Synthesis and Biological Evaluation of (23*R*)- and (23*S*)-24,24-Difluoro-1α,23,25-trihydroxyvitamin D_3

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The syntheses and biological evaluations of (23*R*)- and (23*S*)-24,24-difluoro-1α,23,25-trihydroxyvitamin D_3 (3a and 3b) , new C-24 fluorinated analogs of 1α,25-dihydroxyvitamin D_3, are described. The syntheses of these compounds were achieved in 3 steps from (5*Z*,7*E*,20*R*)-1α,3β-bis-[(*tert*-butyldimethylsilyl)oxy]-20-formylmethyl-9,10-seco-5,7,10(19)pregnatriene (5) which is derived from vitamin D_2. The absolute configuration at the C-23 position of 3a and 3b was determined by the modified Mosher method. The relative affinities of 3a and 3b to the vitamin D receptor were both 10 and 14 times lower than that of 1α,25-dihydroxyvitamin D_3 (1), and to vitamin D binding protein were also both 130 and 40 times lower. The HL-60 cell differentiating activity of 3a was 6 times more potent than that of 1, while there was no remarkable difference in activity between 3b and 1.

Key words 24,24-difluoro-1α,23,25-trihydroxyvitamin D_3; vitamin D analog; Reformatsky reaction; modified Mosher method; binding assay; HL-60 cell differentiation

1α,25-Dihydroxyvitamin D_3 (**1**), the physiologically active form of vitamin D_3, is a hormone which plays a role in serum calcium and phosphorous regulation.[2)] In addition, the possibility of the use of **1** as an antitumor[3)] and antipsoriasis[4)] agent has been recently suggested since a variety of biological activities of **1** such as proliferation and differentiation of human promyeloid leukemia cells (HL-60)[5)] and human keratinocyte[6)] were discovered. However, when **1** is clinically applied to such diseases, there is a possibility that it causes hypercalcemia as a harmful side effect because of its essentially strong calcemic action. Therefore, many non-hypercalcemic analogs of **1** have been synthesized and biologically evaluated for practical use.[7)]

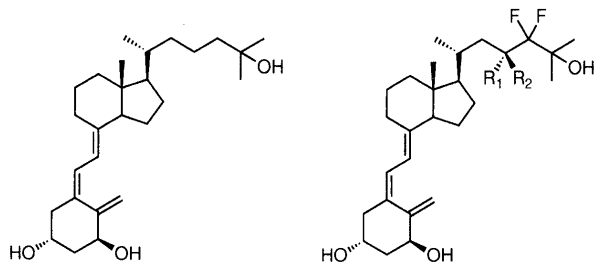
In the meantime, we have investigated the synthesis and carried out biological evaluation of 24,24-difluoro-1α,25-dihydroxyvitamin D_3 (**2**),[8—12)] the fluorinated analog of **1**. This compound is expected to be a highly potent and a long-lasting vitamin D analog because it is an antimetabolic analog of C-24 hydroxylation which is a major metabolic route of vitamin D in various target tissues. Indeed, the potency of **2** is 5 to 10 times that of **1** in the known *in vivo* vitamin D responsive systems, including intestinal calcium transport, bone calcium mobilization, and elevation of serum calcium and phosphorus concentrations in the rat.[13)] Moreover, **2** is 4 to 7 times more potent than **1** in activity, including phagocitosis and C3 rosette formation of HL-60 cells.[12)] Recently, we have synthesized (23*R*)- and (23*S*)-24,24-difluoro-1α,23,25-trihydroxyvitamin D_3 (**3a**, **3b**) as authentic samples to investigate the metabolic routes of **2**. Though neither of these compounds is a major metabolite of **2**,[14)] two or three interesting results were obtained by a preliminary biological evaluation of **3a** and **3b**. In the present paper, we therefore report in detail the synthesis and biological evaluation of these new C-24 fluorinated analogs (Fig. 1).

The synthetic route of **3a** and **3b** is shown as follows (Chart 1). The Reformatsky reaction of aldehyde (**5**), which is available in 11 steps from vitamin D_2 (**4**) as previously reported,[11)] with ethyl bromodifluoroacetate and activated zinc[15)] gave alcohol (**6**) in 93% yield. The alcohol **6** was a diastereomeric mixture at the C-23 position and its production ratio was less polar isomer (**6a**) : more polar isomer (**6b**)=2 : 3. These diastereomers were readily separated by silica gel chromatography.

In order to determine the absolute configuration of **6a** and **6b** at the C-23 position, we applied the modified Mosher method[16)] to these compounds. Four diastereomers ((*S*)-**7a**, (*R*)-**7a**, (*S*)-**7b**, and (*R*)-**7b**) were prepared from **6a** and **6b** by the treatment with (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA chloride) in the presence of 4-dimethylaminopyridine (DMAP). The $\Delta\delta$ values, which were calculated by the measurement of ^{1}H-NMR and H–H COSY of these isomers, are shown as Fig. 2. Thus, it was determined that the less polar **6a** was (23*R*)- and the more polar **6b** was (23*S*)-isomer.

The configurationally clarified **6a** and **6b** were treated with MeMgBr and subsequent deprotection with tetra-*n*-butylammonium fluoride (TBAF) gave **3a** and **3b** in 72% and 66% yield, respectively.

As a preliminary biochemical evaluation of **3a** and **3b**, we investigated the competitive binding affinities of these com-



1 **2** R_1=H, R_2=H

3a R_1=OH, R_2=H

3b R_1=H, R_2=OH

Fig. 1

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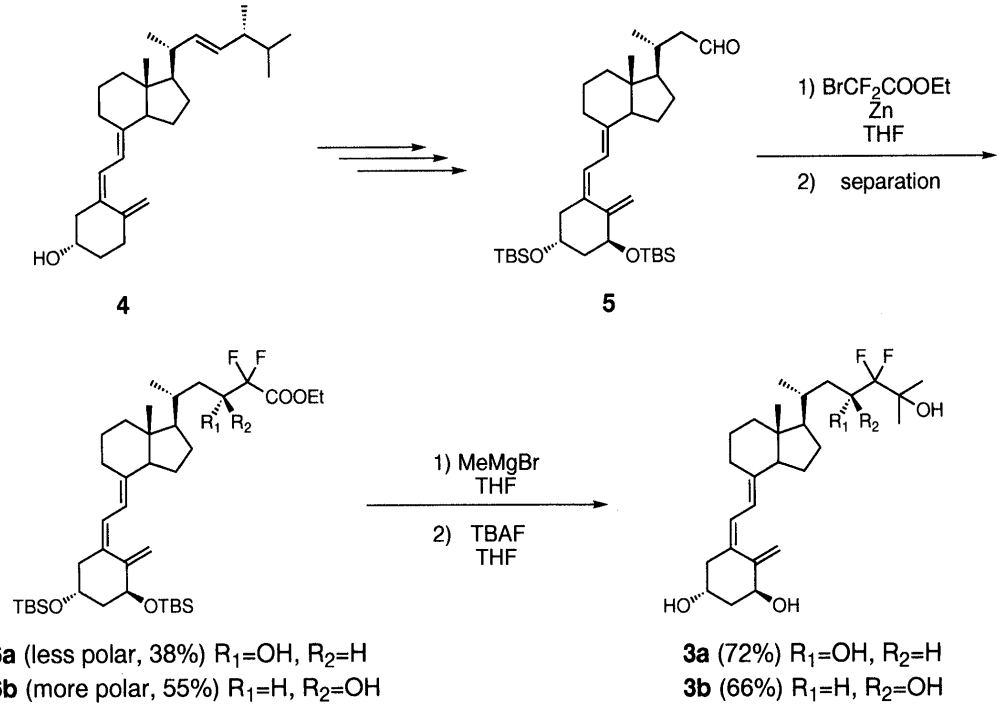

Chart 1

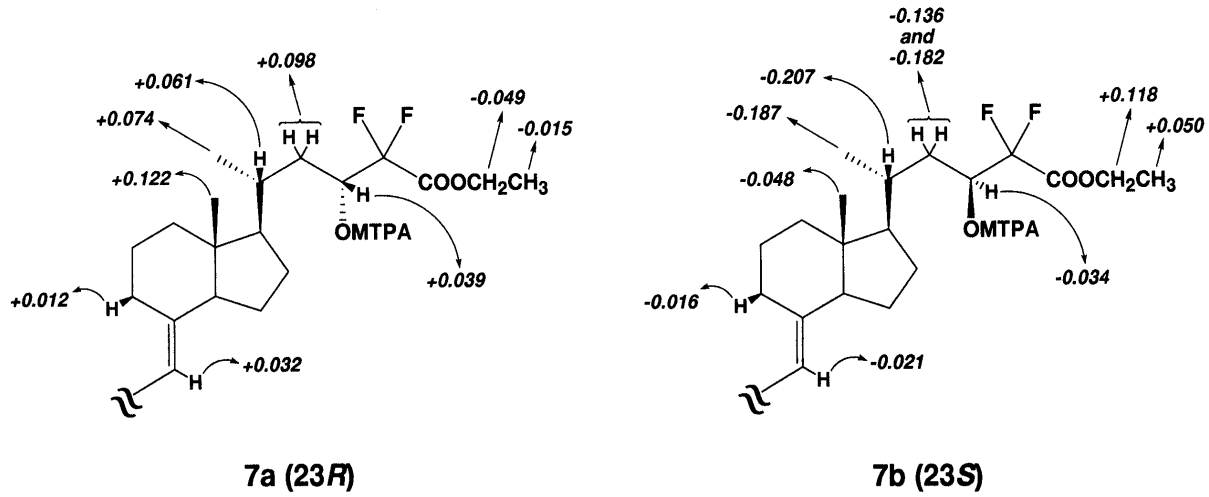

Fig. 2. $\Delta\delta$ Values of **7a** and **7b**.

pounds for **1** to calf thymus vitamin D receptor (VDR) and rat serum vitamin D binding protein (DBP). The results are shown in Fig. 3. The relative affinities (IC_{50}) of **3a** and **3b** to VDR were both 10 and 14 times lower than that of **1**, and to DBP were also both over 130 and 40 times lower. It is remarkable that the affinity of **3a** and **3b** to VDR was almost similar, but to DBP, the affinity of **3a** was obviously lower than that of **3b**.

Next, we investigated the HL-60 cell differentiating effect of these compounds into macrophages. The differentiating activity was evaluated by the induction of superoxide anion production as described previously.[17] As Fig. 4 shows, the differentiating activity of the (23*R*)-isomer **3a** was 6 times more potent than that of **1**. In contrast, there was no remarkable difference in activity between the (23*S*)-isomer **3b** and **1**. These phenomena suggest that the balance between VDR and DBP affinities is one of the important factors which control the *in vitro* cell differentiating activity of vitamin D analogs. It is generally accepted that DBP is a serum factor which affects the cellular uptake of vitamin D derivatives.[18] Vitamin D analogs with lower binding affinity to DBP are more accessible to the cells *in vitro* in the presence of serum. Thus, it can be explained that the low DBP affinity is one reason the differentiating activity of **3a** is much higher than that of **3b** although the affinities of these compounds to VDR are almost similar.

Further biological studies *in vivo* of these compounds are now in progress. The details will be reported elsewhere.

Experimental

^{1}H-NMR spectra were recorded in $CDCl_3$ on a JEOL JNM-EX270 instrument. Chemical shifts are given in ppm (δ), using tetramethylsilane (TMS) as internal standard. Optical rotations were measured on a Horiba SEPA-200 polarimeter. Mass spectra were registered on a JEOL JMS-DX303 instrument. IR spectra were recorded on a JASCO FT/IR-7300 instrument. Tetrahydrofuran (THF) was freshly distilled from benzophenone ketyl. CH_2Cl_2 was distilled from CaH_2 and stored over molecular sieves 4A. Col-

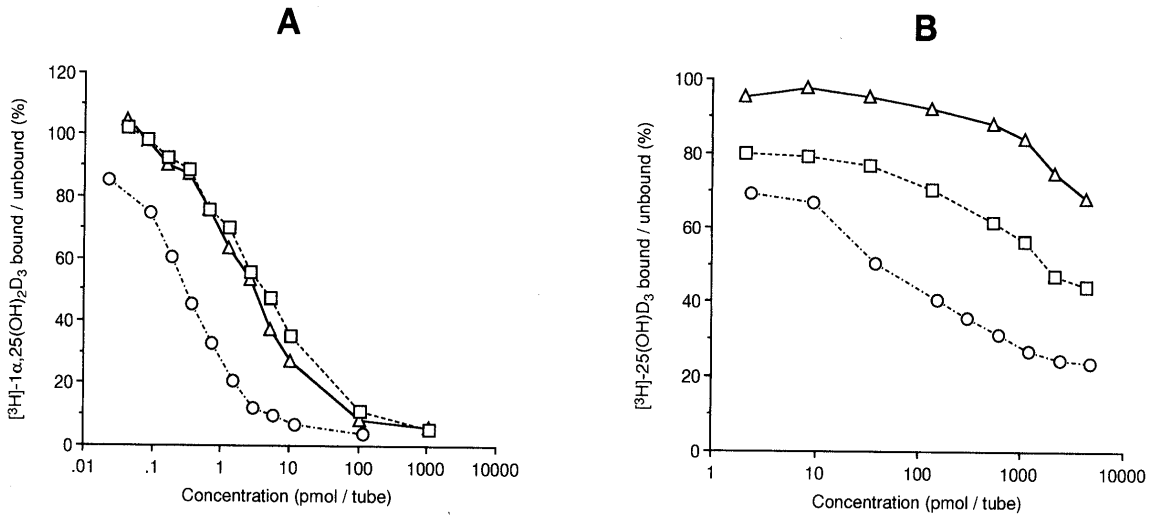

Fig. 3. Competition of the Vitamin D Derivatives for Specific [^{3}H]-1α,25-Dihydroxyvitamin D_3-Binding to Calf Thymus VDR (A) and for Specific [^{3}H]-25-Hydroxyvitamin D_3-Binding to Rat Serum DBP (B)

—△— **3a**, ---□--- **3b**, -·-○-·- **1**.

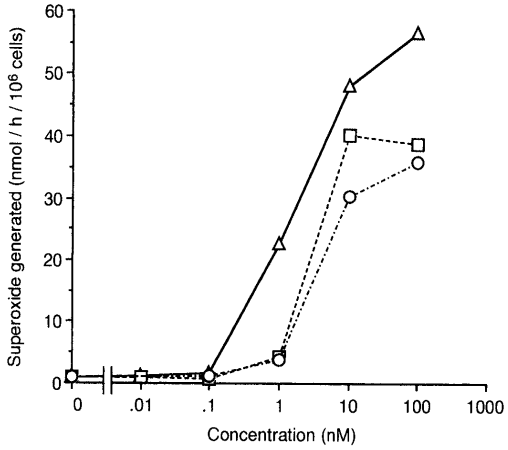

Fig. 4. Effects of the Vitamin D Derivatives on the Induction of Superoxide Production in HL-60 Cells

—△— **3a**, ---□--- **3b**, -·-○-·- **1**.

umn chromatographies were performed with silica gel 60 (70−230 mesh, Merck) and preparative TLC was run on silica gel 60 F_{254} Merck. Preparative HPLC for the purification of **3a** and **3b** was performed on a Tosoh LC system equipped with a CCPS HPLC pump and a UV-8020 variable wavelength detector (Tosoh corporation, Tokyo, Japan) under the following conditions; column: TSK-GEL ODS-80T (21.5 mm i.d.×300 mm, Tosoh corporation, Tokyo, Japan), mobile phase: CH_3CN/H_2O 8 : 2, flow rate: 5 ml/min (pressure: 30 kgf/cm^2). These vitamin D compounds were detected at 265 mn.

(5*Z*,7*E*,20*R*)-24,24-Difluoro-1α,3β-bis[(*tert*-butyldimethylsilyl)oxy]-23-hydroxy-24-ethoxycarbonyl-9,10-seco-5,7,10(19)-cholatriene (6a and 6b) Ethyl bromodifluoroacetate (90 μl, 0.703 mmol) was added to a suspension of freshly activated Zn dust (45 mg, 0.688 mg-atom) in THF (2 ml) and the mixture was heated at reflux for 2 min. A solution of the aldehyde **5** (100 mg, 0.171 mmol) in THF (1 ml) was added to the resulting solution and the refluxing was continued for 2 min. The reaction mixture was poured into 1 M $KHSO_4$ and extracted with AcOEt. The combined organic phases were washed with brine, dried over $MgSO_4$ and evaporated. The residue was purified by chromatography on silica gel (10 g, AcOEt/hexane 1 : 20) to give two diastereomers of the difluoroester **6a** (less polar isomer, 44 mg, 38%) and **6b** (more polar isomer, 63 mg, 55%) as a colorless foam, respectively. **6a**; $[\alpha]_D^{20}$ +52.5° (c=1.21, AcOEt). IR (KBr): 3470, 2953, 1760, 1072 cm^{-1}. ^{1}H-NMR δ: 0.05 (6H, s), 0.06 (6H, s), 0.57 (3H, s), 0.88 (18H, s), 0.99 (3H, d, J=6.3 Hz), 1.20—2.05 (17H, m), 1.37 (3H, t, J=7.3 Hz), 2.21 (1H, dd, J=6.9, 12.5 Hz), 2.45 (1H, dd, J=3.6, 12.5 Hz), 2.83 (1H, dd, J=3.0, 12.5 Hz), 4.05—4.20 (1H, m), 4.19 (1H, tt, J=3.6, 7.6 Hz), 4.60—4.85 (3H, m), 4.86 (1H, d, J=2.6 Hz), 5.17 (1H, d, J=2.0 Hz), 6.02 (1H, d, J=11.2 Hz), 6.24 (1H, d, J=11.2 Hz). FAB-MS m/z: 711 $(M+H)^+$. HR-FAB-MS m/z: 711.4639 (Calcd for $C_{39}H_{69}F_2O_5Si_2$: 711.4651). **6b**; $[\alpha]_D^{20}$ +39.1° (c=1.24, AcOEt). IR (KBr): 3513, 2953, 1760, 1074 cm^{-1}. ^{1}H-NMR δ: 0.06 (12H, s), 0.55 (3H, s), 0.88 (18H, s), 1.07 (3H, d, J=6.6 Hz), 1.20—2.05 (17H, m), 1.37 (3H, t, J=7.3 Hz), 2.22 (1H, dd, J=7.3, 13.2 Hz), 2.45 (1H, dd, J=3.3, 13.2 Hz), 2.83 (1H, dd, J=3.0, 13.9 Hz), 4.05—4.20 (1H, m), 4.19 (1H, tt, J=3.3, 7.3 Hz), 4.30—4.40 (1H, m), 4.36 (2H, q, J=7.3 Hz), 4.86 (1H, d, J=2.3 Hz), 5.18 (1H, d, J=1.7 Hz), 6.02 (1H, d, J=11.2 Hz), 6.23 (1H, d, J=11.2 Hz). FAB-MS m/z: 711 $(M+H)^+$. HR-FAB-MS m/z: 711.4594 (Calcd for $C_{39}H_{69}F_2O_5Si_2$: 711.4651).

(23*R*)-24,24-Difluoro-1α,23,25-trihydroxyvitamin D_3 (3a) A solution of **6a** (43 mg, 0.060 mmol) in THF (2 ml) was treated with MeMgBr (3.0 M in Et_2O) (400 μl, 1.200 mmol) at 0 °C for 30 min, then at room temperature for 3 h. The reaction mixture was cooled to 0 °C, quenched with saturated aqueous NH_4Cl and extracted with AcOEt. The combined organic phases were washed with brine, dried over $MgSO_4$ and evaporated. The residue was dissolved in THF (2 ml) and treated with n-Bu_4NF (1.0 M in THF) (600 μl, 0.600 mmol) at room temperature for 16 h. After evaporating the solvent, the residue was purified by chromatography on silica gel (10 g, CH_2Cl_2/acetone 10 : 1—5 : 1—3 : 1) to give **3a** (21 mg, 72%) as a colorless oil. Further purification of this sample for biological evaluation was performed by HPLC (retention time: 33.33 min). $[\alpha]_D^{20}$ +16.0° (c=0.25, EtOH). IR (KBr): 3355, 2949, 1061 cm^{-1}. ^{1}H-NMR δ: 0.59 (3H, s), 1.00 (3H, d, J=6.6 Hz), 1.38 (3H, s), 1.40 (3H, s), 1.20—2.10 (20H, m), 2.31 (1H, dd, J=6.3, 13.2 Hz), 2.60 (1H, dd, J=3.6, 13.5 Hz), 2.83 (1H, dd, J=3.0, 11.5 Hz), 4.05—4.25 (1H, m), 4.23 (1H, tt, J=3.3, 6.3 Hz), 4.44 (1H, dd, J=4.0, 5.0 Hz), 5.00 (1H, s), 5.32 (1H, s), 6.02 (1H, d, J=11.5 Hz), 6.38 (1H, d, J=11.5 Hz). FAB-MS m/z: 469 $(M+H)^+$. HR-FAB-MS m/z: 469.3169 (Calcd for $C_{27}H_{43}F_2O_4$: 469.3130).

(23*S*)-24,24-Difluoro-1α,23,25-trihydroxyvitamin D_3 (3b) The (23*S*)-isomer **3b** was obtained from **6b** in a similar manner to the preparation of **3a** in 66% yield as a colorless oil. Further purification of this sample for biological evaluation was performed by HPLC (retention time: 29.00 min). $[\alpha]_D^{20}$ +11.5° (c=0.31, EtOH). IR (KBr): 3347, 2948, 1062 cm^{-1}. ^{1}H-NMR δ: 0.57 (3H, s), 1.07 (3H, d, J=6.3 Hz), 1.37 (3H, s), 1.40 (3H, s), 1.20—2.10 (20H, m), 2.31 (1H, dd, J=6.6, 13.5 Hz), 2.60 (1H, dd, J=3.6, 13.5 Hz), 2.83 (1H, dd, J=3.3, 12.2 Hz), 4.05—4.30 (2H, m), 4.43 (1H, dd, J=4.6, 6.6 Hz), 5.00 (1H, d, J=1.3 Hz), 5.33 (1H, t, J=1.7 Hz), 6.02 (1H, d, J=11.2 Hz), 6.38 (1H, d, J=11.2 Hz). FAB-MS m/z: 469 $(M+H)^+$. HR-FAB-MS m/z: 469.3135 (Calcd for $C_{27}H_{43}F_2O_4$: 469.3130).

(*S*)- and (*R*)-MTPA Esters of 6a ((*S*)-7a and (*R*)-7a) (*R*)-MTPA chloride (5.3 μl, 0.028 mmol) and DMAP (3.5 mg, 0.028 mmol) was added to a solution of **6a** (5.0 mg, 0.007 mmol) in CH_2Cl_2 (200 μl) at room temperature. The mixture was allowed to stand at room temperature for 3 h and then kept in a refrigerator (*ca.* 5 °C) for 2 d. The reaction mixture was applied directly on a silica gel preparative TLC (hexane/AcOEt 10 : 1) and purified to give (*S*)-**7a** (1.6 mg, 25%) as a colorless oil. The (*R*)-MTPA ester ((*R*)-**7a**)

was prepared in a similar manner by the treatment with (*S*)-MTPA chloride in 18% yield. (*S*)-**7a**; ^{1}H-NMR δ: 0.06 (6H, s), 0.07 (6H, s), 0.47 (3H, s), 0.88 (18H, s), 1.02 (3H, d, *J*=5.3 Hz), 1.20—2.05 (17H, m), 1.31 (3H, t, *J*=7.3 Hz), 2.22 (1H, dd, *J*=6.9, 12.9 Hz), 2.45 (1H, dd, *J*=3.6, 13.2 Hz), 2.84 (1H, dd, *J*=3.0, 12.5 Hz), 3.49 (3H, s), 4.16—4.25 (1H, m), 4.26 (2H, dq, *J*=1.0, 7.3 Hz), 4.38 (1H, dd, *J*=3.3, 5.9 Hz), 4.87 (1H, d, *J*=2.3 Hz), 5.20 (1H, d, *J*=2.0 Hz), 5.72 (1H, dd, *J*=10.6, 21.8 Hz), 6.00 (1H, d, *J*=11.5 Hz), 6.23 (1H, d, *J*=11.2 Hz), 7.35—7.44 (3H, m), 7.50—7.60 (2H, m). (*R*)-**7a**; ^{1}H-NMR δ: 0.06 (6H, s), 0.07 (3H, s), 0.08 (3H, s), 0.35 (3H, s), 0.88 (9H, s), 0.89 (9H, s), 0.94 (3H, d, *J*=6.3 Hz), 1.00—1.95 (17H, m), 1.32 (3H, t, *J*=7.3 Hz), 2.21 (1H, dd, *J*=6.9, 12.5 Hz), 2.45 (1H, dd, *J*=2.6, 13.2 Hz), 2.81 (1H, dd, *J*=2.3, 12.9 Hz), 3.60 (3H, s), 4.16—4.22 (1H, m), 4.31 (1H, dq, *J*=1.3, 7.3 Hz), 4.38 (1H, dd, *J*=3.6, 6.6 Hz), 4.86 (1H, d, *J*=2.0 Hz), 5.19 (1H, d, *J*=2.0 Hz), 5.69 (1H, dd, *J*=10.2, 21.8 Hz), 5.97 (1H, d, *J*=11.9 Hz), 6.22 (1H, d, *J*=10.9 Hz), 7.33—7.42 (3H, m), 7.50—7.60 (2H, m).

(*S*)- and (*R*)-MTPA Esters of 6b ((*S*)-7b and (*R*)-7b) Difluoroester **6b** was derived to (*S*)-**7b** and (*R*)-**7b** in a similar manner to the preparation of (*S*)-**7a** and (*R*)-**7a** in 48% and 38% yield respectively. (*S*)-**7b**; ^{1}H-NMR δ: 0.06 (6H, s), 0.07 (6H, s), 0.48 (3H, s), 0.86 (3H, d, *J*=6.3 Hz), 0.877 (9H, s), 0.884 (9H, s), 1.10—2.00 (17H, m), 1.32 (3H, t, *J*=7.3 Hz), 2.21 (1H, dd, *J*=6.9, 13.2 Hz), 2.44 (1H, dd, *J*=3.6, 14.2 Hz), 2.80 (1H, dd, *J*=2.3, 12.9 Hz), 3.59 (1H, d, *J*=1.0 Hz), 4.19 (1H, tt, *J*=3.6, 7.3 Hz), 4.29 (2H, q, *J*=7.3 Hz), 4.38 (1H, dd, *J*=4.0, 6.3 Hz), 4.86 (1H, d, *J*=2.3 Hz), 5.19 (1H, d, *J*=1.3 Hz), 5.63 (1H, tt, *J*=4.3, 10.9 Hz), 5.99 (1H, d, *J*=11.2 Hz), 6.22 (1H, d, *J*=11.6 Hz), 7.35—7.43 (3H, m), 7.50—7.60 (2H, m). (*R*)-**7b**; ^{1}H-NMR δ: 0.06 (6H, s), 0.07 (6H, s), 0.53 (3H, s), 0.876 (9H, s), 0.883 (9H, s), 1.05 (3H, d, *J*=6.3 Hz), 1.20—2.17 (17H, m), 1.27 (3H, t, *J*=7.3 Hz), 2.21 (1H, dd, *J*=6.9, 13.9 Hz), 2.44 (1H, dd, *J*=3.3, 12.5 Hz), 2.82 (1H, dd, *J*=3.0, 12.5 Hz), 3.50 (3H, d, *J*=1.0 Hz), 4.17 (2H, q, *J*=7.3 Hz), 4.15—4.25 (1H, m), 4.38 (1H, dd, *J*=4.6, 5.9 Hz), 4.86 (1H, d, *J*=2.3 Hz), 5.19 (1H, d, *J*=1.3 Hz), 5.66 (1H, tt, *J*=5.9, 11.8 Hz), 6.02 (1H, d, *J*=11.2 Hz), 6.23 (1H, d, *J*=11.2 Hz), 7.37—7.45 (3H, m), 7.48—7.58 (2H, m).

Displacement of [26,27-^{3}H]-1α,25-Dihydroxyvitamin D$_3$ from Calf Thymus VDR All procedures were performed as described.[19] A solution of calf thymus VDR (Yamasa Shoyu Ltd.) containing 0.3 M KCl and 5 mM dithiothreitol in 50 mM potassium phosphate buffer, pH 7.4 (500 μl), was added to each vitamin D compound (**3a**, **3b** and **1**) in ethanol (50 μl). After incubation for 40 min at room temperature, [26,27-^{3}H]-1α,25-dihydroxyvitamin D$_3$ (5000 cpm/50 μl ethanol) was added and further incubated overnight at 4 °C. The radioactivity in the supernatant obtained after dextran-coated charcoal extraction was counted.

Displacement of [26,27-^{3}H]-25-Hydroxyvitamin D$_3$ from Rat Serum DBP The serum of male SD rats was diluted with 3.5 mM barbital buffer, pH 8.6, containing 0.1% ovalbumin, then 500 μl was added to a tube containing [26,27-^{3}H]-25-hydroxyvitamin D$_3$ (4800 cpm/30 μl ethanol) and various amounts of each vitamin D compound (**3a**, **3b** and **1**) in ethanol (40 μl). After incubation for 1 h in ice-cold water, dextran-coated charcoal was added and the radioactivity in the supernatant was counted.

HL-60 Cell Differentiating Activity HL-60 cells, purchased from Riken cell bank (Ibaraki, Japan), were cultured in a RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 100 units/ml of penicillin G potassium salt and 100 μg/ml of streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were inoculated at 1×10^5 cells/ml in a growth media (5 ml/dish) and EtOH solutions containing various concentrations of vitamin D analogs (**3a**, **3b** and **1**) were added. After culturing for 4 d, the cells were washed twice with a 0.1% gelatin Hanks' balanced salt solution (HBSS) and were prepared as 2×10^6 cells/ml suspension with/without 1600 units/ml superoxide dismutase (SOD). This suspension (0.5 ml) was added to 0.5 ml of a 0.1% gelatin HBSS without phenol red containing 160 μM ferricytochrome c (Cyt C) (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 1 μg/ml phorbol myristate acetate (PMA) (Wako Pure Chemical Industries, Osaka, Japan) (final concentration: 1×10^6 cells/ml with/without 800 units/ml SOD, 80 μl Cyt C, 500 ng/ml PMA). The mixture was incubated at 37 °C for 60 min and centrifuged for 5 min at 400 ***g*** at 4 °C. The optical density at 550 and 540 nm of the supernatant was measured and the amount of superoxide anion generated was determined as the concentration of Cyt C reduced using a molar extinction coefficient of 21/mM/cm.

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