J. steroid Biochem. Vol. 35, No. 6, pp. 655–664, 1990
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0022-4731/90 \$3.00 + 0.00
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# SYNTHESIS AND BIOLOGICAL ACTIVITY OF (22*E*,25*R*)- AND (22*E*,25*S*)-22-DEHYDRO-1α,25-DIHYDROXY-26-METHYLVITAMIN $D_3$

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(*Received* 18 *August* 1989)

**Summary**—Both 25-epimers of (22*E*)-22-dehydro-1α,25-dihydroxy-26-methylvitamin $D_3$ [22-dehydro-26-methyl-1,25-$(OH)_2D_3$] were synthesized. The biological activity of these compounds was tested in binding affinity to chick intestinal receptor protein of 1α,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$] and in stimulating for intestinal calcium transport and bone calcium mobilization with vitamin D-deficient rats. The relative potency of (25*R*)- and (25*S*)-22-dehydro-26-homo-1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ in competing for the intestinal cytosolic binding was 1.7:1.5:1. A similar order of activity was observed on intestinal calcium transport and bone calcium mobilization. In the ability for stimulation of intestinal calcium transport, (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ were about 3.6 and 2.1 times as active as 1,25-$(OH)_2D_3$, respectively. In bone calcium mobilization tests, (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ were estimated to be 2.2 and 1.6 times as potent as 1,25-$(OH)_2D_3$, respectively.

## INTRODUCTION

Vitamin $D_3$ is synthesized in the skin from 7-dehydrocholesterol in a non-enzymatic reaction by ultraviolet light followed by thermal isomerization. Alternatively, vitamin $D_3$ from dietary source is taken up from the intestine. Vitamin $D_2$ is provided only by dietary source [1]. Vitamin $D_3$ is hydroxylated to 25-hydroxyvitamin $D_3$ in the liver and subsequently in the kidney to 1α,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$], the most potent and hormonal form of vitamin $D_3$ [1]. Vitamin $D_2$ also undergoes metabolic activation in the liver and kidney to 1α,25-dihydroxyvitamin $D_2$ [1,25-$(OH)_2D_2$] [1]. It has been interesting to compare the biological activity of vitamin $D_2$ metabolites with the corresponding vitamin $D_3$ counterparts. Limited biological testing has demonstrated that biological activity of 1,25-$(OH)_2D_2$ is similar to that of 1,25-$(OH)_2D_3$ in mammals [2] whereas in birds it is about one-tenth as potent as the corresponding vitamin $D_3$ compound [3]. In order to study the functional role of the 24-methyl group of vitamin $D_2$, DeLuca and his co-workers synthesized 25-hydroxyvitamin $D_2$ and its epimer, 24-epi-25-hydroxyvitamin $D_2$ (**1**) [4] and investigated their biological activity and metabolism [5]. During those studies, an active new metabolite was obtained in addition to 24-epi-1α,25-dihydroxyvitamin $D_2$ (**3**) and 24-epi-24,25-dihydroxyvitamin $D_2$ (**4**) from the incubation mixture of chick kidney homogenate with 24-epi-25-hydroxyvitamin $D_2$ (**1**), and was subsequently identified as (22*E*)-22-dehydro-1α,25-dihydroxy-26-methylvitamin $D_3$ [22-dehydro-26-methyl-1,25-$(OH)_2D_3$, (**2**)] (Scheme 1). This unexpected metabolite is as active as natural 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ in binding to chick intestinal cytosol receptor [5]. Also of interest is that the metabolite was ten times more potent than 1,25-$(OH)_2D_3$ in inducing cell differentiation against HL-60 cells [6]. Since the stereochemistry at the 25-position of the metabolite has not been elucidated so far, it is therefore of interest to investigate the biological activity of the metabolite in terms of the stereochemistry at 25-position. We undertook synthesis of both 25-epimers of this unique metabolite. In this paper, we described the synthesis and biological activities of both epimers of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$. Steric requirements of the side chain of the vitamin $D_3$ in the binding state to the receptor are also discussed.

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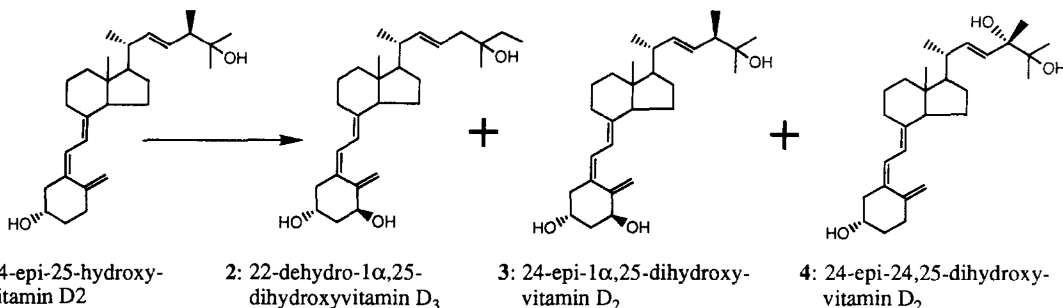
*Abbrevations*: 25-OH-$D_3$; 25-hydroxyvitamin $D_3$, 1,25-$(OH)_2D_3$; 1α,25-dihydroxyvitamin $D_3$, 1,25-$(OH)_2D_2$: 1α,25-dihydroxyvitamin $D_2$, 22-dehydro-26-methyl-1,25-$(OH)_2D_3$; (22*E*)-22-dehydro-1α,25-dihydroxy-26-methylvitamin $D_3$, 22-dehydro-26-methyl-25-OH-$D_3$; (22*E*)-22-dehydro-25-hydroxy-26-methylvitamin $D_3$, THF; tetrahydrofuran.

## EXPERIMENTAL

### General

[$^1$H]NMR spectra were taken with a Hitachi R-24A (60 MHz), JEOL FX-200 (200 MHz) or JEOL




**1**: 24-epi-25-hydroxyvitamin D2 **2**: 22-dehydro-1α,25-dihydroxyvitamin $D_3$ **3**: 24-epi-1α,25-dihydroxyvitamin $D_2$ **4**: 24-epi-24,25-dihydroxyvitamin $D_2$

Scheme 1

GSX-500 (500 MHz) spectrometer in $CDCl_3$ solution with tetramethylsilane as an internal standard. Mass spectra (MS) were obtained with a Shimadzu LKB-9020DF, Shimadzu GCMS-QP1000, or Hitachi M-80B spectrometer. Ultra-violet (u.v.) spectra were recorded with Shimadzu u.v.-200 double beam spectrometer or a Hitachi 228 spectrometer in ethanol solution for vitamin $D_3$ analogues. Infrared (i.r.) spectra were recorded in chloroform with Hitachi 260-10 spectrometer. High-performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-3A or a Hitachi 635A using a Zorbax Sil (DuPont, 4.6 mm × 25 cm) monitoring at 264 nm. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter Model 3255 using an external standard. Preparative thin-layer chromatography (p-TLC) was performed on 20 × 20 cm glass plates coated with a 0.25 mm layer of Kieselgel 60 $F_{254}$ (E. Merck). Column chromatography was carried out on Kieselgel 60 (E. Merck, 70–230 mesh). The usual work-up refers to dilution with water, extraction with the given organic solvent, drying over $MgSO_4$, filtration, and removal of the solvent under reduced pressure. [26,27-$^3$H]1,25-$(OH)_2D_3$ (sp. act. 158 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. [$^{45}$Ca]Calcium chloride (sp. act. 44.56 mCi/mg) was obtained from New England Nuclear Research Products, DuPont.

### Chemical synthesis

*(22E)-3β,25-Diacetoxycholesta-5,22-dien-26-oic acid (6).* 25-Hydroxy-3β-tetrahydropyranyloxy-cholesta-5,22-diene-26-oic acid ethyl ester (**5**) (0.98 g) was dissolved in THF (7 ml) and methanol (7 ml). To this solution was added *p*-toluenesulfonic acid (10 mg) and the mixture was stirred at room temperature for 1.5 h. The usual work-up (ethyl acetate) gave a crude diol, which was dissolved in methanol (14 ml). Aqueous solution (12 ml) of LiOH (785 mg) was added and the mixture was stirred at room temperature for 14 h. The usual work-up (ethyl acetate) gave a crude acid. The acid was then treated with acetic anhydride (1 ml) in pyridine (4 ml) at room temperature for 16 h. The usual work-up (ether) and column chromatography with hexane–ethyl acetate (4:1) containing 1% acetic acid as an eluent afforded the diacetoxy-acid **6** (0.96 g, 99%). [$^1$H]NMR $\delta$: 0.69 (3H, s, 18-H), 1.10 (6H, 19- and 21-H), 1.54 (3H, s, 27-H), 2.04 and 2.07 (6H, each s, acetyl), 5.2–5.5 (3H, m, 6-, 22- and 23-H), 10.03 (1H, m, -COO<u>H</u>). i.r. $\nu_{max}$: 3400 (hydroxyl), 1720 cm$^{-1}$.

*(22′E,5S,25′R and 22′E,5S,25′S)-N-(3′β,25′-Diacetoxycholesta-5′,22′-dien-26′-oyl)-5-isopropyl-3-oxazolid-2-one (7a and 7b).* Oxalyl chloride (0.22 ml, 1.5 eq.) was added to a solution of **6** (888 mg) and dimethylformamide (1 drop) in $CH_2Cl_2$ (10 ml) at 0°C. The mixture was stirred for 15 min at the same temperature, and then at room temperature for 12.5 h. Removal of the solvent gave a crude product as a yellow oil. 5-Isopropyl-3-oxazolid-2-one (271 mg, 2.10 mmol) in THF (8 ml) was treated with n-butyl lithium in hexane (1.3 ml, 1.2 eq.) at −78°C under an argon atmosphere for 30 min. To this solution was added a solution of acid chloride in THF (18 ml), and the mixture was stirred for 30 min at 0°C, and then at room temperature for 5 h. The usual work-up (ether) and column chromatography with hexane–ethyl acetate (20:1–10:1) as an eluant gave the more polar isomer (**7a**) (132 mg, 12%) and the less polar isomer (**7b**) (264 mg, 24%). **7a**: [$^1$H]-NMR $\delta$: 0.69 (3H, s, 18-H), 0.90 and 0.92 (6H, d, $J = 7$ Hz, $CH(CH_3)_2$), 1.01 (3H, d, $J = 7$ Hz, 21-H), 2.03 and 2.06 (6H, each s, acetyl), 2.69 and 2.89 (2H, ABq, $J = 17$ and 8 Hz, 24-H), 4.60 (1H, m, 3-H), 5.1–5.5 (3H, m, 6-, 22- and 23-H). i.r. $\nu_{max}$: 3020, 2940, 2880, 1785, 1728, 1698 cm$^{-1}$. MS $m/z$: 505 ($M^+$-2AcOH), 376, 313, 282, 253 (base peak), 222. **7b**: [$^1$H]NMR $\delta$: 0.68 (3H, s, 18-H), 0.89, 0.91 (6H, each d, $J = 7$ Hz, $CH(CH_3)_2$), 1.00 (3H, d, $J = 7$ Hz, 21-H), 2.03 and 2.06 (6H, each s, acetyl), 2.62 and 2.73 (2H, ABq, $J = 17$ and 8 Hz, 24-$H_2$), 4.60 (1H, m, 3-H), 5.0–5.3 (3H, m, 6-, 22- and 23-H). i.r. $\nu_{max}$: 1780, 1720, 1690 cm$^{-1}$. MS $m/z$: 505 ($M^+$-2AcOH), 490 (505-Me), 376, 313, 282, 253 (base peak), 222.

*(25S and 25R)-Cholest-5-en-3β,25,26-triol 3β,26-di-(+)-MPTA Ester (9a and 9b).* A mixture of oxazolidone (**7a**) (10.2 mg) and 10% Pd/C (4 mg) in ethyl acetate (1 ml) was stirred vigorously at room temperature under a hydrogen atmosphere for 4.5 h. After filtration of catalyst, the filtrate was concentrated to give a residue, which was dissolved in THF (1 ml). $LiAlH_4$ (4 mg) was added and the mixture was stirred at room temperature for 14 h. The usual

work-up (ethyl acetate) and chromatographic purification (benzen–ethyl acetate = 1:1) gave a crude triol. Esterification was carried out by treatment of the crude triol in pyridine (0.5 ml) with (+)-MTPACl (17 drops) at room temperature for 1 day. The usual work-up (ethyl acetate) and preparative TLC (hexane–ethyl acetate = 3:1) gave the MTPA ester (3.2 mg, 22%). [$^1$H]NMR δ: 0.68 (3H, s, 18-H), 0.89 (3H, d, $J$ = 7 Hz, 21-H), 1.01 (3H, s, 19-H), 1.56 (3H, s, 27-H), 3.55 (6H, s, $OCH_3 \times 2$), 409 and 4.26 (2H, each d, $J$ = 11.5 Hz, 26-H), 4.95 (1H, m, 3-H), 5.4 (1H, m, 6-H), 7.3–7.6 (10H, m, phenyl).

The compound (**7b**) was converted to the MTPA ester (**9b**) (6.8 mg, 48%) by the same method as described in **7a**. [$^1$H]NMR δ: 0.67 (3H, s, 18-H), 0.97 (3H, d, $J$ = 7 Hz, 21-H), 1.00 (3H, s, 19-H), 1.56 (3H, s, 27-H), 3.56 (6H, s, $OCH_3 \times 2$), 4.20 (2H, s, 26-H), 4.90 (1H, m, 3-H), 5.40 (1H, m, 6-H), 7.3–7.6 (10H, m, phenyl).

*(22′E,5S,25′S and 22′E,5S,25′R)-N-(25′-Acetoxy-3′β-hydroxycholesta-5′,22′-dien-26′-oyl)-5-iso propyl-3-oxazolid-2-one (**8a** and **8b**).* The compound (**7a**) (110 mg) in THF (10 ml) was treated with 12 N HCl (20 drops) at room temperature for 18 h. The usual work-up (ether) gave a crude residue. A mixture of the residue, chloromethyl methyl ether (0.40 ml), and $N,N$-diethylcyclohexylamine (0.88 ml) in dioxane (5 ml) was stirred at room temperature for 25 h. The usual work-up (ethyl acetate) and column chromatography (hexane–ethyl acetate = 4:1) to give the MOM ether (**8a**) (49.8 mg, 68%). [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 0.90 and 0.91 (6H, d × 2, $J$ = 7 Hz, $CH(C\underline{H}_3)_2$), 1.01 (3H, d, $J$ = 6 Hz, 21-H), 1.01 (3H, s, 19-H), 1.58 (3H, s, 27-H), 2.06 (3H, s, acetyl), 2.69 and 2.89 (2H, ABq, $J$ = 11 and 7 Hz, 24-H), 3.37 (3H, s, $OCH_3$), 3.3–3.5 (1H, m, 3-H), 4.70 (2H, s, $OCH_2O$), 5.2–5.5 (3H, m, 6-, 22- and 23-H). MS $m/z$: 505 ($M^+$-AcOH-MOMOH), 376, 315, 282, 253, 222 (base peak).

The compound (**7b**) (110 mg, 0.18 mmol) was converted into **8b** (50.6 mg, 71%) as the same manner described in **8a**. [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 0.89 and 0.91 (6H, d × 2, $J$ = 7 Hz, $CH(C\underline{H}_3)_2$), 1.00 (3H, d, $J$ = 6 Hz, 21-H), 1.01 (3H, s, 19-H), 1.63 (3H, s, 27-H), 2.06 (3H, s, acetyl), 2.62 and 2.72 (2H, ABq, $J$ = 11 and 7 Hz, 24-H), 3.37 (3H, s, $OCH_3$), 3.2–3.5 (1H, m, 3-H), 4.69 (2H, s, $OCH_2O$), 5.1–5.4 (3H, m, 6-, 22- and 23-H). MS $m/z$: 505 ($M^+$-AcOH-MOMOH), 315, 282, 253, 222 (base peak).

*(22E,25S and 22E,25R)-3β-Methomethyloxy-cholesta-5,22-dien-25,26-diol (**10a** and **10b**).* Lithium aluminum hydride (11.0 mg) was added to a solution of **8a** (49.8 mg) in THF (2 ml) at 0°C and the mixture was stirred at room temperature for 19 h. The usual work-up (ethyl acetate) and chromatography (hexane–ethyl acetate = 2:1) gave the diol (**8a**) (28.2 mg, 77%). [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 1.02 (3H, d, $J$ = 7 Hz, 21-H), 1.00 (3H, s, 19-H), 1.14 (3H, s, 27-H), 3.37 (3H, s, $OCH_3$), 3.40 (3H, m, 3- and 24-H), 4.69 (2H, s, $OCH_2O$), 5.2–5.4 (3H, m, 6, 22, and 23-H). MS $m/z$: 398 ($M^+$-MOMOH), 380, 324, 255, 75 (base peak).

The compound **8b** (93.7 mg, 0.149 mmol) was converted into **10b** as the same method described in **10a** (47.8 mg, 70%). [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 1.00 (3H, d, $J$ = 7 Hz, 21-H), 1.01 (3H, s, 19-H), 1.14 (3H, s, 27-H), 3.37 (3H, s, $OCH_3$), 3.40 (3H, m, 3- and 24-H), 4.69 (2H, s, $OCH_2O$), 5.2–5.4 (3H, m, 6-, 22- and 23-H). MS $m/z$: 398 ($M^+$-MOMOH), 380, 324, 255, 75 (base peak).

*(22E,25S and 22E,25R)-25,26-Epoxy-3β-metho-methyloxycholesta-5,22-diene (**11a** and **11b**).* Methanesulfonyl chloride (29 μl) was added to a solution of **10a** (47.3 mg) in pyridine and the mixture was stirred at room temperature under an argon atmosphere for 1 h. The usual work-up (ethyl acetate) gave a crude mesylate. The mesylate was dissolved in THF (2 ml) and 5% KOH-methanol (0.3 ml) was added. The mixture was stirred at room temperature for 1 h. The usual work-up and chromatography with hexane–ethyl acetate (10:1) afforded the epoxide **11a** (32.2 mg, 94%). [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 1.01 (3H, s, 19-H), 1.02 (3H, d, $J$ = 6 Hz, 21-H), 1.29 (3H, s, 27-H), 2.57 and 2.62 (2H, each d, $J$ = 5 Hz, 26-H), 3.37 (3H, s, $OCH_3$), 3.40 (1H, m, 3-H), 4.69 (2H, s, $OCH_2O$), 5.2–5.4 (3H, m, 6-, 22- and 23-H). MS $m/z$: 380 ($M^+$-MOMOH), 365, 315, 282, 255, 73 (base peak).

The compound (**10b**) (45.5 mg) was converted as the same manner described in **11a** (29.1 mg, 66%). [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 1.01 (3H, s, 19-H), 1.02 (3H, d, $J$ = 6 Hz, 21-H), 1.29 (3H, s, 27-H), 2.57 and 2.62 (2H, each d, $J$ = 5 Hz, 26-H), 3.37 (3H, s, $OCH_3$), 3.40 (1H, m, 3-H), 4.69 (2H, s, $OCH_2O$), 5.2–5.4 (3H, m, 6-, 22- and 23-H). MS $m/z$: 380 ($M^+$-MOMOH), 365, 315, 282, 255, 73 (base peak).

*(22E,25R and 22E,25S)-3β-Methomethyloxy-26-methylcholesta-5,22-dien-25-ol (**12a** and **12b**).* Methylmagnesium bromide (0.17 ml, 1 M solution in THF) was added to a suspension of CuI (2.2 mg, 0.16 eq) in THF (0.5 ml) and the mixture was stirred under an argon atmosphere at 0°C for 10 min. Then a solution of the epoxide (**11a**) (31 mg) in THF (2.5 ml) was added to this Grignard reagent, and stirring was continued at room temperature for 21 h. The usual work-up (ether) and column chromatography with hexane–ethyl acetate (10:1) afforded **12a** (25.8 mg, 77%). [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 0.90 (3H, t, $J$ = 7 Hz, 26-$CH_3$), 1.01 (3H, s, 19-H), 1.03 (3H, d, $J$ = 7 Hz, 21-H), 1.13 (3H, s, 27-H), 3.37 (3H, s, $OCH_3$), 3.40 (1H, m, 3-H), 4.69 (2H, s, $OCH_2O$), 5.3-5.4 (3H, m, 6-, 22- and 23-H). MS $m/z$: 396 ($M^+$-MOMOH), 378, 324, 282, 255, 73 (base peak).

The compound **11b** (29.1 mg) was converted into **12b** as the same manner described in **12a** (27.0 mg, 90%). [$^1$H]NMR δ: 0.69 (3H, s, 18-H), 0.90 (3H, t, $J$ = 7 Hz, 26-$CH_3$), 1.01 (3H, s, 19-H), 1.03 (3H, d, $J$ = 7 Hz, 21-H), 1.12 (3H, s, 27-H), 3.37 (3H, s, $OCH_3$), 3.40 (1H, m, 3-H), 4.69 (2H, s, $OCH_2O$),

5.3–5.4 (3H, m, 6-, 22- and 23-H). MS $m/z$: 378 ($M^+$-MOMOH-$H_2O$), 324, 282, 255, 73 (base peak).

*(22E,25R and 22E,25S)-3β-Acetoxy-26-methylcholesta-5,22-dien-25-ol (**13a** and **13b**).* A solution of **12a** (25.6 mg) in THF (3 ml) was stirred with 6 N HCl (0.5 ml) at 50°C for 1 h. The usual work-up (ether) gave a crude product, which was dissolved in pyridine (1 ml). Acetic anhydride (0.5 ml) was added and the mixture was stirred at room temperature for 6 h. The usual work-up and chromatography with hexane–ethyl acetate (5:1) gave **13a** (25 mg, 99%). [$^1$H]NMR $\delta$: 0.69 (3H, s, 18-H), 0.90 (3H, t, $J = 7$ Hz, 26-$CH_3$), 1.02 (3H, s, 19-H), 1.03 (3H, d, $J = 6$ Hz, 21-H), 1.13 (3H, s, 27-H), 2.03 (3H, s, acetyl), 4.60 (1H, m, 3-H), 5.3–5.4 (3H, m, 6-, 22- and 23-H). MS $m/z$: 396 ($M^+$-AcOH), 378 (396-$H_2O$), 324, 309, 282, 255, 73 (base peak).

The compound **12b** (27.0 mg) was converted into **13b** as the same manner described in **13a** (22.3 mg, 83%). [$^1$H]NMR $\delta$: 0.69 (3H, s, 18-H), 0.91 (3H, t, $J = 7$ Hz, 26-$CH_3$), 1.02 (3H, s, 19-H), 1.03 (3H, d, $J = 7$ Hz, 21-H), 1.13 (3H, s, 27-H), 2.03 (3H, s, acetyl), 4.60 (1H, m, 3-H), 5.3–5.4 (3H, m, 6-, 22- and 23-H).

*(22E,25R and 22E,25S)-22-Dehydro-25-hydroxy-26-methylvitamin $D_3$ (**14a** and **14b**).* A mixture of **13a** (23 mg), *N*-bromosuccinimide (11.6 mg) in $CCl_4$ (3.5 ml) was refluxed under an argon atmosphere for 35 min. After cooling to 0°C, the insoluble materials were filtered off and washed with $CCl_4$. The filtrate and washings were combined and concentrated to give a oily residue, which in THF (4 ml) was treated with $(n\text{-}Bu)_4NBr$ (approx. 5 mg) under an argon atmosphere at room temperature. After 50 min, a solution of $(n\text{-}Bu)_4NF$ (0.17 ml, 1 M solution in THF, 3.4 eq) was added and stirring was continued for 30 min. The usual work-up (ethyl acetate) gave a residue. The resultant residue was dissolved in THF (4 ml) and 5% KOH-methanol (0.5 ml) was added. The mixture was stirred for 17 min at room temperature. The usual work-up (ethyl acetate) and preparative TLC with hexane–ethyl acetate (4:1) afforded the 5,7-diene (3.10 mg, 70%). u.v. $\lambda_{max}$: 293, 282, 271, 263(sh).

A solution of the 5,7-diene (3.1 mg) in benzene (90 ml) and ethanol (40 ml) was irradiated with a u.v. lamp (Hanovia 654A, 200 W) through a Vycor filter under an argon atmosphere at 0°C for 4.5 min. The reaction mixture was then refluxed for 1 h. Removal of the solvent gave a crude product, which was purified by preparative TLC [developed four times with benzene–ethyl acetate (20:1)] to give **14a** (902 μg). [$^1$H]NMR $\delta$: 0.67 (3H, s, 18-H), 0.90 (3H, t, $J = 7$ Hz, 26-$CH_3$), 1.03 (3H, d, $J = 6$ Hz, 21-H), 1.13 (3H, s, 27-H), 3.55 (1H, m, 3-H), 4.81 and 5.05 (2H, each brs, 19-H), 5.38 (2H, m, 22- and 23-H), 6.03 (1H, d, $J = 11.5$ Hz, 7-H), 6.24 (1H, d, $J = 11.5$ Hz, 6-H). MS $m/z$: 412 ($M^+$), 394 ($M^+$-$H_2O$), 379, 340, 271, 253, 211, 136 (base peak), 118, 73. u.v. $\lambda_{max}$ 264 nm, $\lambda_{min}$ 228 nm.

The compound **13b** (22.2 mg) was converted into **14b** (768 μg) in the same manner as described in **14a**. [$^1$H]NMR $\delta$: 0.67 (3H, s, 18-H), 0.90 (3H, t, $J = 7$ Hz, 26-$CH_3$), 1.03 (3H, d, $J = 6$ Hz, 21-H), 1.13 (3H, s, 27-H), 3.55 (1H, m, 3-H), 4.81 and 5.05 (2H, each brs, 19-Hz), 5.39 (2H, m, 22- and 23-H), 6.03 (1H, d, $J = 11.5$ Hz, 7-H), 6.24 (1H, d, $J = 11.5$ Hz, 6-H). MS $m/z$: 412 ($M^+$), 394 ($M^+$-$H_2O$), 379, 340, 271, 253, 211, 136 (base peak), 118, 73. u.v. $\lambda_{max}$ 264 nm, $\lambda_{min}$ 228 nm.

### *1α-Hydroxylation of **14a** and **14b** by chick kidney homogenate*

One-day-old White Leghorn cockerels were fed a vitamin D-deficient diet for 6 weeks [7]. The chicks were sacrificed and their kidneys were taken and 10% tissue homogenate in 0.25 M sucrose was prepared with the aid of a Plotter-Elvehjem homogenizer fitted with a Teflon pestle. To 30 ml of the homogenates (13 mg proteins/ml) in a 300 ml flask was added 70 ml of reaction mixture containing 30 mM Tris–HCl (pH 7.4), 3.6 mM $MgCl_2$, 50 mM sucrose, and 20 mM sodium succinate as described previously [8]. The incubation was initiated by addition of 20 μg of **14a** and **14b** in 0.5 ml of ethanol in each flask. The incubation was carried out at 37°C for 60 min with shaking. 200 ml of chloroform–methanol (1:1) was added to terminate the reaction. The chloroform phase was evaporated, and the residue was dried by ethanol azeotrope and chromatographed. The chloroform extracts of incubation mixture were chromatographed on a Sephadex LH-20 column (1.5 × 25 cm) eluted with chloroform–hexane (65:35). The (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction (**15a**) (fraction No. 25–50, 100–200 ml) was pooled and concentrated. The (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction from the Sephadex LH-20 column was then subjected to HPLC eluted with 10% isopropanol in hexane at a flow rate of 1 ml/min. The eluate was continuously monitored by ultraviolet absorption measured at 264 nm, and the u.v. absorbing peaks were separately collected. The u.v. absorbing peak (24.6–28 min) was collected as a (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction (Fig. 1A). This fraction was chromatographed by HPLC eluted with a 2.5% methanol in dichloromethane at a flow rate of 1 ml/min. (25*R*)-22-dehydro-22-methyl-1,25-$(OH)_2D_3$ fraction was eluted at 19–21.6 min (Fig. 1B). This metabolite was further purified by HPLC eluted with 10% isopropanol in hexane at a flow rate of 1 ml/min. The sole u.v. 264 nm absorbing peak eluted from 25–27.5 min was pooled and used for structural identification. The isolation of (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ (**15b**) was carried out the same procedures as (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$. According to these procedures, the total amounts of isolated metabolites were as follows: 1,25-$(OH)_2D_3$, 14.5 μg; (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ from (25*R*)-22-dehydro-26-methyl-25-

OH-$D_3$, 18.2 μg; (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ from (25*S*)-22-dehydro-26-methyl-25-OH-$D_3$, 18.9 μg.

### *Chick intestinal cytosol 1,25-$(OH)_2D_3$ specific receptor binding affinity of (25R)- and (25S)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$*

The competitive receptor binding assay was performed as described previously [8]. [26,27-$^3$H]1,25-$(OH)_2D_3$ (158 Ci/mmol, 16800 dpm, 20 pg) and various amounts of vitamin $D_3$ analogues to be tested dissolved in 50 μl of absolute ethanol in 12 × 75 mm polypropylene tubes. Chick intestinal cytosol containing 1,25-$(OH)_2D_3$-specific receptor was prepared as described previously [9]. One ml of the chick intestinal cytosol receptor protein 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 tubes were incubated in a shaking water bath for 1 h at 25°C and then chilled in the ice-cold bath. 1 ml of 40% (w/v) polyethylene glycol 6000 in distilled water was added to each, mixed vigorously, and then centrifuged at 4°C. The supernatant was decanted. The bottom of the tube with the pellet was cut off and transferred to a scintillation vial containing 10 ml of dioxane-based scintillation fluid (10% naphthalene and 0.5% Omnifluor in 1,4-dioxane). Radioactivity was measured with a Packard Tri-Carb Model 3255 liquid scintillation counter using an external standard.

### *Assay for intestinal calcium transport and bone calcium mobilization*

Male weanling Wistar rats were fed a vitamin D-deficient, low calcium diet (Ca, 0.0036%: P, 0.3%; Teklad Test Diet, Madison, Wis.) for 6 weeks. At the end of the 6th week, a group of 5 rats (each weighing about 100 g) received intravenous injection of 650 pmol of vitamin D analogues in 0.2 ml of 0.2% Triton X-100-saline solution. The rats were sacrificed at the indicated times after the administration, and the intestinal calcium transport and serum calcium concentrations were measured. The intestinal calcium transport assay using everted duodenal sacs was carried out by the method described by Martin and DeLuca [10]. The serum calcium concentration was determined by OCPC (*O*-cresolphthalein complexone) method [11]. Under the conditions of the assay elevations in serum calcium are a reflection of bone calcium mobilization.

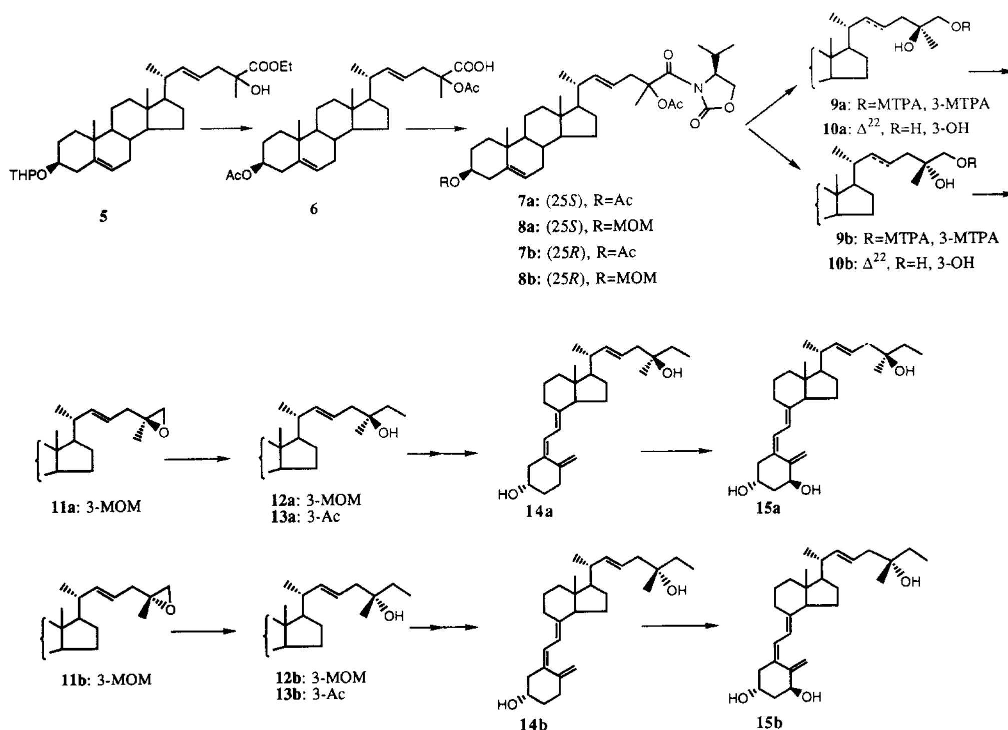
### *Metabolism of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ in vitamin D-deficient rats*

Male weanling Wistar rats were fed a vitamin D-deficient, low calcium diet (Ca, 0.0036%; P, 0.3%; Teklad Test Diet, Madison, Wis.) for 6 weeks. At the end of the 6th week, a group of 5 rats (each weighing about 100 g) received intravenous injection of 650 pmol of vitamin D analogues in 0.2 ml of 0.2% Triton X-100-saline solution. The blood was withdrawn from abdominal aorta at the indicated times after the administration. The blood was centrifuged and the serum was obtained. The 2 ml of serum was diluted with 3 volumes of water and added to 10 pg of [26,27-$^3$H]1,25-$(OH)_2D_3$ (sp. act. 158 Ci/mmol, 8400 dpm) as a tracer and then extracted with 16 ml of chloroform–methanol (1:1) for 30 min. The chloroform phase was evaporated, and the residue was dried by ethanol azeotrope. The chloroform extracts were dissolved in chloroform–hexane (65:35) and filtered through a silica gel Sep-Pak (Waters Associates). 1,25-$(OH)_2D_3$ and 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction was eluted with chloroform–hexane–methanol (75:23:10) and then was further purified by HPLC using a Zorbax Sil column eluted with 10% isopropanol in hexane. In this system, 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were eluted at 25–27.5 min and 28–31 min, respectively. The serum concentrations of 1,25-$(OH)_2D_3$ and 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ were determined by radioreceptor assay as described previously [9].

## RESULTS

The synthetic route of (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-dihydroxyvitamin $D_3$ is outlined in Scheme 2. The starting material, 3β,26-diacetoxycholesta-5,22-dien,26-oic acid **6** derived from the known ester, (22*E*)-25-hydroxy-3β-tetrahydropyranyloxycholesta-5,22-dien-26-oic acid ethyl ester **5** [12, 13] in three steps, was converted into acyl chloride, which was then treated with an anion of (5*S*)-5-isopropyl-3-oxazolid-2-one [14, 15] at −78°C to give the oxazolidone derivatives (**7**) as a diastereomeric mixture due to the 25-position. The mixture was easily separated into the less polar isomer (**7a**) and the more polar isomer (**7b**) in a pure form by a silica gel column chromatography. The stereochemistry at the 25-position of the epimers was determined by conversion of both the epimers to 25,26-dihydroxycholesterol 3,26-di-(+)-MTPA ((+)-α-methoxy-α-trifluoromethylphenylacetic acid) ester, the 25-stereochemistry of which was established by X-ray crystallography [16]. For this purpose each isomer was converted into the corresponding 3,26-di(+)-MTPA esters (**9a** and **9b**) by the following reactions; catalytic hydrogenation, reduction with $LiAlH_4$, and esterification with (+)-MTPA chloride in pyridine. By a comparison of [$^1$H]NMR and HPLC mobility of **9a** and **9b** with the authentic compounds. The less polar compound (**7a**) was found to have 25*R* configuration and to more polar one (**7b**) to 25*S*.

After selective conversion of the protective group at the 3-position of **7a** and **7b** from acetyl group to methoxy methyl group, each oxazolidone isomer (**8a** and **8b**) was reduced with $LiAlH_4$ to afford the 25,26-diol (**10a** and **10b**), respectively. Mesylation of




Scheme 2

the 26-position of **10a** and **10b** with methanesulfonyl chloride in pyridine followed by alkaline treatment with KOH in methanol gave the 25,26-epoxide (**11a** and **11b**), respectively. Grignard reaction of **11a** and **11b** with methylmagnesium bromide in the presence of a catalytic amount of CuI afforded the 22-dehydro-26-methylcholesterol derivatives (**12a** and **12b**), respectively.

After conversion of the protective group at the 3-position of **12a** and **12b** from methoxymethyl group into acetyl group, the precursory cholesterol derivatives (**13a** and **13b**) were converted into the vitamin D form by the same method of the our previous report [5]. Thus, bromination of **13a** and **13b** with *N*-bromosuccinimide, dehydrobromination with tetra-*n*-butylammonium fluoride and alkaline hydrolysis gave the 5,7-diene, respectively. Irradiation of the diene with a medium-pressure mercury lamp through a Vycor in benzene and ethanol at 0°C under an argon atmosphere and subsequent thermal isomerization under reflux provided (25*R* and 25*S*)-22-dehydro-26-methyl-25-OH-$D_3$ (**14a** and **14b**) from the 5-ene derivatives (**13a** and **13b**) in 4.3% and 3.9%, respectively.

The 1α-hydroxylation of **14a** and **14b** were carried out by incubating with kidney homogenates from vitamin D-deficient chicks. The metabolites were extracted with chloroform–methanol (1:1) from the incubation mixture and purified with Sephadex LH-20 column, followed by HPLC on a Zorbax Sil column. The chromatogram is shown in Fig. 1. The (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction was purified to homogeneity by HPLC using two different solvent systems. For the (25*S*)-derivatives, again the 1α-hydroxylation was carried out by the same manner.

Structures of the enzymatically elaborated products were confirmed by u.v. and mass spectra. The u.v. spectra of the isolated (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ displayed the typical u.v. absorptions due to the vitamin $D_3$ *cis*-triene chromophore with $\lambda_{max}$; 264 nm and $\lambda_{min}$; 228 nm. The mass spectra of (25*S*)- and (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ are identical with that of the natural compound.

It should be noted that the side chain moiety of the synthetic intermediates (i.e. **12**–**14**) can be distinguished by the high field [$^1$H]NMR spectroscopy between epimers. The signals of 26-$CH_3$ groups were observed slightly higher field than the corresponding signals of 25*R*-series and the reverse was true for the signals of the 25-$CH_3$ groups. For example, 26-$CH_3$ group of **12a** was observed at 0.90 ppm as a triplet and 25-$CH_3$ at 1.12 ppm as a singlet, but in **12b**, 26-$CH_3$ and 25-$CH_3$ were observed at 0.91 ppm as a triplet and at 1.13 ppm as a singlet, respectively. Therefore, this chemical shift trends would help to elucidate the stereochemistry of the natural metabolite, provided that both isomers are in hand.

Binding affinities of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ and 22-dehydro-26-methyl-25-OH-$D_3$ to chick intestinal cytosol receptor of 1,25-$(OH)_2D_3$ were determined by polyethylene glycol precipitation [8]. In Fig. 2, the binding affinity of 1,25-$(OH)_2D_3$ to its chick intestinal cytosol receptor is

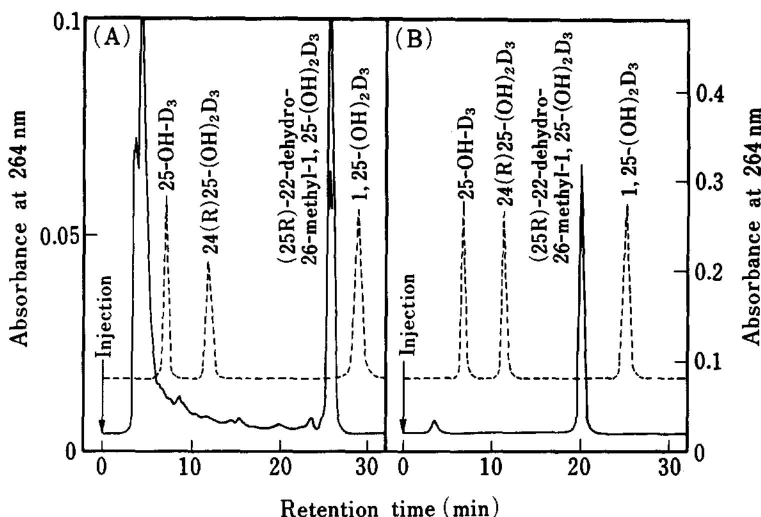



Fig. 1. Column profiles of the high-performance liquid chromatographic purification steps of the 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction from the Sephadex LH-20 column. (A) HPLC profile of the (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction from Sephadex LH-20 column. (B) HPLC profile of (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ fraction (24.6–28 min) shown on Fig. 1A. The dashed lines indicate elution position of authentic 25-OH-$D_3$, 24*R*,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. Two columns (Zorbax Sil; 4.6 × 250 mm) were eluted with a solvent of 10% isopropanol in hexane (A) and 2.5% methanol in dichloromethane (B) at flow rate of 1.0 ml/min.

compared to the binding of (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$, and (25*R*)- and (25*S*)-22-dehydro-26-methyl-25-OH-$D_3$. (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ turned out to bind to the receptor more strongly than 1,25-$(OH)_2D_3$. As determined by 50% displacement of $[^3H]$1,25-$(OH)_2D_3$ from the receptor, the binding affinities of (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ were 1.7 and 1.5 times stronger than that of 1,25-$(OH)_2D_3$, respectively (Table 1). Similarly, the binding affinities of (25*R*)- and (25*S*)-22-dehydro-26-methyl-25-OH-$D_3$ were determined to be 7.0 and 3.1 times stronger than that of 25-OH-$D_3$, respectively (Table 1).

*In vivo* activities of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were assayed in vitamin D-deficient rats fed a low calcium diet. Initially, the chemically synthesized 25-epimeric mixture of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ [6] was used for biological assay to save the previous diastereomeric pure sample for next stage. Single injections of 650 pmol of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ and 650 pmol of 1,25-$(OH)_2D_3$ were tested their intestinal calcium transport activity (Fig. 3). The 1,25-$(OH)_2D_3$ stimulated intestinal calcium transport at 4, 8 and 12 h, reaching a maximum at 8 h after administration, while at 48 h, responses did not significantly stimulate in comparison with the control. In contrast,

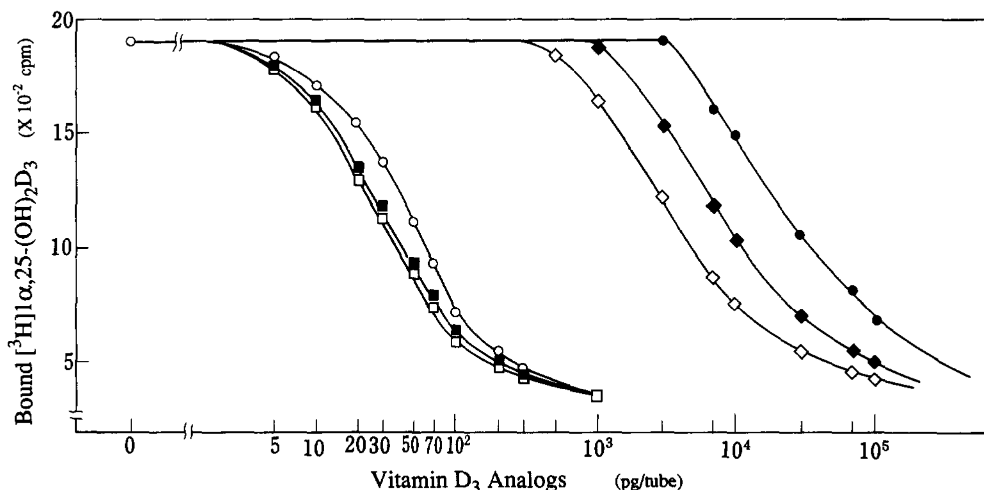



Fig. 2. Chick intestinal cytosol receptor binding assay with (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ (○), (25*R*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ (□), (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ (■), (25*R*)-22-dehydro-26-methyl-25-OH-$D_3$ (◇), (25*S*)-22-dehydro-26-methyl-25-OH-$D_3$ (◆), 25-OH-$D_3$ (●). Points are the mean of triplicate determinations.

Table 1. Ability of 1,25-$(OH)_2D_3$ analogues to compete with $[^3H]$1,25-$(OH)_2D_3$ for the chick intestinal 1,25-$(OH)_2D_3$-specific receptor

| Vitamin $D_3$ analogues | 50% Displacement (pg) | Molar ratio |
|---|---|---|
| 1,25-$(OH)_2D_3$ | 50 | 1 |
| (25*R*)-22-Dehydro-26-methyl-1,25-$(OH)_2D_3$ | 30.5 | 0.59 |
| (25*S*)-22-Dehydro-26-methyl-1,25-$(OH)_2D_3$ | 34 | 0.66 |
| (25*R*)-22-Dehydro-26-methyl-25-OH-$D_3$ | 3750 | 75.7 |
| (25*S*)-22-Dehydro-26-methyl-25-OH-$D_3$ | 8400 | 169.6 |
| 25-OH-$D_3$ | 25500 | 530.4 |

Molar ratio indicates the ratio of mol/l of vitamin $D_3$ analogues over the mol/l of 1,25-$(OH)_2D_3$ required for 50% displacement of the $[^3H]$1,25-$(OH)_2D_3$ from the 1,25-$(OH)_2D_3$-specific receptor.

22-dehydro-26-methyl-1,25-$(OH)_2D_3$ stimulated at all time points intestinal calcium transport to a significant extent than 1,25-$(OH)_2D_3$.

The time-course in activity of calcium mobilization from bone, as measured by an elevation in serum calcium level, was compared after the administration of 650 pmol of 25-epimeric mixture of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ (Fig. 4). Similarly as intestinal calcium transport assay, 1,25-$(OH)_2D_3$ elevated serum calcium level, reaching a maximum 8 h after administration, and then gradually decreased to the control levels. In contrast, 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ increased serum calcium levels at all points studied to a significant extent than 1,25-$(OH)_2D_3$.

Figures 5 and 6 indicate the dose–response relationship of 1,25-$(OH)_2D_3$ and both 25-epimers of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ for *in vivo* activities of intestinal calcium transport and bone calcium mobilization at 8 h after administration, since the activity of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ reached a maximum at 8 h after administration (Figs 3 and 4). Increasing doses of (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ resulted in greater stimulation of intestinal calcium transport. In the stimulation of intestinal calcium transport test, (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ showed 3.6 and 2.1 times activity of 1,25-$(OH)_2D_3$, respectively (Fig. 5). Also as shown in Fig. 6 of bone calcium mobilization test, graded doses of (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ remarkably increased the serum calcium levels. (25*R*)- and (25*S*)-22-Dehydro-26-methyl-1,25-$(OH)_2D_3$ were found to be 2.2 and 1.6 times as potent as 1,25-$(OH)_2D_3$, respectively in bone calcium mobilization test (Fig. 6).

Figure 7 shows time-course of changes in serum concentrations of 1,25-$(OH)_2D_3$ and 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ after intravenous administration of each compound to vitamin D-deficient rats. When 1,25-$(OH)_2D_3$ was administered to rats, its serum concentrations were rapidly decreased until 24 h and then gradually decreased. The same was true, in the case of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$.

## DISCUSSION

Since studies on structure activity relationship between vitamin $D_2$ and vitamin $D_3$ suggested that an

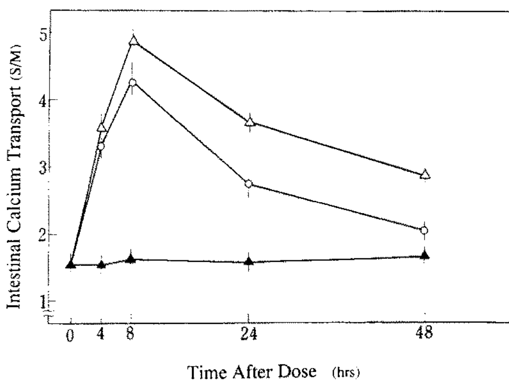



Fig. 3. Time-course response of intestinal calcium transport stimulation. Vitamin D-deficient rats on a low calcium diet received a single dose of ether 650 pmol of 1,25-$(OH)_2D_3$ (○), 650 pmol of 25-epimeric mixture of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ (△), or vehicle (▲) in 0.2 ml of 0.2% Triton X-100-saline solution. At the indicated times, animals were decapitated and their duodena were used for the determination of intestinal calcium transport. The rate of intestinal calcium transport is represented by the ratio of $^{45}Ca$ in the serosal medium to $^{45}Ca$ in the mucosal medium. Each point is the mean ± SE of determinations in five rats.

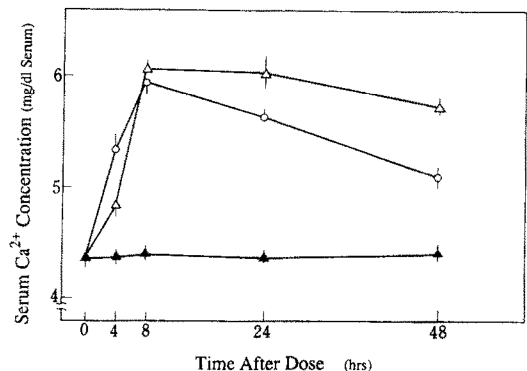



Fig. 4. Time-course response of bone calcium mobilization induced in rats by 650 pmol of 1,25-$(OH)_2D_3$ (○), 650 pmol of 25-epimeric mixture of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ (△), or vehicle (▲). Vitamin D-deficient rats on a low calcium diet received a single intravenous dose of compounds in 0.2 ml of 0.2% Triton X-100-saline solution. At the indicated times, animals were decapitated, blood was collected, and calcium was measured in the serum by OCPC method. Data are expressed as milligrams of Ca/100 ml of serum and the mean ± SE of five determinations.

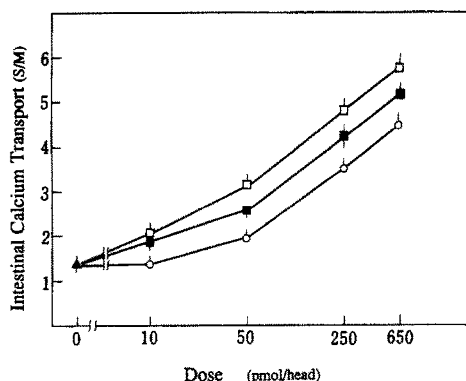



Fig. 5. Dose–response relationship between $1,25\text{-}(OH)_2D_3$ analogues of intestinal calcium transport in vitamin D-deficient rats fed a low calcium diet. After 6 weeks on the vitamin D-deficient, low calcium diet rats received a single dose of either $1,25\text{-}(OH)_2D_3$ (○), (25*R*)-22-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ (□), or (25*S*)-22-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ (■), or vehicle (▲) in 0.2 ml of 0.2% Triton X-100-saline solution. 8 h later, the rats were decapitated, and intestinal calcium transport and serum calcium concentrations were measured as described in Experimental. Data are expressed as mean ± SE.

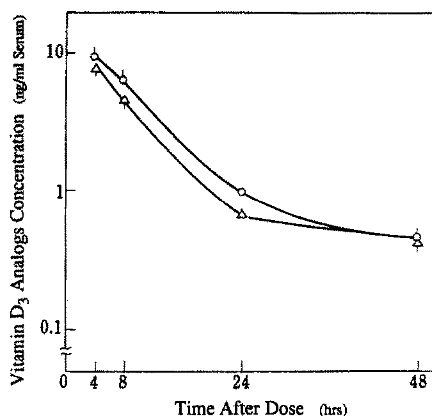



Fig. 7. Time-course of changes in serum concentrations of $1,25\text{-}(OH)_2D_3$ (○) or 22-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ (△) after administration of 650 pmol of each compound to vitamin D-deficient rats. Vitamin D-deficient rats received intravenous injection of 650 pmol of $1,25\text{-}(OH)_2D_3$ or 22-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ in 0.2 ml of 0.2% Triton X-100-saline solution. The blood was collected at the indicated times after administration, and the serum was obtained by centrifugation. The serum concentrations of vitamin D derivatives were determined by radioreceptor assay as described in Experimental.

unsaturation at $C_{22}$–$C_{23}$ was not effective for the biological potency [17], higher activities of 22-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ *in vivo* as well as *in vitro* than $1,25\text{-}(OH)_2D_3$ in the present study, in addition to the investigation on the biological activity of the epimeric 22-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ [5], might be attributable to the presence of 26-methyl group. These results demonstrated that a substitution of methyl group at the 26- or 27-positions of $1,25\text{-}(OH)_2D_3$ increased the biological potency. Furthermore, dimethylation of both the 26- and 27-positions is also effective in comparison the biological activity with $1,25\text{-}(OH)_2D_3$ [18, 19]. These data suggest that

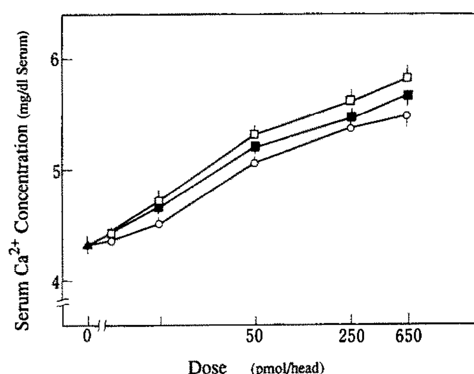



Fig. 6. Dose–response relationship between $1,25\text{-}(OH)_2D_3$ analogues of bone calcium mobilization in vitamin D-deficient rats fed a low calcium diet. After 6 weeks on the vitamin D-deficient, low calcium diet rats received a single dose of either $1,25\text{-}(OH)_2D_3$ (○), (25*R*)-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ (□), or (25*S*)-22-dehydro-26-methyl-$1,25\text{-}(OH)_2D_3$ (■), or vehicle (▲) in 0.2 ml of 0.2% Triton X-100-saline solution. Eight hours later, the rats were decapitated, and intestinal calcium transport and serum calcium concentrations were measured as described in Experimental. Data are expressed as mean ± SE.

homologation of $C_{26}$ and $C_{27}$ of $1,25\text{-}(OH)_2D_3$ by one carbon does not diminish biological activity, but enhances activity. Consideration of the data obtained suggests that the receptor of $1,25\text{-}(OH)_2D_3$ seems not to recognize the 25-dimethyl groups of $1,25\text{-}(OH)_2D_3$ rigorously. In contrast, it was reported that demethylation of one or two methyl groups at 25-position of vitamin $D_3$ derivatives significantly diminish the activity, therefore 25-dimethyl groups of vitamin $D_3$ derivatives were considered to be essential for eliciting activity [20]. These facts are consistent with the assumption when the nature of hydroxyl group is considered to be critical. The existence of dimethyl group at 25-position of vitamin $D_3$ might be essential to maintain the suitable nature of hydroxyl group, e.g. electron density or dissociation constant, in the binding state.

Of interest is that difference of the activities could be observed between the epimers. In all assay methods, the 25*R*-isomer was found to be more potent than the 25*S*-congener. These different activities of *in vivo* experiments might reflect the affinities to the receptor. Although it is not clear why the difference caused in the binding states, one possible explanation is that in the ground states the epimers have different direction of the 25-hydroxyl group by sterically favorite due to the stereochemistry of 25-position, but in the binding states the 25-hydroxyl group of the epimers is most likely to have the same direction. Therefore, the different binding affinity might be attributable to the sterically energy difference between the epimers themselves owing to the same direction of the 25-hydroxyl group, not to an interaction between the vitamin D derivatives and the receptor from the above discussion.

It is worthy to note that the both epimers showed long lasting activity as shown in Figs 3 and 4. Although the serum concentration of 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ gradually decreased as well as 1,25-$(OH)_2D_3$, it is possible that the 22-dehydro-26-methyl-1,25-$(OH)_2D_3$ are degraded more slowly than 1,25-$(OH)_2D_3$ in the cells by steric hindrance against a degradational hydroxylation step of the side chain moiety of the vitamin $D_3$. Other explanations are also possible and further experiment must be needed.

Activity of both epimers was also tested in inhibition of cell growth, induction of phenotypic differentiation and c-*myc* mRNA reduction of HL-60 cells [21]. Both epimers were also more potent than 1,25-$(OH)_2D_3$ in these assay methods. Interestingly, the 25*S*-isomer showed higher activity than the 25*R*-isomer in contrast to the present results. In the assay of the several markers of cell differentiation, (25*R*)- and (25*S*)-22-dehydro-26-methyl-1,25-$(OH)_2D_3$ were 5–10 times and 10–15 times as active as 1,25-$(OH)_2D_3$, respectively.

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