Effect on Carbon Lengthening at the Side Chain Terminal of 1α,25-Dihydroxyvitamin D_3 for Calcium Regulating Activity

Tadashi EGUCHI,[a] Nobuo IKEKAWA*,[a] Koji SUMITANI,[b] Masayoshi KUMEGAWA,[c] Shohei HIGUCHI,[d] and Susumu OTOMO[d]

Iwaki Meisei University,[a] Iwaki, Fukushima 970, Japan, Department of Orthodontics, School of Dentistry, Tokushima University,[b] Kuramoto-cho, Tokushima 770, Japan, Department of Oral Anatomy, Meikai University School of Density,[c] Sakado, Saitama 350-02, Japan, and Research Center, Taisho Pharmaceutical Co., Ltd.,[d] 1-403, Yoshino-cho, Ohmiya, Saitama 330, Japan. *Received September 1, 1989*

A series of analogs of 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$ (1)] with alkyl substitutions in 26- and 27-positions have been tested for their activity 1) in competing with 1,25-$(OH)_2D_3$ for binding to chick intestinal cytosol receptor, 2) in ability for formation of multinucleated cells (MNC) with various osteoclastic cell characteristics from blast cells, and 3) in stimulating bone calcium mobilization in vitamin D-deficient rats. The relative potencies of 1,25-$(OH)_2D_3$, 1α,25-dihydroxy-26,27-dimethylvitamin D_3 (2), 1α,25-dihydroxy-26,27-diethylvitamin D_3 (3), and 1α,25-dihydroxy-26,27-dipropylvitamin D_3 (4) in competing for intestinal cytosolic binding were 1 : 1.1 : 0.25 : 0.05. The similar order of the abilities on formation of the multinucleated cells in the same series was observed. In a bone calcium mobilization test with vitamin D-deficient rats, 1α,25-dihydroxy-26,27-dimethylvitamin D_3 showed slightly less activity than 1,25-$(OH)_2D_3$ at 12 h after administration, but long lasting activity was observed during time course experiments. 1α,25-Dihydroxy-26,27-diethylvitamin D_3, and 1α,25-dihydroxy-26,27-dipropylvitamin D_3 were found to be much less active than 1,25-$(OH)_2D_3$ in a bone calcium mobilization test.

Keywords vitamin D_3 analog; 1α,25-dihydroxyvitamin D_3; 1α,25-dihydroxy-26,27-dimethylvitamin D_3; 1α,25-dihydroxy-26,27-diethylvitamin D_3; 1α,25-dihydroxy-26,27-dipropylvitamin D_3; calcium regulation; binding affinity; multinucleated cell formation; bone calcium mobilization

Introduction

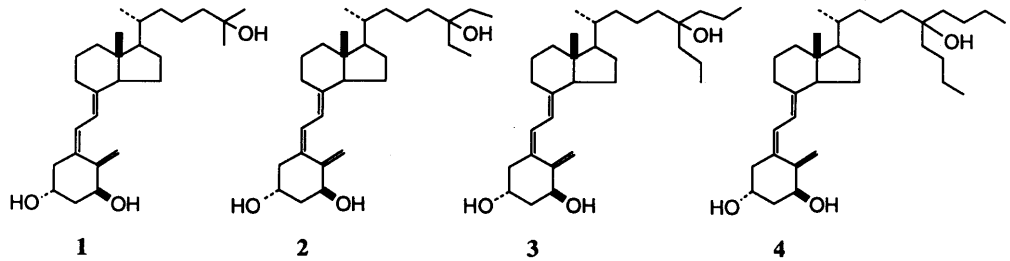
Vitamin D_3 undergoes metabolic activation in the liver and kidney to 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$ (1)], the active principle for the regulation of calcium and phosphorus homeostasis.[1] Many analogs and metabolites of vitamin D_3 have been synthesized and their biological activity have been investigated. Studies on structure–activity relationship have revealed that 1α-hydroxyl group of 1,25-$(OH)_2D_3$ is essential for eliciting activity and that a 25-hydroxyl group and proper side chain length are also important.[2] We recently reported that chain lengthening by one carbon at the 26- and/or 27-position of 1,25-$(OH)_2D_3$ enhance the potency in calcium regulational activity.[3]

Additionally, as an intriguing biological activity, 1,25-$(OH)_2D_3$ was reported to induce differentiation of malignant cells.[4] It was reported that the activity is also greatly enhanced by introduction of an alkyl group at the C-26/27 terminal.[5]

It is therefore of considerable interest to investigate the effect on the carbon lengthening at the side chain terminal of 1,25-$(OH)_2D_3$ systematically. Although we noted the preliminary biological tests of 26,27-dialkylated analogs of 1,25-$(OH)_2D_3$,[3] we describe here the potency of a series of 26,27-dialkylated analogs of 1,25-$(OH)_2D_3$, 26,27-dimethyl- (**2**), 26,27-diethyl- (**3**), and 26,27-dipropyl-1α,25-dihydroxyvitamin D_3 (**4**) in the calcium regulational effect. Steric requirements of the side chain of the vitamin D_3 for the biological activity are also discussed.

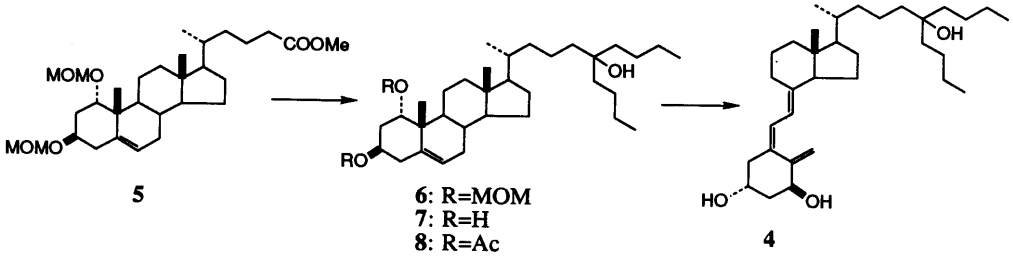
Materials and Methods

Spectroscopy Ultraviolet (UV) spectra were recorded with a Shimadzu UV 200 double-beam spectrometer. Proton nuclear magnetic resonance



1 2 3 4

Chart 1



5 6: R=MOM 7: R=H 8: R=Ac 4

Chart 2

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(^{1}H-NMR) spectra were taken on a JEOL FX-200 spectrometer in $CDCl_3$ solution using tetramethylsilane as an internal standard. Infrared (IR) spectra were obtained with a Jasco DS-701G spectrometer. Electron impact mass spectra (MS) and high resolution mass spectra (HRMS) were obtained with a JEOL DX-303 spectrometer at 70 eV. Radio activity was measured with a Tri-Carb liquid scintillation counter Model 3255 using an external standard.

Compounds 1,25-$(OH)_2D_3$ was obtained from Duphar Co., Ltd. (Amsterdam, the Netherlands). [26,27-^{3}H]1,25-$(OH)_2D_3$ (specific activity 157 Ci/mmol) was purchased from the Radiochemical Center, Amersham. Dimethyl and diethyl analogs of 1,25-$(OH)_2D_3$ (**2** and **3**) were prepared in our laboratory as reported in the previous paper.[3c] 1α,25-Dihydroxy-26,27-dipropylvitamin D_3 (**4**) was synthesized in the present study by a similar method in the case of dimethyl and diethyl analogs as follows.

1α,3β-Bis(methoxymethoxy)-26,27-dipropylcholest-5-ene (6) Butyl bromide (0.21 ml) was added to a suspension of Mg (48 mg) in tetrahydrofuran (THF) (2 ml) under nitrogen. After stirring of 20 min, the mixture was cooled with an ice-bath and a solution of methyl 1α,3β-bis(methoxymethoxy)-26,27-bisnorcholest-5-en-25-oate (**5**) (200 mg) in THF (4 ml) was added. The mixture was stirred at 0 °C for 30 min, and then at room temperature for 4.5 h. Aqueous saturated NH_4Cl solution was added and the mixture was extracted with ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated to dryness. The residue was chromatographed over silica gel with benzene–ethyl acetate (25 : 1) as an eluent, to give the 25-ol (180 mg, 70%) as an oil. ^{1}H-NMR δ: 0.69 (3H, s, 18-H), 0.91 (4H, t, J=7 Hz, CH_2), 0.93 (3H, d, J=6 Hz, 21-H), 1.03 (3H, s, 19-H), 3.35 and 3.40 (6H, each s, OMe), 3.74 (1H, br s, 1-H), 3.85 (1H, m, 3-H), 4.59 and 4.75 (2H, each d, J=7 Hz, OCH_2O), 4.68 (2H, s, OCH_2O), 5.57 (1H, m, 6-H). IR ν_{max} (neat): 3450 cm^{-1}. *Anal.* Calcd for $C_{37}H_{66}O_5$: C, 75.20; H, 11.26. Found: C, 74.70; H, 11.00.

1α,3β,25-Trihydroxy-26,27-dipropylcholest-5-ene (7) A solution of **6** (160 mg) and 6 N HCl (4 ml) in THF (20 ml) was stirred at room temperature for 5 days. The mixture was diluted with ether and the whole was washed with aqueous $NaHCO_3$ solution and brine, successively. The organic phase was dried over Na_2SO_4, filtered and evaporated. The residue was chromatographed over silica gel with hexane–ethyl acetate (5 : 1) as an eluent to give the triol (121 mg, 82%). mp 75—77 °C (acetone–hexane). ^{1}H-NMR δ: 0.69 (3H, s, 18-H), 0.91 (4H, t, J=7 Hz, CH_2), 0.93 (3H, d, J=6 Hz, 21-H), 1.03 (3H, s, 19-H), 3.82 (1H, br s, 1-H), 3.97 (1H, m, 3-H), 5.60 (1H, m, 6-H). *Anal.* Calcd for $C_{33}H_{58}O_3$: C, 78.83; H, 11.63. Found: C, 78.77; H, 11.61.

1α,3β-Diacetoxy-25-hydroxy-26,27-dipropylcholest-5-ene (8) A solution of **7** (91 mg) and Ac_2O (0.2 ml) in pyridine (1 ml) was stirred at room temperature overnight. Water was added and the mixture was stirred for 30 min. Extractive work-up gave a crude product, which was chromatographed over silica gel with hexane–ethyl acetate (10 : 1) to give the diacetate as an oil. ^{1}H-NMR δ: 0.67 (3H, s, 18-H), 0.91 (4H, t, J=7.1 Hz, CH_2), 0.92 (3H, d, J=6 Hz, 21-H), 1.08 (3H, s, 19-H), 2.02 and 2.05 (6H, each s, acetyl), 4.91 (1H, m, 3-H), 5.07 (1H, br s, 1-H), 5.53 (1H, m, 6-H). IR ν_{max} (neat): 1735 cm^{-1}. MS *m/z*: 466 ($M^+ - 2AcOH$), 271, 253. HRMS Calcd for $C_{33}H_{54}O$ ($M^+ - 2AcOH$): 466.4175. Found: 466.4080.

1α,25-Dihydroxy-26,27-dipropylvitamin D_3 (4) A solution of **8** (51.3 mg) and *N*-bromosuccinimide (21.8 mg) in CCl_4 (2 ml) was refluxed for 20 min under argon. After cooling with ice-water, the insoluble materials were filtered out and washed with CCl_4. The filtrate and washings were combined and evaporated to dryness. The residue was dissolved in THF (5 ml) and a catalytic amount of $(n\text{-}Bu)_4NBr$ was added and the mixture was stirred for 1 h at room temperature. A solution of $(n\text{-}Bu)_4NF$ in THF (0.31 ml, 1 M solution) was added and stirring was continued for 30 min. Extractive work-up with ethyl acetate gave a crude 5,7-diene diacetate. This was dissolved in THF (5 ml), and a solution of 5%-KOH in methanol (2 ml) was added. The mixture was stirred for 14 h at room temperature. Extractive work-up with ethyl acetate gave a crude product, which was purified by reverse phase chromatography (Merck. Lobar column RP-8, size A, methanol : water = 9 : 1) to afford 1α,3β,25-trihydroxy-26,27-dipropylcholesta-5,7-diene (8.5 mg). A solution of the 5,7-diene (8.5 mg) in benzene (90 ml) and ethanol (40 ml) was irradiated with an ultraviolet lamp (Hanovia 654A) through a Vycor filter under argon at 0 °C for 3 min. Then, the solution was refluxed for 1 h. After removal of the solvent, the residue was purified by preparative thin-layer chromatography (TLC) (benzene : ethyl acetate = 1 : 1, developed three times) to give the dipropyl derivatives of 1,25-$(OH)_2D_3$ (0.61 mg, 2%). ^{1}H-NMR δ: 0.54 (3H, s, 18-H), 0.92 (4H, t, J=7 Hz, CH_2), 0.93 (3H, d, J=6 Hz, 21-H), 4.27 (1H, m, 3-H), 4.44 (1H, m, 1-H), 5.01 (1H, br s, 19*Z*-H), 5.34 (1H, br s, 19*E*-H), 6.02 (1H, d, J=11 Hz, 7-H), 6.38 (1H, d, J=7 Hz, 6-H). UV (ethanol) λ_{max}: 265 nm, λ_{min}: 228 nm. MS *m/z*: 500 (M^+), 482, 107, 273, 251, 152, 143, 134. HRMS Calcd for $C_{33}H_{56}O_3$: 500.4230. Found: 500.4226.

Binding Assay of Vitamin D_3 Analogs for Chick Intestinal Cytosol 1,25-$(OH)_2D_3$-Specific Receptor The competitive receptor binding assay was performed as described previously.[6] [26,27-^{3}H]1,25-$(OH)_2D_3$ (157 Ci/mmol, 13300 dpm, 20 pq) and various amounts of vitamin D_3 analogs to be tested were dissolved in 50 μl of absolute ethanol in 12 × 75 mm polypropylene tubes. Chick intestinal cytosol receptor containing 1,25-$(OH)_2D_3$-specific receptor was prepared as described previously. One ml of the chick intestinal cytosol receptor protein was diluted to 0.3 mg protein/ml in phosphate buffer A (25 mM KH_2PO_4, 0.1 M KCl, 1 mM dithiothreitol, pH = 7.4) and 1 mg of gelatin were added to each tube in an ice-cold bath. The assay tube were incubated in a shaking water bath for 1 h at 25 °C and then chilled in the ice-cold bath. One ml of 40% (w/v) polyethylene glycol 6000 in distilled water was added to each, mixed vigorously, and then centrifugated at 4 °C. The supernant was decanted. The bottom of the tube with pellet was cut off and tranfered to a scintillation vial.

Assay of Vitamin D_3 Analogs for Multinucleated Cell (MNC) Formation Six-week old female BDF_1 mice were obtained from the Sizuoka Experimental Animal Center (Sizuoka, Japan) and were administered 5-fluorouracil (5-FU, F. Hoffmann-La Roche Co., Ltd., Basel, Switzerland) at a dosage of 150 mg/kg of body weight through a tail vein. Spleen cells were harvested at 4 d after the injection, and a single-cell suspensions were prepared from pooled spleens of 2 mice. Samples of 1.8×10^6/ml spleen cells were plated in 35 mm nontissue culture dishes (Falcon, Oxnard, California) in 1 ml of α-minimum essential medium (α-MEM, Flow Laboratories, Mclean, Virginia) containing 1.2% methylcellulose (Aldrich Chemical Company, Milwaukee, Wisconsin), 50 unit/ml of interleukin-3 (IL-3), 10 mg of deionized bovine serum albumin (Fraction V, Sigma, St. Louis, Missouri), and 30% fetal bovine serum (FBS, Flow Laboratories, Mclean, Virginia). Blast cell colonies appeared after about 5 d and were lifted with a Eppendorf micropipet. Pooled blast cells were washed several times with α-MEM and cell number was counted using a hemocytometer. Fifteen μl of medium containing 800 blast cells, 5% FBS, and 100 unit/ml of granulocyte macropharge colony stimulating factor (GM-CSF) was spotted into each of 48 wells of a microplate (Coaster, Cambridge, Massachusetts). After 12 h of incubation in a CO_2 incubator the cells were supplied with 250 μl of the same medium and were then cultured for one more day. The medium of the cultures was replaced with fresh medium containing GM-SCF and various concentrations of vitamin D_3 derivatives. The blast cells were further incubated for 4 d. The cells adherent to the plates were washed with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline, dried, and immediately stained with acid phosphatase kit in the presence of 25 mM L-tartrate (Sigma, St. Louis, Missouri). The cells were viewed under an inverted phase-contrast microscope, and those containing 3 or more nuclei were counted as MNC's. Results are expressed as the mean ± S.D. for triplicate determinations.

Assay of Vitamin D_3 Analogs for Bone Calcium Mobilization Male weanling rats were purchased from Charles River Japan (Atsugi, Japan) and were fed a vitamin D-deficient, low calcium diet (calcium 0.02%; phosphorus 0.3%) for 3 weeks. At the end of the 3rd week, a group of 6—7 rats received intravenous injection of various concentrations of vitamin D_3 derivatives in 0.05 ml of ethanol. At the time point as shown in Table I blood of the rats was drawn from the external iliac vein to measure serum calcium concentration. Serum was prepared by centrifugation of clotted blood and the serum calcium concentration was determined by the OCPC (*o*-cresolphthalein complexone) method.[7] Under the conditions of the assay elevations in serum calcium are a reflection of bone calcium mobilization.

Results and Discussion

The binding of 1,25-$(OH)_2D_3$ to its intracellular receptor is thought to be a preliminary step for eliciting biological action. The 26,27-dialkylated analogs were tested for their relative abilities to compete with [26,27-^{3}H]1,25-$(OH)_2D_3$ for the receptor from chick intestine. The results, shown in Fig. 1, indicated that the 26,27-dimethyl analog had almost the same affinity as 1,25-$(OH)_2D_3$ for the chick 1,25-$(OH)_2D_3$-specific receptor. While the diethyl and the

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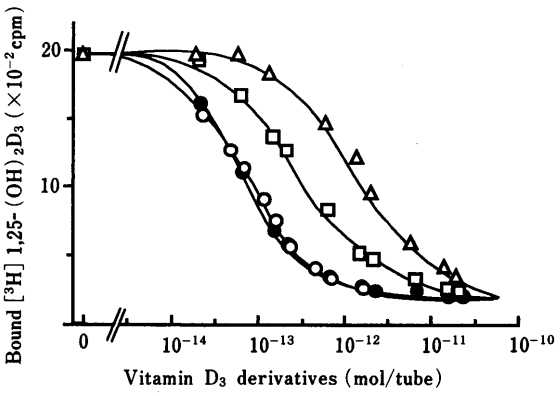

Fig. 1. The Binding Affinity of 26,26-Dialkylated Analogs of 1,25-$(OH)_2D_3$ to the Chick Intestinal 1,25-$(OH)_2D_3$-Specific Receptor

1,25-$(OH)_2D_3$ (○); 26,27-dimethyl-1,25-$(OH)_2D_3$ (●); 26,27-diethyl-1,25-$(OH)_2D_3$ (□); and 26,27-dipropyl-1,25-$(OH)_2D_3$ (△). Points are the mean of duplicate determinations.

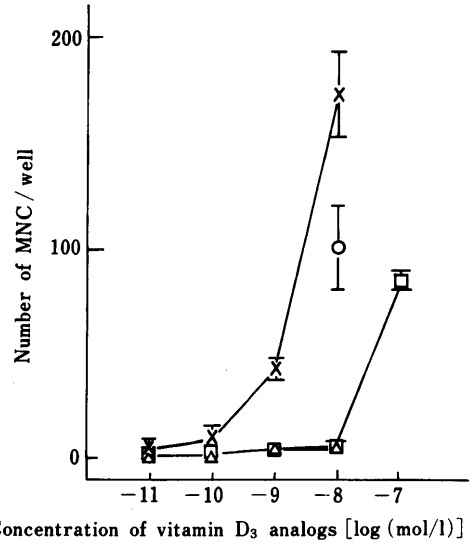

Fig. 2. Dose Response of the Ability of 26,27-Dialkylated Analogs of 1,25-$(OH)_2D_3$ for Formation of MNC from Blast Cells

1,25-$(OH)_2D_3$ (○); 26,27-dimethyl-1,25-$(OH)_2D_3$ (×); 26,27-diethyl-1,25-$(OH)_2D_3$ (□); and 26,27-dipropyl-1,25-$(OH)_2D_3$ (△). Results are expressed as the mean ± S.D. for triplicate determinations.

dipropyl analogs had weaker affinity for the receptor than that of 1,25-$(OH)_2D_3$. As determined by 50% displacement of [^{3}H]1,25-$(OH)_2D_3$ from the receptor, 26,27-dimethyl-, 26,27-diethyl-, and 26,27-dipropyl-1,25-$(OH)_2D_3$ were estimated to have about 1.1, 0.25, 0.05 times more affinity than 1,25-$(OH)_2D_3$, respectively.

Recently a culture system for osteoclast formation from blast cell colonies was established,[8] these colonies were derived from the spleen cells of 5-FU treated mice. These blast cells had specific binding protein for 1,25-$(OH)_2D_3$.[9] When these blast cells were cultured in medium containing IL-3 or GM-SCF and 1,25-$(OH)_2D_3$, MNC's with various osteoclastic cells were formed from the cells. 1,25-$(OH)_2D_3$ acts on the MNC formation in a dose-related manner. Therefore, this system is considered to be a useful *in vitro* model for osteoclast formation.[8] The 26,27-dialkylated analogs were tested in this system and the results are shown in Fig.2. The dimethyl and diethyl analogs induced the MNC's from blast cells in a dose-related manner, but the dipropyl compound did not show the ability to form the

TABLE I. Ability of 1,25-$(OH)_2D_3$ Analogs for Bone Calcium Mobilization in Vitamin D-Deficient Rats

	Dose (pmol/100 g of body weight)	Serum calcium concentration (mg/dl) 12 h	24 h
Experiment 1			
Vehicle		4.75±0.08	5.06±0.14
1,25-$(OH)_2D_3$	500	6.39±0.17[b]	5.60±0.19
	1000	7.16±0.21[b]	6.55±0.33[b]
26,27-Dimethyl	500	6.11±0.15[b]	6.57±0.17[c,b]
1,25-$(OH)_2D_3$	1000	6.60±0.18[b]	7.00±0.12[b]
	2000	7.38±0.20[b]	7.29±0.20[b]
Experiment 2			
Vehicle		3.94±0.13	4.35±0.18
1,25-$(OH)_2D_3$	500	4.43±0.16[a]	4.95±0.17[a]
	5000	5.20±0.27[b]	5.54±0.13[b]
26,27-Diethyl 1,25-$(OH)_2D_3$	5000	4.34±0.14	4.87±0.48
26,27-Dipropyl 1,25-$(OH)_2D_3$	5000	3.51±0.16	4.58±0.09

Significantly different from vehicle group (*a*) $p<0.05$, *b*) $p<0.01$). *c*) Significantly different from the same dose of 1,25-$(OH)_2D_3$. $p<0.01$.

MNC at the concentrations tested. Among the derivatives tested, including 1,25-$(OH)_2D_3$, the dimethyl analog showed the highest ability to induce the MNC formation. On the other hand, diethyl analog was much less active than 1,25-$(OH)_2D_3$.

The results of the bone calcium mobilization test in the vitamin D-deficient rats are shown in Table I. The 1,25-$(OH)_2D_3$ and the dimethyl analog significantly increased the serum calcium concentration in a dose-related manner. Since preliminary experiments exhibited that diethyl and dipropyl analogs showed no response at a same dose level as 1,25-$(OH)_2D_3$, the diethyl and dipropyl analogs were tested at a much higher dose (experiment 2). However, these compounds did not show any increase at this dosage. Of interest is that the dimethyl analog showed slightly less effectiveness than 1,25-$(OH)_2D_3$ at 12 h after administration, but at 24 h the reverse is the case. This may be due to the longer life of dimethyl analog by prevention of the deactivation step of metabolic hydroxylation at 26 or 27-positions as is the case with multi-fluoro derivatives of 1,25-$(OH)_2D_3$.[10] Other explanations are also possible and further experimentation is needed. In conclusing the biological activities, the dimethyl analog has the same or a slightly higher effectiveness than 1,25-$(OH)_2D_3$. The diethyl analog was less active and the dipropyl analog was significantly less active. The activity pattern can be explained on the basis of the affinity of these analogs for the 1,25-$(OH)_2D_3$-specific receptor.

It was reported that demethylation of one or two methyl groups at the 25-position of vitamin D_3 derivatives significantly diminish the activity, therefore 25-dimethyl groups of vitamin D_3 were considered to be essential for eliciting activity.[11] But, the present results, in addition to the previous data of studies on the 26-homo analogs[3a,d] and 26,27-dimethyl analog of 1,25-$(OH)_2D_3$,[3b,c,12] suggest that the lengthening by one carbon at 26- and/or 27-position enhance the activity rather than diminish activity. These data suggest that the receptor does not recognize the 25-dimethyl group of 1,25-$(OH)_2D_3$ rigorously in the binding

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states. Therefore we postulate that another factor in the side chain, for example, the nature of a 25-hydroxyl group such as electron density, is essential for the binding. The existence of a dimethyl group at the 25-position of vitamin D_3 might be essential to maintain the suitable nature of a 25-hydroxyl group in the binding state. When ethyl or propyl groups are substituted at the 26- and 27-positions, the binding affinity may be decreased ultimately by steric hindrance of those bulky groups.

As we reported previously, the diethyl analog has a preferential activity in inducing cell differentiation of human leukemia cells HL-60.[5b] It is not clear why this analog shows a high potency in cell differentiation since the mechanism of cell differentiation has not been clarified. It could be noted that a modification in the side chain, such as increasing side chain carbons by adding onto the terminal 26- and 27-positions and carbon homologation of 1,25-$(OH)_2D_3$,[5a] is likely to yield a therapeutically interesting compound.

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