steric perturbation because the van der Waals radius of the fluorine atom (1.47 Å) is very similar to that of hydrogen (1.20 Å), and the C–F bond length (1.26—1.41 Å) is not much greater than the C–H bond length (1.08—1.11 Å). The ^{19}F chemical shift range is much larger than that of a proton, and the chemical shift is sensitive to changes in the local van der Waals and electrostatic interactions. The most important feature for studying biological systems by ^{19}F-NMR is that there is no fluorine background signal to interfere with the objectives. For this purpose, we synthesized fluorinated vitamin D analogs as probe compounds: 4,4-Difluoro-1α,25-dihydroxyvitamin D_3 [4,4-F_2-1,25-$(OH)_2D_3$, **2a**][33,34] and two geometrical isomers of 19-fluoro-1α,25-dihydroxyvitamin D_3 **3a** [(10*Z*)-19-F-1,25-$(OH)_2D_3$] and **4a** [(10*E*)-19-F-1,25-$(OH)_2D_3$].[35—37] The syntheses of these compounds have been reported in the previous papers.[33—37]

In this paper, we investigated in detail the static and dynamic properties of the fluorovitamin D derivatives (**2a**, **3a**, **4a**) by ^{19}F- and ^{1}H-NMR spectroscopy. The results in combination with their potency in binding to VDR suggest that the fluorovitamin D compounds **2a** and **4a** are appropriate compounds to probe the conformation of vitamin D in its VDR complex.

^{19}F-NMR Studies of Fluorovitamin D Derivatives We studied the static and dynamic ^{19}F-NMR of fluorovitamin D derivatives **2**—**4** in detail, to investigate whether 4,4-F_2-1,25-$(OH)_2D_3$ or 19-F-1,25-$(OH)_2D_3$ can be a useful tool to probe the A-ring and/or the seco-B-ring conformation in the vitamin D-receptor complex.

4,4-Difluoro-1,25-dihydroxyvitamin D_3 (2a) The ^{19}F-NMR spectra of 4,4,-difluorovitamin D (**2a**) (in CD_2Cl_2/CD_3OD, 2 : 1) at room temperature showed the two signals of the geminal fluorine atoms as a doublet pair (Fig. 3A, Table 1). On cooling, the two signals were broadened gradually (0—−40 °C) and then separated into clear signals of two sets of doublet pairs at −80—−95 °C (Fig. 3A): The doublet at δ −115.1 (F-4β) at 25 °C was split into a pair of doublets at δ −108.2 (J=229 Hz) and −120.2 (J=241 Hz) in a 51 : 49 ratio, and the doublet at δ −104.5 (F-4α) into a pair of doublets at δ −119.9 (J=229 Hz) and −92.0 (J=241 Hz) (51 : 49). Similarly, in the spectrum of **2b**, which lacks the 1α-hydroxyl group, the signals at δ −113.6 (F-4β) and −109.5 (F-4α) at 25 °C were separated into two sets of doublet pairs at δ −108.1 and −119.8 (ratio, 79 : 21) and at δ −121.2 and −92.9 (79 : 21), respectively (Fig. 3D). These results indicate that both **2a** and **2b** are in a rapid equilibrium between two conformations giving rise to an average spectrum of the two conformers at room temperature, but at −80—−95 °C, because of their slow exchange rate, these two conformers were observed separately. The two conformers were assigned to the well-known α- and β-forms of the vitamin D A-ring conformations on the basis of their ^{1}H-NMR spectra. The ^{1}H-NMR spectrum of **2a** below −75 °C was nearly the same as the spectrum at 25 °C except that the signals of H-6 and H-18 were separated from the twin peaks at −75 °C: H-6, δ 6.97 (d, J=11.4 Hz) at 25 °C into two broad peaks at δ 6.83 and 6.96 (about 1 : 1) below −75 °C; H-18, δ 0.53 (s) at 25 °C to two singlets at δ 0.35 and 0.38 (about 1 : 1) (Fig. 5A). In the ^{1}H-NMR spectrum of **2b** at −95 °C, the two conformers were separately observed in the signals of H-3 (δ 3.72, 3.90), the ratio (79 : 21) being in accord with that observed in the ^{19}F-NMR spectrum. In the spectrum of **2b**, we assigned the larger signal appearing at the higher field to the axial H-3 in the α-form and the smaller at the lower field to the equatorial H-3 in the β-form.

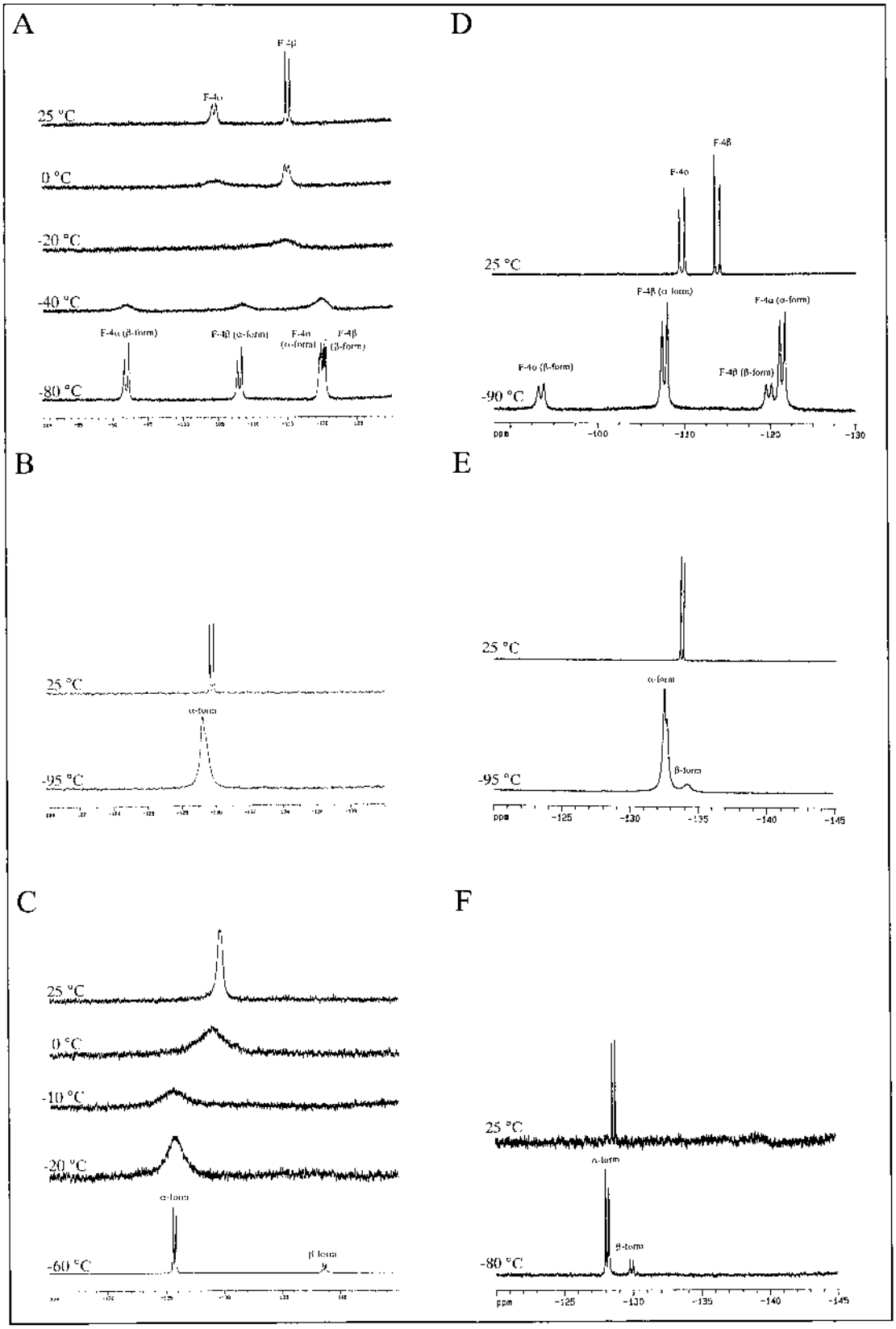

Fig. 3. Variable Temperature ^{19}F-NMR Spectra of Fluorovitamin D_3 Analogs **2a** (A), **3a** (B), **4a** (C), **2b** (D), **3b** (E) and **4b** (F) in CD_2Cl_2–CD_3OD (2 : 1)

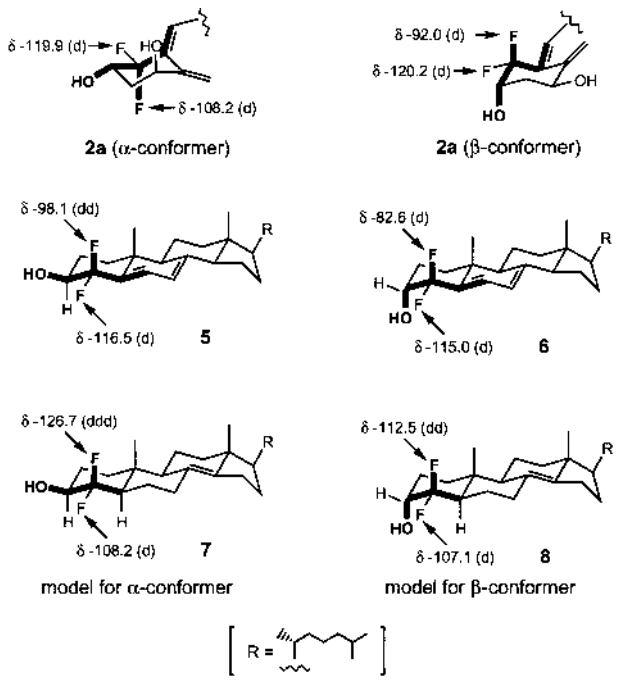

Fig. 4. ^{19}F Signal Assignment of the Two Conformers of 4,4-Difluoro-1α,25-dihydroxyvitamin D_3 and Model Steroids

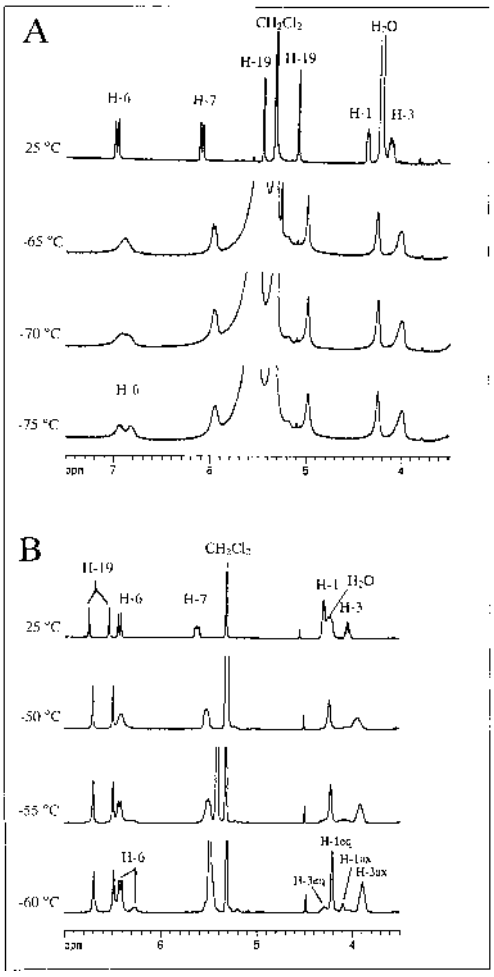

Fig. 5. Temperature Dependence of ^{1}H-NMR Spectra of **2a** (A) and **4a** (B) in CD_2Cl_2–CD_3OD (2 : 1)

It is generally accepted that the signal of an axial proton on a cyclohexane ring appears at a higher field than the corresponding signal of an equatorial proton.[38]

Thus we were able to detect the two conformers of the vitamin D A-ring as definitely resolved clear signals using the 4,4-difluorovitamin D derivative **2a** as an NMR probe. The results of this ^{19}F-NMR study definitely showed that the A ring of the vitamin D derivative (**2a**) adopts just two conformations: No other conformations whose contributions were assumed by computational studies[39] were observed in this study. The detection of the two A-ring conformations of vitamin D has been rather difficult by ^{1}H-NMR spectroscopy: Only Eguchi and Ikekawa have successfully observed the two A-ring conformers of 1,25-$(OH)_2D_3$ as a pair of broad signals of H-3.[40] In most cases, the dynamics between the two A-ring conformers were analyzed by the coupling constants of the H-3α. Thus, we showed 4,4-difluoro-1,25-$(OH)_2D_3$ **2a** to be a potential probe compound to monitor the VDR-bound conformation: If **2a** is bound to VDR adopting the α-form, the two fluorine peaks with about 12 ppm chemical shift difference would be observed, and if it bound with its β-form, a twin peak with a much larger chemical shift difference (28 ppm) would be detected.

The fluorine signals in compound **2a** were assigned using the conformationally rigid fluorosteroids **5**—**8** as models. The two $\Delta^{8,14}$-steroid derivatives **7** and **8** were prepared by catalytic reduction of **5** and **6**, respectively. The geminal fluorine atoms couple with each other with a large coupling constant (J=237—252 Hz) and with the protons (J=15—29 Hz) on the carbons adjacent to the fluorine bearing carbon, C(3) and C(5), when the coupling partners occupy an antiparallel position (Table 1). Based on these criteria, the fluorine signals of **5**, **7** and **8** were unambiguously assigned as shown in Table 1 and Fig. 4. In the ^{19}F-NMR of **6**, which has no proton antiparallel to the geminal fluorine atoms, the lower field signal at δ −82.6 (d) was assigned to the axial F-4β in analogy with **5**. In summarizing the ^{19}F signal data of the model compounds **5** and **6**, the following chemical shift order (from lower to higher) was found: (1) axial fluorine with an antiparallel α-hydroxyl group and a parallel π-bond orbital suffers the least shielding and appears at the lowest field (δ −82.6 ppm), (2) axial fluorine with a *cis*-α-hydroxyl and a parallel π-bond orbital experiences the next lowest shielding and appears at the next lower field (δ −98.1 ppm), and (3) equatorial fluorine with a *cis*-α-hydroxyl and an orthogonal π-bond orbital and equatorial fluorine with a *trans*-α-hydroxyl and an orthogonal π-bond orbital appear at the highest field (δ −115.0—−116.5 ppm). With these spectral data in hand, we assigned the ^{19}F signals of the two conformations of the 4,4-fluorovitamin D (**2a**, **b**) as shown in Fig. 4 and Table 1. Thus, in the β-form of **2a**, the signal at the lower field was assigned to the axial F-4α, and in the α-form of **2a**, the signal at the lower field was assigned to the axial F-4β.

From these ^{19}F-NMR studies, we noticed that (1) an axial fluorine resonates at a higher field than the equatorial counterpart; (2) a hydroxyl group with an antiparallel orientation has a deshielding effect of about 14 ppm; (3) an adjacent π-orbital parallel to the axial fluorine has a deshielding effect of about 30 ppm; (4) a vicinal coupling between a fluorine and a proton in an antiparallel relationship is sensitive to the changes in the torsional angle between them: we did not ob-

Table 1. ^{19}F-NMR Spectra of 4,4-Difluorovitamin D (**2**) and Model Steroids (**5**—**8**)

Compd.	Temp. (°C)	F-4α δ ppm (splitting, Hz)	F-4β δ ppm (splitting, Hz)	A-Ring conformation (Ratio)	$\Delta\delta$ ppm (F-4α−F-4β)
2a	25[a]	−104.5 (br d, J=235)	−115.1 (d, J=235)	$\alpha+\beta$ (50.6 : 49.4)[c]	10.6
	−80[a]	−119.9 (d, J=229)	−108.2 (d, J=229)	]α (51) : β (49)	−11.7
		− 92.0 (d, J=241)	−120.2 (d, J=241)		28.2
	$\Delta\delta$ ppm[d] ($\alpha-\beta$)	− 27.9	12		
2b	25[a]	−109.5 (br d, J=232)	−113.6 (dd, J=232, 12)	$\alpha+\beta$ (69 : 31)[c]	4.1
	−90[a]	−121.2 (d, J=226)	−108.1 (d, J=226)	]α (79) : β (21)	−13.1
		− 92.9 (d, J=236)	−119.8 (d, J=236)		26.9
	$\Delta\delta$ ppm[d] ($\alpha-\beta$)	− 28.3	11.7		
5	25[b]	−116.5 (d, J=244)	− 98.1 (dd, J=244, 15)		
6	25[b]	−115.0 (d, J=260)	− 82.6 (d, J=260)		
7	25[b]	−108.2 (d, J=237)	−126.7 (ddd, J=237, 28, 21)		
8	25[b]	−107.0 (d, J=252)	−112.5 (dd, J=252, 29)		

^{19}F-NMR spectra were recorded *a*) in CD_2Cl_2–CD_3OD (2 : 1) or *b*) in $CDCl_3$. *c*) Conformer ratio calculation based on that observed at −80—−90 °C. *d*) Chemical shift differences estimated from the two fluorine peaks, F-4α (α-form)−F-4α (β-form) and F-4β (α-form)−F-4β (β-form) at −80—−90 °C.

Table 2. ^{19}F-NMR Data for 19-Fluorinated Vitamin D_3[a]

Compd.	Temp. (°C)	F-19 δ (splitting, Hz)	A-Ring conformation
3a	25	−129.8 (d, J=87)	α
	−95	−129.3 (br s)	α
3b	25	−133.8 (d, J=88)	$\alpha+\beta$ (78 : 22)[b]
	−95	−132.6 (d, J=79)	]α (88) : β (12)
		−134.2 (br s)	
	$\Delta\delta$ ppm ($\alpha-\beta$)	1.6	
4a	25	−129.6 (br s)	$\alpha+\beta$ (77 : 23)[b]
	−60	−125.8 (d, J=83)	]α (84) : β (16)
		−138.7 (d, J=81)	
	$\Delta\delta$ ppm ($\alpha-\beta$)	12.9	
4b	25	−128.8 (d, J=86)	$\alpha+\beta$ (77 : 23)[b]
	−80	−128.1 (d, J=86)	]α (87) : β (13)
		−129.9 (d, J=89)	
	$\Delta\delta$ ppm ($\alpha-\beta$)	1.8	

a) ^{19}F-NMR spectra were recorded in CD_2Cl_2–CD_3OD (2 : 1). *b*) Conformer ratio calculation based on that observed at −60—−95 °C.

serve this type of vicinal coupling in all the conformers of the fluorovitamin D compounds, though these couplings were observed in the model fluorosteroid derivatives. This indicates that the A-ring of **2a** adopts a conformation somewhat distorted from the normal chair conformation.

(10*Z*)- and (10*E*)-19-Fluoro-1,25-dihydroxyvitamin D_3 (3a, 4a) The ^{19}F-NMR spectrum of 19-fluorovitamin D **3a** displayed a single doublet at δ −129.8 at 25 °C (Fig. 3B, Table 2). On cooling, the signal became broadened but no separated signals were observed down to −95 °C. On the other hand, in the spectrum of **3b**, a single doublet observed at 25 °C was split at −95 °C into two broad peaks (δ −134.2 and −132.6, 12 : 88) with a small chemical shift difference (1.6 ppm) (Fig. 3E). The single conformer of **3a** and the major conformer of **3b** were assigned to the α-form based on their ^{1}H-NMR. The ^{1}H-NMR spectra of **3a** and **3b** at −95 °C are nearly the same as those recorded at 25 °C, and no separation of signal due to conformation freezing was observed (data not shown). Thus the (10*Z*)-19-fluoro-1,25-$(OH)_2D_3$ **3a** was shown not to be an appropriate probe for studying A-ring dynamics.

The ^{19}F-NMR spectra of **4a** showed a broad singlet (δ −129.6) at 25 °C, and at −60 °C the signal was separated into two sharp doublets (δ −138.7 and −125.8, 16 : 84) with a big chemical shift difference (12.9 ppm) (Fig. 3C). It is also likely that the exchange rate of the two conformers of **4a** is already slow at 25 °C. Homolog **4b** without the 1α-hydroxyl group showed a sharp doublet at 25 °C, which was separated at −80 °C into two sharp doublets (δ −129.9 and −128.1, 13 : 87) with a chemical shift difference of only 1.8 ppm (Fig. 3F). Clearly, the introduction of a 1α-hydroxyl group yields a large chemical shift difference between the two conformers of **4a**.

Temperature-dependent changes in the ^{1}H-NMR spectra of **4a** were also examined (Fig. 5B). Each signal of H-1β, H-3α and H-6 distinctly separated into two signals due to the two rigid conformers at −60 °C, each with a population ratio (16 : 84) similar to that observed in their low temperature ^{19}F-NMR spectrum. The fact that the coupling constant of H-6 (J=11 Hz) at this temperature is identical with that observed at room temperature indicates that the stereochemistry around the 6,7-bond did not significantly change in the two rigid conformers. A pair of signals for H-3α was detected at δ 3.90 and 4.30 (84 : 16), the former signal having a wider half-width (W/2=25 Hz) than the latter. A set of H-1β signals was observed at δ 4.10 and 4.22 (16 : 84), and the latter major signal has a narrower W/2 (10 Hz) than the former. These features indicate that, in the major conformer, H-1β and H-3α adopt an equatorial and axial orientation, respectively; namely, the major conformer adopts the α-form and the minor adopts the β-form. The fluorine chemical shift difference between the α- and β-conformers is large (about 13 ppm) in **4a**. In **4a**, the α-form predominates ($\Delta G°$=0.702 kcal/mol) over the β-form, probably because the hydrogen at C-19 produces steric congestion with the 1α-OH group in the β-form.

Like **4a**, the larger signals in **4b** can be assigned to the α-conformer. The population ratio of the A-ring conformers in **3b**, **4a** and **4b** was also calculated based on the coupling constants observed in the H-3α and H-4β signals ($J_{\text{H-3}\alpha,\ \text{H-4}\beta}$) in the ^{1}H-NMR spectra at 25 °C: The α- and β-conformer ratios of **3b**, **4a** and **4b** were determined to be 76 : 24, 74 : 26 and 74 : 26, respectively (standard coupling constant used for the calculation: $J_{\text{axial, axial}}$=11.1 Hz and $J_{\text{equatorial, axial}}$=2.7 Hz).

Table 3. Solvent Effect on ^{19}F Chemical Shift of **2a**

Solvent	Temp. (°C)	F-4α δ ppm	F-4β δ ppm	A-Ring conformation	$\Delta\delta$ ppm[a] F-4α	$\Delta\delta$ ppm[a] F-4β
CD_2Cl_2/CD_3OD 2 : 1	25	−104.5	−115.1	$\alpha+\beta$ (51 : 49)[e]		
	−80	−119.9	−108.2	α		
		−92.0	−120.2	β		
THF-d_8	25	−102.4	−119.3	$\alpha+\beta$ (34 : 66)[e]	+2.1	−4.2
	−80	−122.2	−110.9	α	−2.3	−2.7
		−94.0	−122.2	β	−2.0	−2.0
$CDCl_3$	25	−107.6	−116.3	ND	−3.1	−1.2
CD_3OD	25	−107.4	−117.7	$\alpha+\beta$ (54 : 46)[e]	−2.9	−2.6
	−80	−122.8	−111.8	α	−2.9; −0.6[b]	−3.6; −0.9[b]
		−94.7	−122.7	β	−2.7; −0.7[b]	−2.5; −0.5[b]
Buffer[f]	25	−103.2	−115.0	ND	+1.3; −0.8[b] +4.4[c]; +4.2[d]	+0.1; +4.3[b] +1.3[c]; +2.7[d]

Chemical shift difference: *a*) (δ in the respective solvent)−(δ in CD_2Cl_2/CD_3OD); *b*) (δ in the respective solvent)−(δ in THF-d_8); *c*) (δ in the respective solvent)−(δ in $CDCl_3$); *d*) (δ in the respective solvent)−(δ in CD_3OD). *e*) Conformer ratio calculation based on that observed at −80 °C in the same solvent (ND, not determined). *f*) Phosphate buffer (20 mM NaH_2PO_4, 100 mM NaCl, 10% D_2O) containing 3 eq of TMβCD as a solubilizing agent.

These conformer ratios are in good agreement with those calculated from the signal ratio at the low temperature ^{19}F-NMR; 78 : 22 for **3b**, 77 : 23 for **4a** and 77 : 23 for **4b**.

Again, only two conformers, which were assigned to the α- and β-conformations of the A-ring, were observed in the 19-fluorovitamin D analogs **3** and **4** by the ^{19}F-NMR study. In the 19-fluorovitamins, however, the α-conformer was greatly preferred. Based on these NMR studies, (10*E*)-19-fluorovitamin D **4a** was shown to be a useful probe for studying the A-ring dynamics, because **4a** provides a set of fluorine signals due only to the α- and β-conformers, and the chemical shift difference between the two conformers is large (about 13 ppm).

Effect of Solvents on the Chemical Shifts and Conformational Proportion Our purpose was to investigate the dynamics of vitamin D conformation complexed with VDR by analyzing the ^{19}F-NMR spectra of the fluorovitamin D derivatives described in this paper. As the present results show, we can use only ^{19}F chemical shifts to monitor the A-ring conformation. So we need to determine how the ^{19}F chemical shift of the probe compounds change when the chemical environment changes. Also to record NMR spectra of a complex of fluorovitamin D with VDR, it must be dissolved in phosphate buffer at high concentration (1—2 mM). Therefore, it is also necessary to select the proper conditions for recording **2a** and **4a** in phosphate buffer. For this purpose, we examined the ^{19}F-NMR spectra of fluorovitamin D derivatives **2a**, **3a** and **4a** in THF-d_8, $CDCl_3$, CD_3OD, and phosphate buffer.

To dissolve the fluorinated vitamin D **2a** and **4a** in phosphate buffer, addition of solubilizing agents is necessary. We examined ethanol (5%), polyethylene glycol (1—2%), polyvinyl alcohol (1—2%), gelatin (0.01%) and sodium lauryl sulfate (SDS, 2 eq) as additives. Of these, only SDS was effective in obtaining a clear solution and satisfactory ^{19}F-NMR spectra. The ^{13}C-NMR spectrum of [^{13}C]-labeled 25-hydroxyvitamin D_3 recorded in buffer solution in the presence of SDS has been reported.[41] We also examined cyclodextrins (CDs) as the solubilizing agent, because CDs have been known to form a stable inclusion complex with vitamin D_3 and its metabolites. CDs have also been successfully used in the microbial transformation of vitamin D_3 to 25-OHD_3 and 1,25-$(OH)_2D_3$.[42] We tested several CDs and found 2,3,6-tri-*O*-methyl-β-CD (TMβCD) to be the best for both fluorovitamin D derivatives tested (**2a**, **4a**). In the presence of 3 equivalents of TMβCD, we obtained a clear 1 mM solution of the fluorovitamin D derivatives, and the ^{19}F-NMR spectra were even better than that obtained using SDS. Though we did not investigate the inclusion mode of our compounds in TMβCD, it has been reported in the studies of the complex of vitamin D_3 with β-CD that both the A-ring and the side chain parts are included in the cavity of β-CD.[43]

Table 4. Solvent Effect on ^{19}F Chemical Shift of **4a** and **3a**

Compd.	Solvent	Temp. (°C)	F-19 δ ppm	A-Ring conformation	$\Delta\delta$ ppm[a]
4a	CD_2Cl_2/CD_3OD 2 : 1	25	−129.6	$\alpha+\beta$ (77 : 23)[e]	
		−60	−125.8	α	
			−138.7	β	
	THF-d_8	25	−132.4	$\alpha+\beta$ (69 : 31)[e]	−2.8
		−80	−127.0	α	−1.2
			−139.3	β	−0.6
	$CDCl_3$	25	−128.0	$\alpha+\beta$ (71 : 29)[f]	+1.6
	CD_3OD	25	−129.4	$\alpha+\beta$ (77 : 23)[e]	+0.2
		−60	−125.5	α	+0.3; +1.5[b]
			−138.4	β	+0.3; +0.9[b]
	Buffer[f,g]	25	−131.0	ND	−1.4; +1.4[b] −3.0[c]; −1.6[d]
3a	THF	25	−133.7	ND	
	$CDCl_3$	25	−129.6	ND	
	CD_3OD	25	−132.8	ND	
	Buffer[f,g]	25	−129.6	ND	

Chemical shift difference: *a*) (δ in the respective solvent)−(δ in CD_2Cl_2/CD_3OD); *b*) (δ in the respective solvent)−(δ in THF-d_8); *c*) (δ in the respective solvent)−(δ in $CDCl_3$); *d*) (δ in the respective solvent)−(δ in CD_3OD). *e*) Conformer ratio calculation based on the coupling constants of H-3 in its ^{1}H-NMR spectrum at 25 °C solvent (ND, not determined). *f*) Phosphate buffer (20 mM NaH_2PO_4, 100 mM NaCl, 10% D_2O) containing 3 eq of TMβCD as a solubilizing agent. *g*) ^{19}F Chemical shift in the same phosphate buffer containing 2 eq of SDS: **4a**, −125.7 ppm; **3a**, −129.9 ppm.

As Tables 3 and 4 show, the ^{19}F chemical shifts do not significantly change on changing the solvent nature: With pure conformers of **2a** and **4a**, the solvent effect on the ^{19}F chemical shift was at maximum 0.9 and 1.5 ppm, respectively,

when the solvent was switched from THF to CH_3OH. These values are much smaller than the chemical shift difference between the different conformers (12—28 ppm in **2a** and 12.9 ppm in **4a**). Chemical shift differences observed in the averaged ^{19}F-NMR spectra recorded at 25 °C are still small ranging from +2.1 to −4.2 ppm for **2a** and +1.6 to −2.8 ppm for **4a** and are largely due to the changes in the conformer population. The solvent effect on the conformation ratio of the fluorovitamin D compounds in comparison with the natural hormone (**1**) is summarized in Table 5. The natural hormone (**1**) and the 4,4-difluorovitamin analog **2a** adopt the α- and β-conformers in approximately a 1 : 1 ratio in CH_3OH, but the population of the β-conformer considerably increased in THF. In all the 19-fluorovitamin D analogs **3** and **4**, the α-conformer predominates over the β-conformer: The α-conformer was the only one observed in (10*Z*)-isomer **3a**, and the α-conformer predominates (77—69%) in the (10*E*)-isomer **4a**.

Dynamic NMR Studies of Fluorovitamin D Analogs We investigated the kinetic properties of **2** and **4** by a variable temperature NMR technique. For this purpose, ^{1}H-NMR was more appropriate than ^{19}F-NMR, because in ^{19}F-NMR, the frequency difference between the two components is too large to determine the coalescence temperature (*Tc*), the temperature where the two peaks due to the two components coalesced to one. We used modified Eyring equations[44] (Eqs. 1, 2) to calculate the free energy of activation ($\Delta G^{\ddagger}$) for the interconversion between the α- and β-forms of the substrate:

$$\Delta G^{\ddagger}_{\alpha\beta} = 4.57Tc(10.62 + \log X/2\pi(1-\Delta P) + \log Tc/\Delta\nu) \quad \text{(cal/mol)} \qquad (1)$$

$$\Delta G^{\ddagger}_{\beta\alpha} = 4.57Tc(10.62 + \log X/2\pi(1+\Delta P) + \log Tc/\Delta\nu) \quad \text{(cal/mol)} \qquad (2)$$

where $\Delta G^{\ddagger}_{\alpha\beta}$ and $\Delta G^{\ddagger}_{\beta\alpha}$: Free energy of the activation for the interconversions of conformers α to β and β to α, respectively. *Tc*: Coalescence temperature; $\Delta\nu$: Chemical shift difference in Hz at *Tc*; ΔP: Population difference between the two conformers; *X* is value defined by $\Delta P = [(X^2-2)/3]^{3/2}/X$ and by $X = 2\pi\delta\nu\tau$ where τ is defined by $1/\tau = (1/\tau\alpha) + (1/\tau\beta)$.

Table 5. Solvent Effect in A-Ring Conformation

Compd.	Conformer ratio in each solvent			
	THF $\alpha:\beta$	$CDCl_3$ $\alpha:\beta$	CD_3OD $\alpha:\beta$	CD_2Cl_2–CD_3OD $\alpha:\beta$
1	38 : 62[a]	45 : 55[a]	46 : 54[a]	—
2a	34 : 66[b]	—	54 : 46[b]	51 : 49[b]
3a	100 : 0[a]	100 : 0[a]	100 : 0[a]	100 : 0[a]
3b				78 : 22[b]; 76 : 24[a]
4a	69 : 31[b]	71 : 29[b]	77 : 23[b]	77 : 23[b]; 74 : 26[a]
4b				77 : 23[b]; 74 : 26[a]

a) Conformer ratio calculation based on the coupling constants of H-3α at 25 °C. *b*) Conformer ratio at 25 °C calculation based on the peak area of ^{19}F signal at slow exchange of the conformers.

The results are summarized in Table 6. The activation energies for the interconversion of the α- and β-conformers of **2a** were 9.8—9.9 kcal/mol. This value is slightly (0.3—4 kcal/mol) larger than that of 1,25-$(OH)_2D_3$ (**1**) (9.5 kcal/mol)[40] and smaller by about 1 kcal/mol than that of 4,4-dimethyl-1α-OHD_3 (11.0 kcal/mol).[45] The activation energy of **2b** was smaller than that of **2a** by about 1.0 kcal/mol; namely, the 1α-hydroxyl group elevates the activation energy about 1.0 kcal/mol. The two conformers of 19-fluoro-1,25-$(OH)_2D_3$ (**4a**) have larger activation energies which are comparable to that of 4,4-dimethyl-1α-OHD_3. The difference in the activation energies between the two conformers of **4a** is 0.7 kcal/mol which corresponds to the difference in the free energy ($\Delta G°$) between the two conformers. Here again, the effect of the 1α-hydroxyl group on the energy of activation is 1.0 kcal/mol. The large increase in the $\Delta G^{\ddagger}$ in (10*E*)-19-fluorovitamin D **4a** is probably due to an unfavorable interaction between H-7 and F-19 at the transition state of the A-ring inversion.

Biological Evaluation of Fluorovitamin D Analogs (2a, 3a, 4a) We next evaluated the ability of the fluorovitamin D compounds (**2a**, **3a**, **4a**) to bind to the VDR and to activate gene transcription. Both are necessary to evaluate static and dynamic behavior of a ligand in binding to VDR. The affinity for VDR was determined by a competitive binding assay between radioactive 1,25-$(OH)_2D_3$ and the substrates. The 4,4-difluoro-analog **2a** was 100 times less active than the natural ligand. In general, substitution of a single fluorine for a hydrogen produces a small steric perturbation, but it is also known that replacement of both hydrogen atoms of a methylene group with two fluorine atoms causes a rather dramatic effect. The LBP of VDR is narrow at the place where the A-ring part is harbored: distances between C(4) and the two closest residues Cys288 and Ser278 are 3.618 Å (C–S distance) and 3.782 Å (C–O distance), respectively. Therefore, 4,4-difluorination of 1,25-$(OH)_2D_3$ might produce a severe crash: in a model where two hydrogen atoms on C(4) were simply replaced with two fluorine atoms, the distances between the fluorine atoms and Cys288 and Ser278 were 2.632 Å (F–S distance) and 2.862 Å (F–O distance), respectively, at the shortest. These unfavorable interactions may be the reason for the decreased affinity of **2a** for VDR. Both 19-fluorovitamins **3a** and **4a** have a significant affinity for VDR, about 1/10 that of the natural ligand. In the crystal structure of mutant VDR-LBD, 1,25-$(OH)_2D_3$ harbored in the LBP adopts the β-conformation at the A-ring. In the β-form,

Table 6. Free Energy of Activation during Interconversion between Two A-Ring Conformations

Compd.	Signal	*Tc* (K)	$\Delta\nu$ (Hz)	Population $\alpha:\beta$ ($\Delta G°_{Tc}$, kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol) Major conformer	$\Delta G^{\ddagger}$ (kcal/mol) Minor conformer
2a	H-6	203	54.1	51 : 49 (0.015)	9.8	9.8
	H-18	193	11.5	51 : 49 (0.015)	9.9	9.9
2b	H-3	183	74.1	78 : 22 (0.469)	9.1	8.7
4a	H-6	223	58.0	83 : 17 (0.702)	11.5	10.8
4b	H-3	213	193.2	85 : 15 (0.729)	10.5	9.8

Table 7. VDR Affinity and Transcriptional Activity of Fluorovitamin D Analogs[a)]

Compd.	VDR affinity	Transactivation[b)]
1	100	100
2a	1	42
3a	8	53
4a	9	27

a) Activities are presented as % effect of 1,25-$(OH)_2D_3$. *b*) Compounds were assayed at a concentration of 10^{-8} M.

C(19) methylene has close contact with Leu233 (C–C distance 3.544 Å) and Ser237 (C–O distance 3.348 Å). A fluorine atom introduced at C(19) might then cause steric congestion between these amino acid residues. However, 19-fluorovitamins **3a** and **4a** prefer the α-form and in this conformation, the steric congestion mentioned above may be relieved.

Because the native ligand 1,25-$(OH)_2D_3$ binds to the VDR with very high affinity ($K_d = 10^{-11}$ M), the fluorinated vitamin D analogs still maintain a high affinity for VDR, K_d for **2a**, 10^{-9} M; for **3a** and **4a**, 10^{-10} M. The results show that these fluorovitamin D derivatives present mostly as a complex with VDR when a sufficient amount of VDR exists.

The ability of the fluorinated vitamin D to activate gene transcription was evaluated by a luciferase assay using a chimera vector composed of a GAL4 DNA-binding domain and human VDR-LBD (GAL4-VDR), which are transfected in CV-1 cells. All the tested analogs showed significant potency, 27—53% of the potency of the native hormone. The transactivation potency of these analogs (**2a**, **3a**, **4a**) is not parallel to their affinity for VDR. However, it has also been known that the dissociation constants (K_d) of ligands and their transcriptional activities are not always parallel,[2)] although this discrepancy has not been clearly explained. Based on the generally accepted transcription mechanism of nuclear receptors, it is assumed that the VDR adopts mostly the inactive conformation in the apo-form where the carbon-terminal helix 12 is extended, but in the holo-form it mainly takes the active conformation with the helix 12 holding back to the body of the receptor. For the transactivation, the stability or the concentration of the latter conformation is important, whereas a complex equilibrium among the all-possible conformations of its apo- and holo-forms is involved in the determination of the affinity for VDR. We assume this may be a reason why the affinity for VDR and the transactivation potency are not necessarily parallel. In the present examples, the 4,4-difluoro-analog **2a** has a low affinity for VDR (1/100) but its transcriptional potency is considerably high (42%). Probably **2a** has difficulty in entering into the LBP due to the bulky geminal fluorine atoms. However, once in the LBP, **2a** can have intense hydrophobic interaction with the amino acid residues lining the LBP *via* the difluoro group stabilizing the transcriptionally active conformation of the VDR-ligand.

In conclusion, we showed, for the first time, the dynamic property of the vitamin D A-ring conformation by ^{19}F-NMR using six fluorovitamin D derivatives (**2a**, **b**, **3a**, **b**, **4a**, **b**) synthesized previously in this laboratory. We also demonstrated that, among these, the two compounds **2a** and **4a** are useful probes for investigating the interaction of vitamin D ligand with VDR in solution, in particular the dynamics of the A-ring conformation in the VDR complex. Biological studies provided supporting evidence that these compounds have sufficient affinity for VDR to study the NMR of the VDR complex. ^{19}F-NMR studies of the fluorovitamin D compounds complexed with VDR-LBD are now progressing in our laboratory.

Experimental

Materials 1,25-$(OH)_2D_3$ was a gift from Mercian Co. (Tokyo, Japan). [26,27-Methyl-^{3}H]-1,25-$(OH)_2D_3$ (specific activity 6.62 TBq/mmol) was purchased from Amersham (Buckinghamshire, U.K.). 4,4-F_2-1,25-$(OH)_2D_3$ (**2a**), 4,4-F_2-vitamin D_3 (**2b**), (10*Z*)-19-F-1,25-$(OH)_2D_3$ (**3a**), (10*E*)-19-F-vitamin D_3 (**3b**), (10*E*)-19-F-1,25-$(OH)_2D_3$ (**4a**), and (10*Z*)-19-F-vitamin D_3 (**4b**) were synthesized in our laboratory as reported. VDR binding assays were carried out using a bovine thymus receptor kit (Yamasa Shoyu Co., Ltd., Chiba, Japan). ACS-II scintillation fluid was obtained from Amersham (Buckinghamshire, U.K.).

Methods. 4,4-Difluoro-8(14)-cholesten-3β-ol (7) A solution of **5** (20 mg, 0.05 mmol) in $CHCl_3$–AcOH (1.5 ml; 2 : 1; v/v) was hydrogenated at 1 atmosphere in the presence of catalytic PtO_2 (4 mg) for 1 h at room temperature. After filtration through a Celite pad, the filtrate was diluted with ethyl acetate (AcOEt). The organic phase was washed with 5% $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated to dryness. The residue was chromatographed on silica gel (Wakogel C-200, 5 g) with 5% AcOEt in hexane to give **7** as the major product and its double bond isomer ($\Delta^{7,8}$) in a ratio of 10 : 1 (18.7 mg, 93%).

7: ^{1}H-NMR ($CDCl_3$) δ: 0.81 (3H, s, H-18 or 19), 0.84 (3H, s, H-18 or 19), 0.86, 0.87 (each 3H, d, J=6.6 Hz, H-26, 27), 0.93 (3H, d, J=6.6 Hz, H-21), 3.71 (1H, m, H-3). ^{19}F-NMR ($CDCl_3$) δ: −126.7 (ddd, J=236.8, 27.6, 20.7 Hz, F-4β), −108.2 (d, J=236.8 Hz, F-4α). MS *m/z* (%): 422 (M^+, 100), 407 (46), 309 (43), 283 (22), 267 (34).

4,4-Difluoro-8(14)-cholesten-3α-ol (8) A mixture of **6** (17.5 mg, 0.042 mmol) and PtO_2 (3.5 mg) in $CHCl_3$–AcOH (1.5 ml, 2 : 1; v/v) was hydrogenated at atmospheric pressure for 21 h at room temperature and was filtered *in vacuo* on a pad of Celite. The same work-up as described above gave the residue, which was purified by chromatography on silica gel (Wakogel C-200, 5 g). The column was eluted with 5% AcOEt in hexane to afford **8** and its double bond isomer ($\Delta^{7,8}$) in a 1 : 1 ratio (10.0 mg, 57%).

8: ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, H-18 or 19), 0.84 (3H, s, H-18 or 19), 0.86, 0.87 (each 3H, d, J=6.6 Hz, H-26, 27), 0.93 (3H, d, J=6.6 Hz, H-21), 3.91 (1H, m, H-3). ^{19}F-NMR ($CDCl_3$) δ: −112.5 (dd, J=251.6, 28.7 Hz, F-4β), −107.1 (d, J=251.6 Hz, F-4α). MS *m/z* (%): 422 (M^+, 100), 407 (44), 309 (65), 282 (18), 267 (41).

^{1}H- and ^{19}F-NMR Studies NMR spectra were recorded in $CDCl_3$, CD_2Cl_2, CD_3OD, THF-d_8, a mixture of CD_2Cl_2–CD_3OD (*ca.* 2 : 1), and phosphate buffer (20 mM NaH_2PO_4, 100 mM NaCl) containing 10% D_2O on a Bruker ARX-400 MHz spectrometer, operating at 400 MHz for ^{1}H, and 376 MHz for ^{19}F. Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane as an internal standard (δ 0 ppm) for ^{1}H-NMR and trifluorotoluene as an external standard (δ −63 ppm) for ^{19}F-NMR. Temperature was controlled with a variable temperature unit B-VT2000 (Burker) equipped with liquid nitrogen reservoirs.

Binding Assay for VDR The radio receptor assay was carried out according to the manufacturer's instructions. Freeze-dried receptors were dissolved in phosphate buffer (40—45 ml, 0.3 M KCl, 0.05 M K_2HPO_4–KH_2PO_4, pH 7.4) and 500 μl each was pipetted into glass tubes. A solution containing an increasing amount of 1,25-$(OH)_2D_3$ (1—512 pg/tube) or the fluorovitamin D analogs in 50 μl of ethanol was added to the receptor solution in each tube. The mixture was incubated for 1 h at room temperature, [^{3}H]-1,25-$(OH)_2D_3$ (10000 dpm, in 50 μl ethanol) was added, and the whole mixture was then allowed to stand for 18 h at 4 °C. At the end of the second incubation, 200 μl of dextran-coated charcoal suspension (used as received) was added and the sample was vortexed. After 30 min at 4 °C, bound and free [^{3}H]-1,25-$(OH)_2D_3$ were separated by centrifugation (3000 rpm) for 15 min at 4 °C. Aliquots (500 μl) of the supernatant were mixed with ACS-II scintillation fluid (10 ml) and submitted for radioactivity counting. Each assay was performed at least twice in duplicate.

Transfection and Luciferase Reporter Assay CV-1 cells were cultured in 24-well plates and grown in (phenol-red-free) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (dextran-coated charcoal treated) fetal bovine serum (FBS) for 8—12 h at 37 °C. Expression vectors were generated by insertion of a human VDR-LBD fused with the yeast transcription factor GAL4 DNA-binding domain (DBD). CV-1 cells were

transfected with the above expression vector and a luciferase reporter plasmid containing the GAL4 upstream activation sequence fused to a tk promoter. Transfections were performed using calcium phosphate according to the manufacturer's instructions. A β-galactosidase expression plasmid was included in each transfection for use as a normalization control. After 6—8 h, the cells were washed twice with phosphate buffer saline, and 10% FBS DMEM containing 1,25-$(OH)_2D_3$ or its fluorinated analogs were added at a concentration of 10^{-8} M and incubated for 36 h at 37 °C. The luciferase activities of the cell lysates were measured with a luciferase assay system. Transcription measured by luciferase activity was standardized with luciferase activity of the same cells. Each assay was performed at least twice.

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