BIOORGANIC CHEMISTRY 17, 294–307 (1989)

Synthesis and Biological Activities of 22-Hydroxy and 22-Methoxy Derivatives of 1α,25-Dihydroxyvitamin D_3: Importance of Side Chain Conformation for Biological Activities

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Received November 2, 1988

Both 22-epimers of 22-hydroxy and 22-methoxy derivatives of 1α,25-dihydroxyvitamin D_3 **(1)** were synthesized from 22-aldehyde **(6)** to clarify the precise structural requirement for exerting various biological activities. While the synthetic vitamin D derivatives did not show any increase of serum calcium concentration in rats by oral administration, all derivatives induced into nitroblue tetrazolium (NBT)-positive cells at a concentration more than 1 μg/ml in the NBT reduction test for induction of differentiation of HL-60 cells. Especially noteworthy was that 22*S*-isomers (**3** and **5**) were at least 30 times more effective than corresponding 22*R*-isomers (**2** and **4**), respectively. Binding affinities of the (22*S*)-22-methoxy derivatives **(5)** to the chick intestinal receptor for **1** was also about 3 times as potent as 22*R*-isomer **(4)**. A major structural difference was their side chain conformations, which were elucidated by molecular mechanics calculation (MM2 force field) and NMR studies. A zig-zag conformation turned out to be sterically most favorable for 22*S*-isomers, whereas such a zig-zag conformation is energetically unfavorable for 22*R*-isomers due to the interaction between the 22-substituent and the 16-methylene group. The side chain conformation seems to be responsible for the difference of their biological activity and the zig-zag conformation plays an important role for the activities.

INTRODUCTION

It is now well established that 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$, **1**][3] is a hormonal metabolite of vitamin D_3, which mediates calcium and phosphorus metabolism (*1*, *2*). Recently, a monoclonal antibody to a chick intestinal cytosolic receptor was generated and the primary structure of the receptor protein was determined (*3*). However, topological features of the receptor as a whole and of the binding site(s) of 1,25-$(OH)_2D_3$ are still unknown. In order to obtain insight

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[3] Abbreviations used: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; NBT, nitroblue tetrazolium; THF, tetrahydrofuran; THP, tetrahydropyran.

0045-2068/89 $3.00

into the structure of the receptor binding site, actual conformations of 1,25-$(OH)_2D_3$ and its derivatives must be prerequisite.

Since its discovery, a number of analogs of vitamin D_3 and its metabolites have been synthesized to increase and separate biological activities (*4*). Studies on the structure–activity relationships of these compounds have revealed that the 1α-hydroxyl group was essential for biological activities and that the hydroxyl groups at the 3β- and 25-positions and proper side chain length contributed as well (*4*). Conformation of A-ring of vitamin D derivatives has been discussed in terms of biological activities (*5, 6*). The side chain structure and conformation are important for biological activities.

Although the sterol side chain bears zig-zag conformation generally, there are several evidences suggesting that C-22 epimers with a substituent at C-22 position have different side chain conformations depending on their configuration (*7*). Thus, introduction of a substituent into the C-22 position of the side chain of 1,25-$(OH)_2D_3$ seems to induce conformational changes and this may serve as a clue in examining the importance of the side chain conformation for vitamin D activity. Previously, we synthesized 22-epimers of 22,25-dihydroxy- and 25-hydroxy-22-methoxyvitamin D_3, but neither vitamin D nor antivitamin D activity was observed (*8*). This may be due not only to the lack of the 1α-hydroxyl group but also the oxygenation on the C-22 position and the conformational changes of the side chain as well.

Additionally, as an intriguing biological action 1,25-$(OH)_2D_3$ has been reported to induce differentiation of malignant cells (*9*). For the most of analogs of 1,25-$(OH)_2D_3$, activities of the induction of differentiation seem to be proportional to the binding affinity to the chick intestinal receptor of 1,25-$(OH)_2D_3$ (*10, 11*).

It is therefore of interest to see the effect of the side chain conformation from the chemical as well as biological viewpoints for the activities of the 22-substituted analogs. We describe here the synthesis of (22*R*)- and (22*S*)-1α,22,25-trihydroxyvitamin D_3 (**2** and **3**) and (22*R*)- and (22*S*)-1α,25-dihydroxy-22-methoxy vitamin D_3 (**4** and **5**) and the results of biological activities including vitamin D activity, affinity to the chick intestinal receptor of 1,25-$(OH)_2D_3$, and induction of differentiation of HL-60 cells. The side chain conformations of these compounds were also investigated by means of molecular mechanics calculation (MM2 force field) and ^1H NMR studies.

RESULTS AND DISCUSSION

The syntheses of 22-substituted vitamin D_3 derivatives **(2–5)** were achieved by essentially the same method for the previously described synthesis of 22,25-dihydroxy- and 25-hydroxy-22-methoxyvitamin D_3 (*8*). The 22-aldehyde **(6)** (*12*) was coupled with lithium acetylide derived from 2-methyl-2-(2-tetrahydropyranyloxy)-but-3-yne to give a ca. 1 : 1.2 mixture of the less polar 22*S* compound **(7a)** and the more polar 22*R* compound **(7b)** (the stereochemistry of which is discussed below). After separation by the medium pressure chromatography, both isomers were separately converted to 1,3,22-triacetate (**8a** and **8b**) through the following reac-

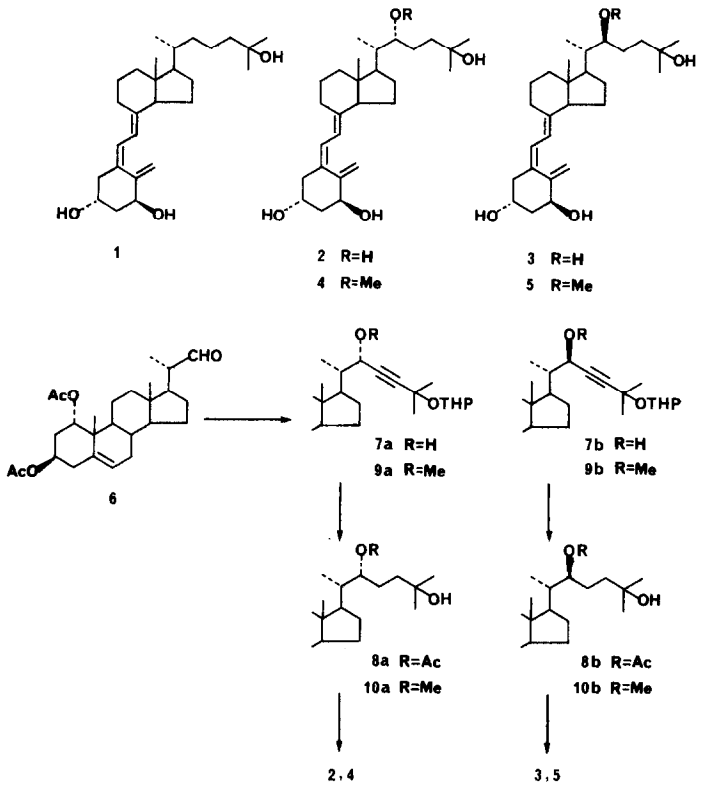

FIG. 1. Structures of **1–5** and synthetic scheme for **2–5.**

tions of acid hydrolysis, catalytic hydrogenation over Pd–C, and acetylation (Fig. 1).

The 22-methoxy derivatives (**9a** and **9b**) were prepared by treatment of **7a** and **7b** with methyl iodide and KOH in dimethyl sulfoxide followed by acidic hydrolysis and catalytic hydrogenation over Pd–C.

In order to determine the stereochemistry at the 22-position, acetylenic compounds (**7a** and **7b**) were converted to the allylic benzoates (**11a** and **11b**). Thus, partial hydrogenation of **7a** and **7b** over Pd–$CaCO_3$ in the presence of quinoline followed by acylation with *p*-bromobenzoyl chloride and acid hydrolysis afforded **11a** and **11b,** respectively. The C-22 stereochemistry of **11a** and **11b** was determined with a induced circular dichroic (CD) exciton chirality method (*13, 14*). As shown in Figs. 2A and 2B, *anti*-relationships between the 22- and 23-hydrogens for **11a** and **11b** were apparent from the coupling constant ($J_{22,23}$ = 10.5 and 8.6 Hz, respectively). In the CD spectra (in CH_3OH), **11a** showed a Cotton effect at 244 nm ($\Delta\varepsilon$ + 7.27), while **11b** showed a negative Cotton effect at 247 nm ($\Delta\varepsilon$ − 5.85). These results clearly indicated that **11a** and **11b** had 22*R* and 22*S* configuration, respectively (Fig. 2). Therefore, the C-22 stereochemistry of the less polar compound **(7a)** was established as *S* and the more polar compound **(7b)** as *R*.

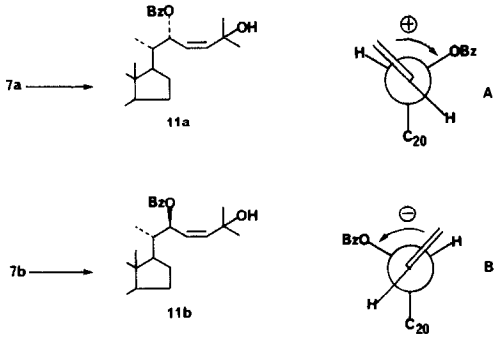

FIG. 2. Synthetic scheme and Newman projections of allylic benzoates **11a** and **11b**.

The above-mentioned precursory sterol acetates (**8a, 8b, 9a,** and **9b**) were transformed into the corresponding vitamin D form (**2, 3, 4,** and **5**) by the same method of our previous report (*8*).

An initial attempt to determine the side chain conformation of the compounds **(2–5)** by means of ^{1}H NMR [e.g., measurement of coupling constant for H_{20}–H_{22} and nuclear Overhauser effect (nOe)] was unsuccessful, because the signals due to 22-H showed complex multiplet and no significant nOe was observed around the C-22 position for these compounds. We decided then to apply the conformational analysis of these compounds by using MM2 force field calculation (*15*). Calculations were actually carried out for the model structures (**12a, 12b, 13a,** and **13b**). In all cases, the rotational driver was used incrementally in steps of 5° for the C17–C20–C22–C23 dihedral angles (steroidal numbering) from −180° to +180° (positive sign means clockwise rotation through the axis C20–C22). According to the results of MM2 calculations, the most stable conformer of the 22*S*-isomers (**12b** and **13b**) was found to have about −170° of the dihedral angle for C17–C20–C22–C23, at least 1 kcal/mol lower energy than the second lowest. On the other hand, in the case of the 22*R*-isomers (**12a** and **13a**), the dihedral angle for C17–C20–C22–C23 of the rotamers showing the lowest energy (at least 2 kcal/mol lower energy than the other local minima) was found to be about 70° (see Figs. 3C and 3D). As shown in Fig. 3B, the most stable side chain conformation was zig-zag conformation for the 22*S*-isomers. For the 22*R*-isomers, such a zig-zag conformation was sterically unfavorable, so the conformation like Fig. 3A was the most energetically stable. The difference of the side chain conformation between 22-stereoisomers can be attributed to an unfavorable interaction between the 22-substituent and the 16-methylene group.

To confirm the results of calculations, we synthesized 23,23,24,24-tetradeuterio derivatives (**14a** and **14b**) to simplify the ^{1}H NMR coupling patterns of the 22-H signals. Deprotection of the THP group of the compounds (**7a** and **7b**), followed by hydrogenation over Pd–C under $^2H_2$, afforded 23,23,24,24-$^2H_4$-labeled derivatives (**14a** and **14b**), respectively (Fig. 4). Compound **(14a)** showed a doublet at 3.63 ppm of the 22-H (J = 3.1 Hz). On the other hand, a broad singlet (J ≃ 0 Hz) was observed at 3.62 ppm for **14b.** Dihedral angles (H20–C20–C22–H22) were esti-

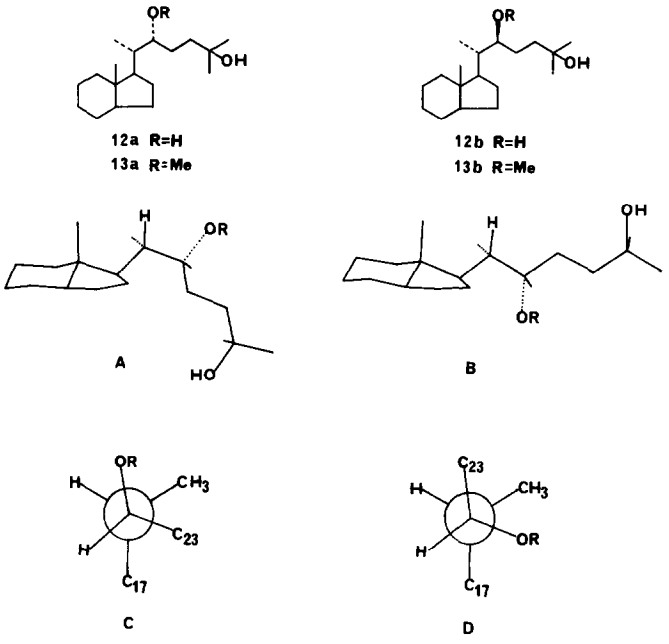

FIG. 3. Structures of the model compounds **12a, 12b, 13a,** and **13b,** wire models, and Newman projections for stable conformations of **12a, 12b, 13a,** and **13b.**

mated to be about 50° for 22*R*-isomer and to be nearly 90° for 22*S*-derivative according to the Karplus correlation. The conformations suggested on the basis of these ^1H NMR data were well coincided with the prediction from the force field calculations (see Newman projections in Figs. 3C and 3D). Consequently, the actual conformations can be illustrated as shown in Figs. 3A and 3B.

Biological tests of these compounds **(2–5)** for the vitamin D activity indicated no significant hypercalcemic effect in normal rats by oral administration. As shown in Table 1, even at 1 μg/kg dose, 1α-hydroxyvitamin D_3 (1α-OH-D_3) signifi-

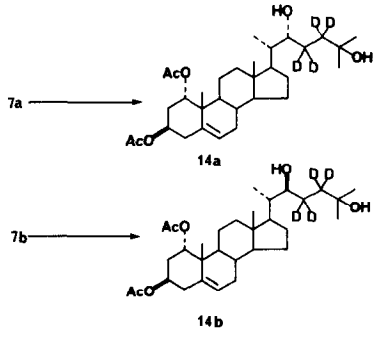

FIG. 4. Synthetic scheme for 23,23,24,24-$^2H_2$-labeled derivatives (**14a** and **14b**).

TABLE 1

Activity of Vitamin D_3 Derivatives for Increase of Serum Calcium Concentration in Rats

Compound	Dose (μg/kg)	Serum Ca^{2+} (mmol/liter)[a]
Control	—	1.44 ± 0.01
1α-OH-D_3	1	1.48 ± 0.01[b]
	3	1.53 ± 0.01[b]
	10	1.64 ± 0.01[b]
1α,22*R*,25-$(OH)_2D_3$ **(2)**	3	1.45 ± 0.01
	30	1.46 ± 0.01
22*R*-OMe-1α,25-$(OH)_2D_3$ **(3)**	30	1.41 ± 0.01
	100	1.46 ± 0.01
1α,22*S*,25-$(OH)_2D_3$ **(4)**	3	1.41 ± 0.01
	30	1.46 ± 0.01
22*S*-OMe-1α,25-$(OH)_2D_3$ **(5)**	30	1.43 ± 0.01
	100	1.41 ± 0.01

[a] The value represents mean ± standard deviation.
[b] Significantly different from control group $P < 0.01$.

cantly increased the serum calcium concentration in rats, but increase of the serum calcium concentration was not observed in much higher doses of **2–5.**

Figure 5 shows the dose–response curves of the percentage ratio of NBT-positive cells induced by various concentrations of vitamin D_3 derivatives. This assay method is one of the earliest differentiation markers expressed in maturing HL-60 cells (*16*). Among the derivatives tested, 1,25-$(OH)_2D_3$ showed the highest activity to induce the NBT-positive cells and the ED_{50} value (the concentration in μg/ml required for the induction of 50% NBT-positive cells) was estimated to be 0.018 μg/ml. Interestingly, upon comparison of the ED_{50} values, (22*S*)-isomers **(3)** and **(5)** were at least 30 times as effective as their corresponding 22*R*-isomers **(2)** and **(4).** The ED_{50} values of 22*S*-isomers **(3)** and **(5)** were 0.24 and 0.07 μg/ml, respectively, but >7.0 and 3.0 μg/ml for 22*R*-isomers **(2)** and **(4)**, respectively.

Furthermore, to clarify vitamin D activities on the receptor level, we determined the binding affinities of methoxy derivatives (**4** and **5**) to the 1,25-$(OH)_2D_3$ receptor of a chick intestinal cytosol, since the binding of hormone to its receptor must be a primary step for biological actions. Figure 6 shows the logalithmic concentration response curves of the vitamin D derivatives required for competitive inhibition against binding of [^{3}H]1,25-$(OH)_2D_3$ to the receptor. The quantity of the 22*S*-isomer **(5)** required to displace 50% of the bound 1,25-$(OH)_2D_3$ to the receptor was about a third of that of the 22*R*-isomer **(4).** The relative molar ratios for **(4)** and **(5)** to show binding affinity of equivalent to one molar 1,25-$(OH)_2D_3$ are estimated to be 95 and 35, respectively. In any case, the 22*S* derivatives with a substituent on C-22 showed significantly higher biological activities than the 22*R* counterparts. Further investigations for the biological activities of these compounds are now in progress.

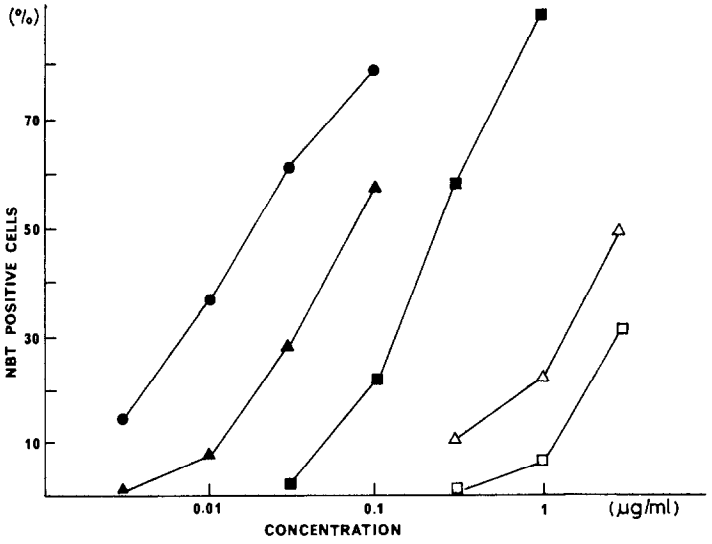

FIG. 5. Logarithmic dose–response curves of the percentage ratio of NBT-positive cells induced by various concentrations of (●) 1,25-dihydroxy- **(1)**, (□) (22*R*)-1,22,25-trihydroxy- **(2)**, (■) (22*S*)-1,22,25-trihydroxy- **(3)**, (△) (22*R*)-1,25-dihydroxy-22-methoxy- **(4)**, and (▲) (22*S*)-1,25-dihydroxy-22-methoxyvitamin D_3 **(5)**.

Generally, the zig-zag conformation for the steroidal side chain was thought to be energetically favored from the studies of X-ray crystallography of several steroids (*17*). 25-Hydroxyvitamin D_3 was also reported to have a similar zig-zag conformation in a crystalline form (*18*). Although it is not clear why the aforemen-

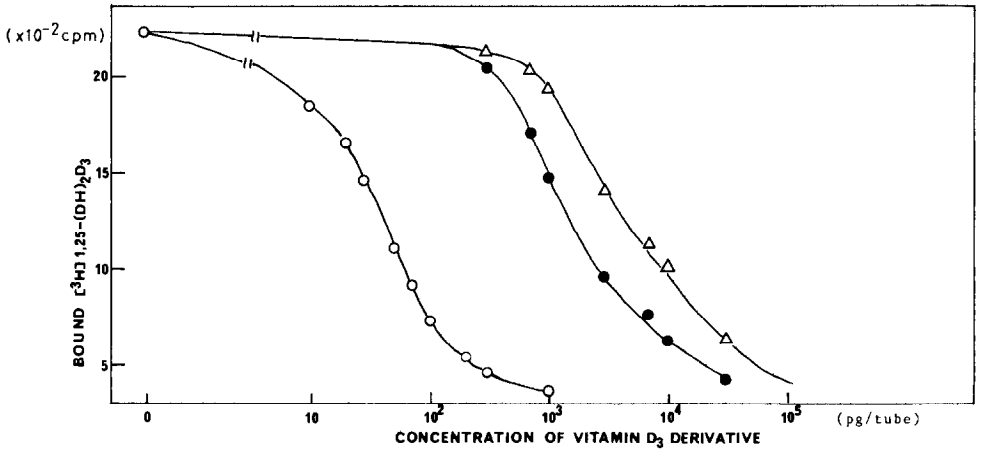

FIG. 6. Dose–response curves of binding affinity to chick intestinal cytosol receptor of (○) 1,25-dihydroxy- **(1)**, (△) (22*R*)-1,25-dihydroxy-22-methoxy- **(4)**, and (●) (22*S*)-1,25-dihydroxy-22-methoxyvitamin D_3 **(5)**.

tioned synthetic derivatives showed no activities in *in vivo* experiments, the present results of the *in vitro* biological assays clearly indicated that the 22*S*-isomers have greater activities than the 22*R*-isomers. Taking into account the conformational studies described above, the difference of biological activities may be attributed to their side conformation. The zig-zag conformation seems to be an important role in exerting their biological functions. The concept of conformational optimization for biological activities, especially elucidating an actual binding conformation of 1,25-$(OH)_2D_3$ to the receptor, is clearly one that should be further examined.

EXPERIMENTAL

Synthesis

Melting points were determined on a hot stage with a microscope and are uncorrected. Ultraviolet spectra were obtained in an ethanol solution with a Shimadzu double-beam spectrometer. ^{1}H NMR spectra were taken on a JEOL FX-200 spectrometer and/or a JEOL GX-270 spectrometer in $CDCl_3$ solution using tetramethylsilane as an internal standard unless otherwise stated. Electron impact mass spectra (MS) were obtained with a Shimadzu 9020-DF spectrometer or JEOL DX-303 spectrometer at 20 or 70 eV. Circular dichroism spectra were determined in a methanol solution with a JASCO J-500C spectrometer. Preparative thin layer chromatography (p-TLC) was performed on precoated glass plates (E. Merck, silica gel 60 F_{254}, 0.25 mm thickness). Column chromatography was carried out on silica gel 60 (E. Merck, 70–230 mesh). Extractive workup refers to dilution of the reaction mixture with water (or indicated solvent), extraction with the given organic solvent, washing of the extracts to neutrality with brine, drying over Na_2SO_4, filtration, and removal of the solvent under reduced pressure.

(22S and 22R)-1α,3β-Diacetoxy-25-(2-tetrahydropyranyloxy)-cholest-5-en-23-yn-22-ol (***7a*** *and* ***7b***). A solution of *n*-butyl lithium (1.97 ml, 1.56 M solution in hexane, 2.97 mmol) was added to a solution of 2-methyl-2-(2-tetrahydropyranyloxy)-but-3-yne (0.61 ml, 2.97 mmol) with ice-cooling under argon. After 5 min, the mixture was cooled to −78°C and a solution of **6** (912 mg, 2.12 mmol) in THF (5 ml) was added. The mixture was stirred at −78°C for 20 min. Addition of saturated aqueous NH_4Cl solution and extractive workup (ethyl acetate) gave an oily residue. The residue was applied to a column chromatography over silica gel with hexane–ethyl acetate (5 : 1) as an eluant to give a mixture of 22-isomers. Further purification was carried out by medium pressure column chromatography (Lobar column, E. Merck, Lichroprep Si60, size B) with hexane–ethyl acetate (2 : 1) as an eluant to give the less polar isomer **7a** (213 mg, 17%) and the more polar **7b** (270 mg, 21%).

(22R and 22S)-1α,3β,22-Triacetoxycholest-5-ene-25-ol (***8a*** *and* ***8b***). A solution of **7a** (109 mg) and 2 N HCl (three drops) in methanol (3 ml) and THF (3 ml) was stirred for 3 h at room temperature. Addition of saturated aqueous $NaHCO_3$ solution and extractive workup (ethyl acetate) gave oily residue. A suspension of

the residue and 10% Pd–C (10 mg) in ethyl acetate (10 ml) was stirred for 4 h under an atmosphere of hydrogen at room temperature. The catalyst was filtered off and washed several times with ethyl acetate. The filtrate and washings were combined and concentrated to dryness to give an oily residue, which was dissolved in pyridine (0.5 ml). Acetic anhydride (0.1 ml) was added and the mixture was stirred overnight at room temperature. Extractive workup (ethyl acetate) and chromatography over silica gel with hexane–ethyl acetate (4 : 1) as an eluant gave the acetate **8a** (72 mg, 70%).

The compound **7b** (154 mg) was converted to **8b** (108 mg, 75%) by the same manner described for **8a.**

*(22R and 22S)-1α,22,25-Trihydroxyvitamin D_3 (**2** and **3**).* A mixture of **8a** (18.4 mg) and *N*-bromosuccinimide (8.2 mg, 1.4 eq) in carbon tetrachloride (2 ml) was refluxed under argon for 20 min. After being cooled with ice water, the insoluble materials were filtered off and washed with carbon tetrachloride. The filtrate and washings were combined and evaporated to dryness. The residue was dissolved in THF (5 ml) and a catalytic amount of tetra-*n*-butylammonium bromide was added. The mixture was stirred under argon at room temperature. After 50 min, a solution of tetra-*n*-butylammonium fluoride in THF (0.97 ml, 3.5 eq, 1 M solution) was added and stirring was continued for 30 min. Extractive (ethyl acetate) workup gave a crude product. This was dissolved in acetone (10 ml) and a catalytic amount of *p*-toluenesulfonic acid was added. The mixture was stirred at room temperature for 14 h. Extractive (ethyl acetate) workup gave a crude product, which was purified by p-TLC with hexane–ethyl acetate (2 : 1, five times development) as a developing solvent to afford the 5,7-diene (1.75 mg). A solution of 5,7-diene (1.75 mg) in benzene (90 ml) and ethanol (40 ml) was irradiated by an ultraviolet lamp (Hanovia 654A; 200 W) through a Vycor filter under argon with ice-cooling for 2.5 min. Then, the solution was refluxed for 1 h. After removal of the solvent, the residue was purified by p-TLC with hexane–ethyl acetate (2 : 1, five times development) as a developing solvent to give a vitamin D_3 triacetate derivative, which was dissolved in THF (5 ml) and 5% KOH–methanol solution (2 ml) was added. The mixture was stirred at room temperature overnight. Extractive workup and purification by high-performance liquid chromatography (Zorbax Sil, 4.6 mm × 25 cm, flow rate 2 ml/min) with 5% methanol in CH_2Cl_2 as an eluant gave **2** (0.12 mg, 0.8% from diacetate **8a**).

The compound **8b** (14 mg) was converted to **4** (0.13 mg, 1.2%) by the same manner described for **2.**

*(22S and 22R)-1α,3β-Diacetoxy-22-methoxy-25-(2-tetrahydropyranyloxy)-cholest-5-en-23-yne (**9a** and **9b**).* To a solution of **7a** (100 mg) in dimethyl sulfoxide (1.5 ml) was added powdered KOH (13.2 mg, 2 eq), followed by MeI (31 μl, 4 eq). The mixture was stirred at room temperature for 3.5 h. Extractive workup (ethyl acetate) and chromatography over silica gel with hexane–ethyl acetate (7 : 1) as an eluant gave the methyl ether **9a** (92 mg, 90%).

The compound **7b** (103 mg) was converted to **9b** (70 mg, 67%) by the same manner described for **9a.**

*(22R and 22S)-1α,3β-Diacetoxy-22-methoxycholest-5-en-25-ol (**10a** and **10b**).* A solution of **9a** (105 mg) and 2 N HCl (one drop) in THF (2 ml) and methanol (2 ml)

was stirred at room temperature for 1.5 h. Extractive workup (ethyl acetate) gave an oily residue, which was dissolved in methanol (10 ml) and 10% Pd–C (80 mg) was added. The mixture was stirred under an atmosphere of hydrogen at room temperature for 40 min. The catalyst was filtered off and washed several times with methanol. The combined filtrate and washings were concentrated to give an oily residue, which was applied to column chromatography over silica gel with hexane–ethyl acetate (5 : 1) to give **10a** (57.7 mg, 63%).

The compound **9b** (79 mg) was converted to **10b** (51 mg, 73%) by the same manner described for **10a.**

*(22R and 22S)-1α,25-Dihydroxy-22-methoxyvitamin D_3 (**4** and **5**).* The compound **10a** (26 mg) was converted to **4** (1.02 mg, 4.8%) by the same manner described for **2.**

The compound **10b** (33 mg) was converted to **5** (0.74 mg, 2.7%) by the same manner described for **2.**

*(22S,23Z and 22R,23Z)-1α,3β-Diacetoxycholesta-5,22-diene-22, 25-diol 22-p-Bromobenzoate (**11a** and **11b**).* A mixture of **7a** (30 mg), 5% Pd–$CaCO_3$ (15 mg), and quinoline (20 μl) in methanol (5 ml) was stirred under an atmosphere of hydrogen at room temperature for 6 h. The catalyst was filtered off and washed several times with methanol. The filtrate and washings were combined and concentrated to give an oily residue, which was dissolved in pyridine (2 ml). To this solution was added *p*-bromobenzoyl chloride (55 mg) and the resulting mixture was stirred at room temperature overnight. Extractive workup (ether) gave a residue, which was dissolved in THF (3 ml) and methanol (3 ml). To this solution was added 2 N HCl (one drop) and the mixture was stirred at room temperature for 1 h. Extractive workup and p-TLC with hexane–ethyl acetate (10 : 1, developed six times) gave the *cis*-benzoate **11a** (12 mg).

The compound **7b** (30 mg) was converted to **11b** (4.0 mg) by the same manner described for **11a.**

*(22S and 22R)-(22,23,24,24-$^2H_4$)1α,3β-Diacetoxy-22,25-dehydroxycholest-5-ene (**14a** and **14b**).* A solution of **7a** (42 mg) and 2 N HCl (one drop) in THF (1 ml) and methanol (1 ml) was stirred at room temperature for 3 h. Extractive workup (ethyl acetate) gave an oily residue, which was dissolved in ethyl acetate (5 ml). Ten percent Pd–C (10 mg) was added and the mixture was stirred under an atmosphere of $^2H_2$ at room temperature for 3 h. The catalyst was filtered off and washed several times with ethyl acetate. The filtrate and washings were combined and concentrated to dryness. The residue was purified by column chromatography over silica gel with hexane–ethyl acetate (2 : 1) as an eluant to afford the deuterio compounds **14a** (16 mg). This sample was analyzed by ^1H NMR without further purification.

The compound **7b** (57 mg) was converted to **14b** (30 mg) by the same manner described for **14a.**

Measurement of Increment for Serum Calcium Concentration

Wistar rats (5 weeks) were divided into groups. Each group contained six rats. The corresponding doses of vitamin D derivatives dissolved in 2 ml/kg (rat weight)

of corn oil were administered orally to the rats. After 24 h, the rats received the second same dose. Rats in the control group received the vehicle alone. Nineteen hours after the second dose, blood from all rats was drawn from the external iliac vein to measure calcium concentration. The blood was centrifuged to yield serum and the serum calcium concentrations were measured by means of an atomic absorption spectrometer.

NBT Reduction Tests

The activity inducing the differentiation of HL-60 human promyelocytic leukemia cells was determined by a modified method of the previously reported (*11*), which was adopted from the method of the NBT reduction test modified by Bonder *et al.* (*19*) and Tarella *et al.* (*20*). Microwell plates (A/S nunc, roskild, Denmark) were used throughout for spectrophotometric measurement with a multiwell photometer (Bio-Rad Model 2550 EIA reader, Richmond, CA). HL-60 cells were placed into 96 wells at 3.5×10^5 cells/ml in the RPMI 1640 medium supplemented with 15% fetal blood serum, and vitamin D_3 derivatives were added to each well at various concentrations. After being grown for 2 days at 37°C in 5% CO_2 in air, the cells were washed with 0.15 M Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 0.1 ml of serum-free culture medium and 0.1 ml of 0.2% NBT (Sigma Chemical Co., St. Louis, MO) dissolved in DPBS, in the presence of 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate. Following incubation for 30 min at 37°C the media from the plates were aspirated completely after centrifugation and 0.1 ml of 10% sodium dodecyl sulfate solution was added to each well. The plates were allowed to stand overnight at room temperature and a blue formazan product was measured at 540 nm with a multiwell photometer.

Determination of Binding Affinity of Vitamin D Derivatives for the Chick Intestinal Cytosol

The competitive receptor binding assay was performed as described previously (*21*) by the method of Eisman *et al.* (*22*). Tritium-labeled 1,25-$(OH)_2D_3$ (0.0036 μCi) and vitamin D derivatives were added to 1 ml of a cytosol fraction (300 μg protein). The suspension mixture was incubated with shaking for 60 min at 25°C and then chilled in the ice bath. One milliliter of 40% (w/v) polyethylene glycol 6000 was added to the mixture with stirring. The precipitate was obtained by a centrifugation at 2260 *g* for 60 min (at 4°C). By measuring radioactivity in the precipitate using a liquid scintillation counter, the binding volume of [^{3}H]1,25-$(OH)_2D_3$ to the receptor was determined.

APPENDIX

Physicochemical Properties of the Synthetic Compounds

7a: mp 116–118°C (ether–hexane); ^{1}H NMR δ 0.70 (3H, s, 18-H), 1.04 (3H, d, J = 6 Hz, 21-H), 1.09 (3h, s, 19-H), 1.50 and 1.54 (6H, each s, 26- and 27-H), 4.47

(1H, d, J = 3.7 Hz, 22-H), 4.95 (1H, m, 3-H), 5.07 (2H, m, 1-H and 2′-H of THP). *Anal.* Calcd for $C_{36}H_{54}O_7$: C, 72.21; H, 9.09. Found: C, 72.20; H, 9.21.

7b: oil; ^{1}H NMR δ 0.68 (3H, s, 18-H), 1.08 (3H, s, 19-H), 1.09 (3H, d, J = 6 Hz, 21-H), 1.48 and 1.52 (6H, each s, 26- and 27-H), 4.48 (1H, d, J = 1 Hz, 22-H), 4.93 (1H, m, 3-H), 5.07 (2H, m, 1-H and 2′-H of THP). *Anal.* Calcd for $C_{36}H_{54}O_7$: C, 72.21; H, 9.09. Found: C, 72.07; H, 9.21.

8a: mp 174–176°C (from ether–hexane); ^{1}H NMR δ 0.67 (3H, s, 18-H), 0.93 (3H, d, J = 7 Hz, 21-H), 1.08 (3H, s, 19-H), 1.21 and 1.22 (6H, each s, 26- and 27-H), 4.95 (2H, m, 3- and 22-H), 5.03 (1H, m, 1-H). *Anal.* Calcd for $C_{33}H_{52}O_7$: C, 70.68; H, 9.35. Found: C, 70.40; H, 9.26.

8b: oil; ^{1}H NMR δ 0.67 (3H, s, 18-H), 0.97 (3H, d, J = 7 Hz, 21-H), 1.08 (3H, s, 19-H), 1.21 (s, 6H, 26- and 27-H), 4.92 (2H, m, 3- and 22-H), 5.06 (1H, m, 1-H). *Anal.* Calcd for $C_{33}H_{52}O_7$: C, 70.68; H, 9.35. Found: C, 70.63; H, 9.41.

2: uv λ_{max} 265 nm, λ_{min} 228 nm; ^{1}H NMR δ 0.57 (3H, s, 18-H), 0.86 (3H, d, J = 6 Hz, 21-H), 1.22 (6H, s, 26- and 27-H), 3.63 (1H, m, 22-H), 4.22 (1H, m, 3-H), 4.43 (1H, m, 1-H), 5.01 (1H, br.s, 19*E*-H), 5.33 (1H, br.s, 19*Z*-H), 6.03 (1H, d, J = 11 Hz, 7-H), 6.38 (1H, d, J = 11 Hz, 6-H); MS m/z 432 (M^+), 414, 396, 269, 251, 152, 134. High resolution mass spectrum, Calcd for $C_{27}H_{44}O_4$:432.3239. Found: m/z 432.3219.

3: uv λ_{max} 265 nm, λ_{min} 228 nm; ^{1}H NMR δ 0.56 (3H, s, 18-H), 0.89 (3H, d, J = 6 Hz, 21-H), 1.22 and 1.23 (6H, each s, 26- and 27-H), 3.64 (1H, m, 22-H), 4.23 (1H, m, 3-H), 4.46 (1H, m, 1-H), 5.03 (1H, br.s, 19*E*-H), 5.35 (1H, br.s, 19*Z*-H), 6.05 (1H, d, J = 11 Hz, 7-H), 6.40 (1H, d, j = 11 Hz, 6-H); MS m/z 432 (M^+), 414, 396, 269, 251, 152, 134. High resolution mass spectrum, Calcd for $C_{27}H_{44}O_4$: 432.3239. Found: 432.3209.

9a: oil; ^{1}H NMR δ 0.69 (3H, s, 18-H), 1.01 (3H, d, J = 6.5 Hz, 21-H), 1.09 (3H, s, 19-H), 1.52 and 1.55 (6H, each s, 26- and 27-H), 3.37 (3H, s, OCH_3), 3.97 (1H, d, J = 3.5 Hz, 22-H), 4.92 (1H, m, 3-H), 5.05 (2H, m, 1-H, and 2′-H of THP). *Anal.* Calcd for $C_{37}H_{56}O_7$: C, 72.51; H, 9.21. Found: C, 72.25; H, 9.01.

9b: oil; ^{1}H NMR δ 0.68 (3H, s, 18-H), 1.08 (3H, d, J = 6.5 Hz, 21-H), 1.08 (3H, s, 19-H), 1.50 and 1.54 (6H, each s, 26- and 27-H), 3.34 (3H, s, OCH_3), 3.98 (1H, s, 22-H), 4.92 (1H, m, 3-H), 5.05 (2H, m, 1-H and 2′-H of THP). *Anal.* Calcd for $C_{37}H_{56}O_7$: C, 72.51; H, 9.21. Found: C, 72.51; H, 9.21.

10a: oil; ^{1}H NMR δ 0.71 (3H, s, 18-H), 0.87 (3H, d, J = 6 Hz, 21-H), 1.09 (3H, s, 19-H), 1.21 (6H, s, 26- and 27-H), 3.05 (1H, m, 22-H), 3.27 (3H, s, OCH_3), 4.92 (1H, m, 3-H), 5.02 (1H, br.s, 1-H). *Anal.* Calcd for $C_{32}H_{52}O_6$: C, 72.14; H, 9.84. Found: C, 72.14; H, 9.83.

10b: oil; ^{1}H NMR δ 0.67 (3H, s, 18-H), 0.89 (3H, d, J = 6 Hz, 21-H), 1.08 (3H, s, 19-H), 1.23 (6H, s, 26- and 27-H), 3.10 (1H, m, 22-H), 3.36 (3H, s, OCH_3), 4.92 (1H, m, 3-H), 5.05 (1H, br.s, 1-H). *Anal.* Calcd for $C_{32}H_{52}O_6$: C, 72.14; H, 9.84. Found: C, 72.01; H, 10.02.

4: uv λ_{max} 265 nm; λ_{min} 228 nm; ^{1}H NMR δ 0.59 (3H, s, 18-H), 0.89 (3H, d, J = 6.6 Hz, 21-H), 1.22 (6H, s, 26- and 27-H), 3.07 (1H, m, 22-H), 3.29 (3H, s, OCH_3), 4.21 (1H, m, 3-H), 4.43 (1H, m, 1-H), 5.01 (1H, br.s, 19*E*-H), 5.32 (1H, br.s, 19*Z*-H), 6.02 (1H, d, J = 11 Hz, 7-H), 6.39 (1H, d, J = 11 Hz, 6-H); M/S m/z 446 (M^+), 428 (M^+-H_2O), 383, 366, 290, 271, 251, 152, 134. High resolution mass spectrum, Calcd for $C_{28}H_{46}O_4$: 446.3395. Found: 446.3321.

5: uv λ_{max} 265 nm; λ_{min} 228 nm; ^{1}H NMR δ 0.55 (3H, s, 18-H), 0.91 (3H, d, J = 6.6 Hz, 21-H), 1.24 and 1.25 (6H, each s, 26- and 27-H), 3.11 (1H, m, 22-H), 3.38 (3H, s, OCH_3), 4.21 (1H, m, 3-H), 4.45 (1H, m, 1-H), 5.01 (1H, br.s, 19*E*-H), 5.34 (1H, br.s, 19*Z*-H), 6.03 (1H, d, J = 11 Hz, 7-H), 6.39 (1H, d, J = 11 Hz, 6-H); MS *m/z* 446 (M^+), 428 (M^+-H_2O), 383, 366, 290, 271, 251, 152, 134. High resolution mass spectrum, Calcd for $C_{28}H_{46}O_4$: 446.3395. Found: 446.3325.

11a: mp 132–134°C (from methanol); ^{1}H NMR (CD_3OD) δ 0.67 (3H, s, 18-H), 1.04 (3H, s, 19-H), 1.09 (3H, d, J = 6 Hz, 21-H), 1.23 (6H, s, 26- and 27-H), 5.36 (1H, dd, J = 10.5 and 12.0 Hz, 23-H), 5.48 (1H, m, 6-H), 5.65 (1H, d, J = 12.0 Hz, 24-H), 6.34 (1H, dd, J = 3.3 and 10.5 Hz, 22-H); uv λ_{max} 245 nm (ε 23,000); CD Δε +7.27 (244 nm).

11b: mp 179–181°C (from acetone–hexane); ^{1}H NMR (CD_3OD) δ 0.68 (3H, s, 18-H), 1.04 (3H, s, 19-H), 1.12 (3H, d, J = 6 Hz, 21-H), 1.27 and 1.38 (6H, each s, 26- and 27-H), 4.96 (1H, m, 1-H), 5.35 (1H, dd, J = 8.6 and 12.0 Hz, 23-H), 5.45 (1H, m, 6-H), 5.50 (1H, d, J = 12.0 Hz, 24-H), 6.41 (1H, dd, J = 1 and 8.6 Hz, 22-H); uv λ_{max} 245 nm (ε 22,000); CD Δε −5.85 (247 nm).

14a: ^{1}H NMR δ 0.69 (3H, s, 18-H), 0.94 (3H, d, J = 6 Hz, 21-H), 1.11 (3H, s, 19-H), 1.24 (6H, s, 26- and 27-H), 3.63 (1H, d, J = 3.1 Hz, 22-H).

14b: ^{1}H NMR δ 0.69 (3H, s, 18-H), 0.94 (3H, d, J = 6 Hz, 21-H), 1.11 (3H, s, 19-H), 1.23 (6H, s, 26- and 27-H), 3.62 (1H, s, 22-H).

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (61124007) from the Ministry of Education, Science, and Culture, Japan. The authors thank Dr. S. Ishizuka, Teijin Institute for Bio-Medical Research, for the measurement of binding affinity and also Central Research Laboratory, Yamanouchi Pharmaceutical Co., Ltd., for a part of the measurement of biological activity.

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