

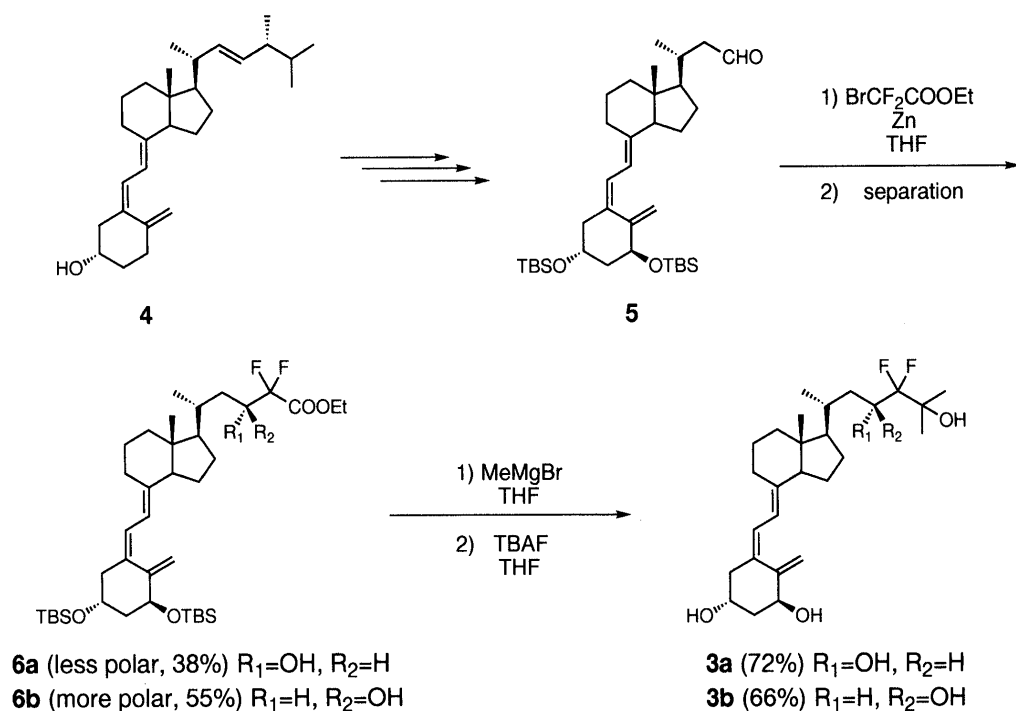
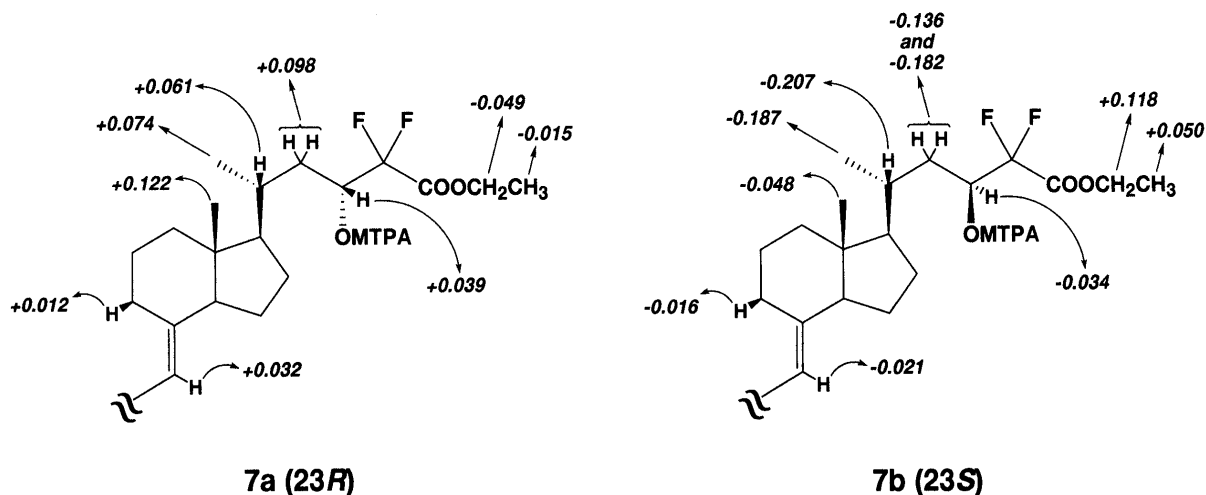


Chart 1

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

¹H-NMR spectra were recorded in CDCl₃ 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. CH₂Cl₂ was distilled from CaH₂ and stored over molecular sieves 4A. Col-

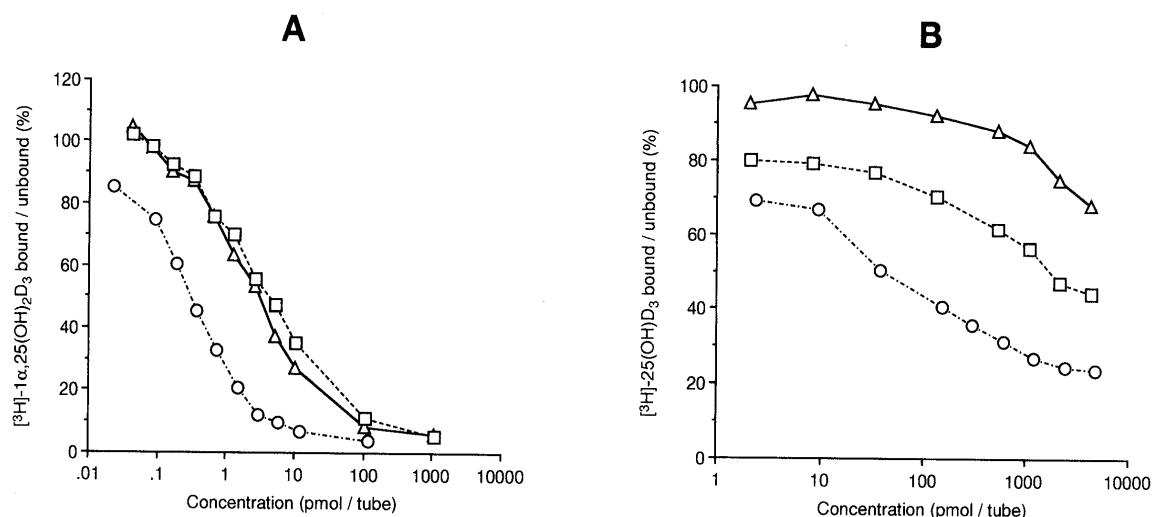


Fig. 3. Competition of the Vitamin D Derivatives for Specific [^3H]-1 α ,25-Dihydroxyvitamin D_3 -Binding to Calf Thymus VDR (A) and for Specific [^3H]-25-Hydroxyvitamin D_3 -Binding to Rat Serum DBP (B)

— Δ — **3a**, --- $\square$ — **3b**, ... $\circ$ — **1**.

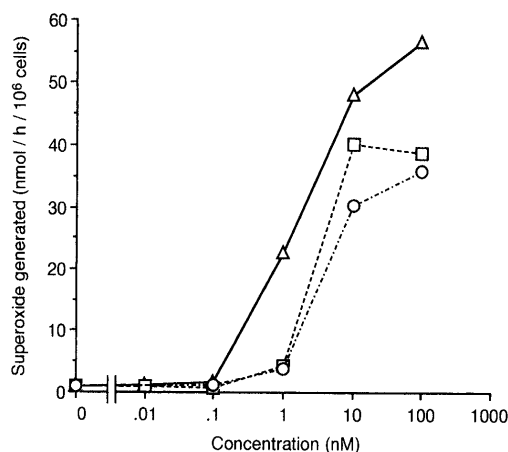


Fig. 4. Effects of the Vitamin D Derivatives on the Induction of Superoxide Production in HL-60 Cells

— Δ — **3a**, --- $\square$ — **3b**, ... $\circ$ — **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. $\times$ 300 mm, Tosoh corporation, Tokyo, Japan), mobile phase: $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 8:2, flow rate: 5 ml/min (pressure: 30 kgf/cm 2). These vitamin D compounds were detected at 265 nm.

(5Z,7E,20R)-24,24-Difluoro-1 α ,3 β -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 KH_2SO_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**: $[\alpha]_D^{20} + 52.5^\circ$ ($c=1.21$, AcOEt). IR (KBr): 3470, 2953, 1760, 1072 cm^{-1} . $^1\text{H-NMR}$ 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 ($\text{M}+\text{H}$) $^+$. HR-FAB-MS m/z : 711.4639 (Calcd for $\text{C}_{39}\text{H}_{69}\text{F}_2\text{O}_5\text{Si}_2$: 711.4651). **6b**: $[\alpha]_D^{20} + 39.1^\circ$ ($c=1.24$, AcOEt). IR (KBr): 3513, 2953, 1760, 1074 cm^{-1} . $^1\text{H-NMR}$ 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 ($\text{M}+\text{H}$) $^+$. HR-FAB-MS m/z : 711.4594 (Calcd for $\text{C}_{39}\text{H}_{69}\text{F}_2\text{O}_5\text{Si}_2$: 711.4651).

(23R)-24,24-Difluoro-1 α ,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 $n\text{-Bu}_4\text{NF}$ (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). $[\alpha]_D^{20} + 16.0^\circ$ ($c=0.25$, EtOH). IR (KBr): 3355, 2949, 1061 cm^{-1} . $^1\text{H-NMR}$ 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 ($\text{M}+\text{H}$) $^+$. HR-FAB-MS m/z : 469.3169 (Calcd for $\text{C}_{27}\text{H}_{43}\text{F}_2\text{O}_4$: 469.3130).

(23S)-24,24-Difluoro-1 α ,23,25-trihydroxyvitamin D_3 (3b) The (23S)-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). $[\alpha]_D^{20} + 11.5^\circ$ ($c=0.31$, EtOH). IR (KBr): 3347, 2948, 1062 cm^{-1} . $^1\text{H-NMR}$ 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 ($\text{M}+\text{H}$) $^+$. HR-FAB-MS m/z : 469.3135 (Calcd for $\text{C}_{27}\text{H}_{43}\text{F}_2\text{O}_4$: 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**: ¹H-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**: ¹H-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**: ¹H-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**: ¹H-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 [26,27-³H]-1 α ,25-Dihydroxyvitamin D₃ 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 μ l), was added to each vitamin D compound (**3a**, **3b** and **1**) in ethanol (50 μ l). After incubation for 40 min at room temperature, [26,27-³H]-1 α ,25-dihydroxyvitamin D₃ (5000 cpm/50 μ l 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 [26,27-³H]-25-Hydroxyvitamin D₃ 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 μ l was added to a tube containing [26,27-³H]-25-hydroxyvitamin D₃ (4800 cpm/30 μ l ethanol) and various amounts of each vitamin D compound (**3a**, **3b** and **1**) in ethanol (40 μ l). 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 μ g/ml of streptomycin sulfate at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were inoculated at 1 $\times$ 10⁵ 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 $\times$ 10⁶ 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 μ M ferricytochrome c (Cyt C) (Sigma Chemical

Co., St. Louis, MO, U.S.A.) and 1 μ g/ml phorbol myristate acetate (PMA) (Wako Pure Chemical Industries, Osaka, Japan) (final concentration: 1 $\times$ 10⁶ cells/ml with/without 800 units/ml SOD, 80 μ l Cyt C, 500 ng/ml PMA). The mixture was incubated at 37°C for 60 min and centrifuged for 5 min at 400g 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.

References and Notes

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