24-Homologated 1,25-Dihydroxyvitamin D_3 Compounds: Separation of Calcium and Cell Differentiation Activities†

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ABSTRACT: A series of 24-homologated 1,25-dihydroxyvitamin D_3 compounds have been chemically synthesized and studied with regard to their activity in inducing differentiation of human promyelocyte HL-60 cells to monocytes and in calcium mobilizing activity in vitamin D deficient rats. Homologation of 1,25-dihydroxyvitamin D_3 or its Δ^{22} analogue by one or two carbons increases by 10-fold and three-carbon homologation reduces by half the activity in causing differentiation of HL-60. On the other hand, homologation causes a substantial decrease in in vivo calcium mobilization activity. The addition of each carbon at the 24-position decreases binding to the HL-60 receptor or rat intestinal receptor by 5–10-fold so that binding affinity of the trihomo compound for the receptors is 130 times less that of 1,25-dihydroxyvitamin D_3. Thus, binding affinity for the receptor cannot account for the preferential activity of the 24-homologated compounds in inducing cell differentiation.

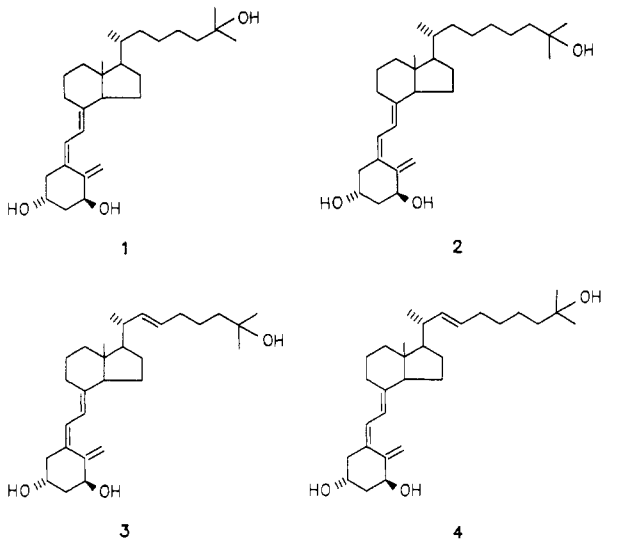
Since the discovery of the active form of vitamin D, namely 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$],[1] considerable expansion in our understanding of the role of vitamin D in physiology has been realized. For example, 1,25-$(OH)_2D_3$ not only stimulates intestinal calcium absorption, the mobilizing of calcium from bone, and renal reabsorption of calcium (DeLuca & Schnoes, 1983; DeLuca, 1988) but also appears to suppress parathyroid gland function (Russell et al., 1986), plays a role in the biological activity of the islet cells of the pancreas (Chertow et al., 1983), causes differentiation of promyelocytes to monocytes (Abe et al., 1981; Tanaka et al., 1982), stimulates differentiation of keratinocytes (Hosomi et al., 1983), and is required for reproduction (Halloran & DeLuca, 1980; Kwiecinski et al., 1989). It is therefore, apparent that the biological functions of vitamin D are much broader than previously realized.

In the investigation of the activities of vitamin D compounds in causing differentiation of human promyelocytes, it was learned that 24-homo-1,25-$(OH)_2D_3$ appears to have a higher activity than 1,25-$(OH)_2D_3$ in this function (Ostrem et al., 1987a,b). To exploit this finding, we have devised a new synthetic approach that permits side-chain homologues of 1,25-$(OH)_2D_3$ to be rapidly prepared (Kutner et al., 1988). We have now prepared 24,24-dihomo-, Δ^{22}-24,24-dihomo-, and Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$ and studied their activities in HL-60 and in the calcium mobilizing systems. The results demonstrate very clearly that 24-homologation increases activity in causing differentiation of HL-60 while markedly diminishing activity in mobilization of calcium in vivo.

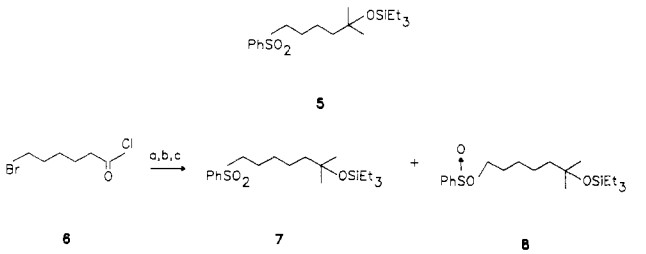
Experimental Procedures

Preparation of the Vitamin D Homologues. 24-Homo-1,25-$(OH)_2D_3$ (**1**) and 24,24-dihomo-1,25-$(OH)_2D_3$ (**2**) were prepared as described previously (Kutner et al., 1988; Tanaka et al., 1986), whereas Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$ (**3**) and

Scheme I: Compounds Tested for Biological Activity



Scheme II: Construction of Side-Chain Components



[a] MeMgBr. [b] $PhSO_2Na$, DMF. [c] Et_3SiCl, imidazole, DMF.

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; THF, tetrahydrofuran; Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$, (22*E*)-22-dehydro-24,24-dihomo-1,25-dihydroxyvitamin D_3; Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$, (22*E*)-22-dehydro-24,24,24-trihomo-1,25-dihydroxyvitamin D_3.

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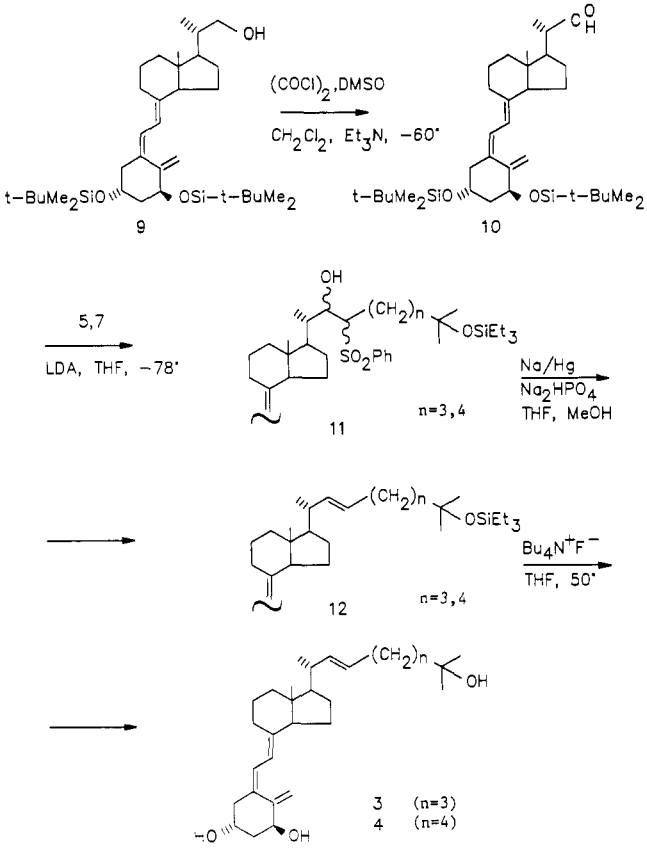
Scheme III: Condensation of Side-Chain Components with Steroid Nucleus To Produce Desired Compounds Shown in Scheme I



Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$ (**4**) were prepared as shown in Scheme I.

Compound **9** prepared previously (Kutner et al., 1988) was oxidized by Swern oxidation to the vitamin D C-22 aldehyde **10** as our precursor for homologated Δ^{22}-1,25-$(OH)_2D_3$ analogues (Scheme III). The protected sulfone derivatives **5** and **7** were deprotonated by *n*-BuLi in tetrahydrofuran (THF) at −78 °C and added to the 1α-hydroxyvitamin D C-22 aldehyde **10** to afford the corresponding 22-hydroxy-23-phenylsulfonyl derivative **11**. Reductive desulfonylation with sodium amalgam gave the protected Δ^{22}-1,25-$(OH)_2D_3$ homologue **12**. This was followed by protecting group removal to give the final products **3** and **4**.

Side-chain fragment **5** was synthesized as described before (Kutner et al., 1988). For the synthesis of phenyl sulfone fragment **7**, 6-bromohexanoyl chloride (**6**) served as the C-6 synthon (Scheme II). This was treated with methylmagnesium bromide to give the tertiary alcohol, followed by reaction with benzenesulfinic acid sodium salt in dimethylformamide. Under these conditions in addition to the expected product **7**, a phenylsulfinate ester **8** is formed as a byproduct which was separated from the phenyl sulfone **7** after the formation of the triethylsilyl ether using triethylsilyl chloride and imidazole in anhydrous dimethylformamide. The two products were easily separated by silica gel column chromatography.

Lithiation of phenyl sulfone derivatives **5** and **7** (LDA, THF, −78 °C, 30 min) followed by the addition of aldehyde **10** (THF, −78 °C, 1 h) gave **11** as a mixture of diastereoisomers in fair to good yields (Scheme III). Desulfonylation of **11** with fresh 5% Na–Hg (THF, MeOH, Na_2HPO_4) gave the ether **12** with some 22-hydroxylated byproducts. Deprotection with Bu_4NF^- in THF at 60 °C gave the final Δ^{22}-1,25-$(OH)_2D$ homologues in 60% yield.

2-Methyl-7-(phenylsulfonyl)-2-[(triethylsilyl)oxy]heptane (**7**). A solution of 6-bromohexanoyl chloride (**6**) (3.8 g, 2.8 mL) (18 mmol) in anhydrous THF (10 mL) was added dropwise with vigorous stirring over 15–20 min under argon atmosphere to a solution of methylmagnesium bromide (14 mL of 3 M solution in ether) in anhydrous THF (15 mL) at −10 °C. The mixture was stirred at room temperature for 2 h, cooled to 0 °C, and carefully decomposed with 1:1 diluted hydrochloric acid. The mixture was extracted with ether, and the combined organic layers were washed with water, dried over anhydrous $MgSO_4$, and evaporated to give 3.6 g (94%) of the bromo alcohol as a colorless oil.

The bromo alcohol (3.4 g) (16 mmol) was treated with benzenesulfinic acid sodium salt (3.3 g) (20 mmol) in anhydrous dimethylformamide at 70 °C for $4^1/_2$ h. The mixture was poured on ice, extracted with dichloromethane, washed with 1 N HCl, water, and 10% $NaHCO_2$, dried over anhydrous $MgSO_4$, filtered, and evaporated. The resulting sulfone was purified by flash chromatography on silica gel and eluted with 40–50% ethyl acetate in hexane to give 4.18 g of sulfone (98%) containing some sulfinate ester. Mass spectrum 270 (M^+), 255 (M^+, −15), 77, 59.

To a stirred solution of the sulfone (4 g) (14 mmol) and imidazole (3.8 g, 55 mmol) in anhydrous dimethylformamide (13 mL) was added triethylsilyl chloride (4.6 g, 5.1 mL) (30 mmol). The reaction mixture was stirred at room temperature for 2 h, poured on ice water, extracted with ether, dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by flash chromatography. Hexaethyldisiloxane was first eluted with hexane. The protected sulfinate ester was eluted with 3% ethyl acetate in hexane, and 10% ethyl acetate in hexane eluted the protected pure sulfone **7** (3.4 g, 60%).

Anal. Calcd for $C_{20}H_{36}O_3SSi$: C, 62.45; H, 9.43; S, 8.34. Found: C, 61.97; H, 9.45; S, 8.33. Mass spectrum, *m/z* (rel intensity), 355 (M^+ −29) (100), 227 (15), 173 (35), 103 (43), 75 (95), 55 (23). ^1H NMR (400 MHz, $CDCl_3$) δ 0.54 (6 H, q, J = 7 Hz, $SiCH_2$), 0.94 (9 H, t, J = 8 Hz, $SiCCH_3$), 1.15 (6 H, s, CH_3), 1.31–1.36 (4 H, m), 3.08–3.12 (2 H, m, H-2), 7.57 (2 H, t, J = 6.8 Hz, Ar H meta), 7.66 (1 H, t), Ar H para), 7.92 (2 H, d, J = 6.8 Hz, Ar H ortho).

(5Z,7E)-(1S,3R,20S)-1,3-Bis[(tert-butyldimethylsilyl)oxy]-9,10-seco-22,23-dinor-5,7,10(19)-cholatrien-24-al (**10**). A solution of 15 μL (0.17 mmol) of oxalyl chloride in 0.75 mL of anhydrous dichloromethane was added dropwise to a stirred solution of 25 μL (0.36 mmol) of dimethyl sulfoxide in 0.25 mL of anhydrous dichloromethane at −60 °C under argon atmosphere. After the mixture was stirred for 10 min at −60 °C, the solution of 20.3 mg (0.035 mmol) of alcohol **9** in 0.5 mL of anhydrous dichloromethane was slowly added, and the flask was rinsed with an additional 0.2 mL of anhydrous dichloromethane. The mixture was stirred for 30 min at −60 °C, and 0.3 mL (2.15 mmol) of triethylamine was added at −60 °C. The mixture was stirred for 5 min, warmed to 0 °C, and extracted with ether. The ether phase was washed with brine and dried ($MgSO_4$). Silica gel SepPak filtration afforded **10** as a colorless oil, which was purified by HPLC (Zorbax-Sil column, 9.4 × 25 cm, 10% EtOAc in hexane) to give the pure aldehyde **10** (19 mg, 96%); only a trace (0.12 mg) of alcohol **9** was recovered.

Preparation of Hydroxy Sulfone **11**. To a stirred solution of 31 mg (84 μmol) of 2-methyl-6-(phenylsulfonyl)-2-[(triethylsilyl)oxy]hexane (**5**) in 300 μL of anhydrous THF (containing 1,10-phenanthroline as an indicator) under argon at −78 °C was added 13 μL (90 μmol) of diisopropylamine followed by 70 μL of *n*-BuLi (91 μmol) (1.30 M in hexane).

The solution was stirred under argon at −78 °C for 30 min, and then 6 mg of aldehyde **10** (10 μmol) in 300 μL of anhydrous THF was added; the solution was stirred at −78 °C for 1 h. The mixture was decomposed by the addition of 1 mL of saturated NH_4Cl, warmed to 0 °C, and extracted with ethyl acetate. The ethyl acetate was washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. Preparative HPLC (Zorbax-Sil column, 9.6 × 25 cm; solvent system, 10% ethyl acetate in hexane) gave 0.6 mg of unreacted aldehyde **10** and 6.6 mg (77%) of the hydroxy sulfone **11** as a mixture of epimers.

Δ^{22}-24,24-Dihomo-1,25-$(OH)_2D_3$ (**3**). A saturated solution of Na_2HPO_4 in methanol (1.0 mL) was added to a stirred solution of hydroxy sulfones **11** (3.3 mg) in 1.0 mL of anhydrous THF followed by powdered anhydrous Na_2HPO_4 (160 mg). The mixture was stirred under argon for 30 min and cooled to 0 °C. Fresh 5% sodium amalgam (ca. 400 mg) was then added, and the mixture was stirred for 16 h at 5 °C. The mixture was diluted with 5 mL of hexane, and stirring was continued for 15 min. Solvents were decanted, and the solid material was washed with hexane (3 × 5 mL). Ice and saturated NaCl solution were added to the combined organic solution. The organic layer was separated and passed through a SepPak cartridge in hexane. HPLC purification (Zorbax-Sil, 9.4 × 25 column, 10% EtOAc in hexane) gave 2.0 mg (71%) of protected Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$ and some 22-hydroxylated product. Protected triol (2 mg) was dissolved in 1.0 mL of anhydrous THF, and to this solution was added tetrabutylammonium fluoride in THF (50 μL, 1 M solution). The mixture was stirred under argon for 1 h at 50 °C.

Ether (8 mL) was then added, and the organic phase was washed with saturated NaCl. Solvents were removed, and the residue was dissolved in 10% 2-propanol in hexane and filtered through silica SepPak. HPLC (Zorbax-Sil 9.4 × 25 cm column, 20% 2-propanol in hexane) gave the triol **3** (592 μg). UV (EtOH) λ_{max} 264 nm, λ_{min} 228 nm, A_{264}/A_{228} = 1.87; ^1H NMR ($CDCl_3$) δ 0.55 (3 H, s, 18-CH_3), 1.0 (3 H, d, J = 6.6 Hz, 21-CH_3), 1.23 (6 H, s, 26,27-CH_3), 4.23 (1 H, m, 3-H), 4.43 (1 H, m, 1-H), 5.00 (1 H, br s, 19*Z*-H), 5.32 (1 H, br s, 19*E*-H), 5.29 (2 H, m, 22-H and 23-H), 6.01 (1 H, d, J = 11.3 Hz, 7-H), 6.36 (1 H, d, J = 11.2 Hz); MS, m/z (rel intensity) 442 (M^+, 15), 424 (23), 406 (33), 391 (7), 287 (11), 285 (10), 269 (27), 251 (23), 152 (33), 134 (100), 116 (6), 59 (20). Exact mass calcd for $C_{29}H_{46}O_3$: 442.3446. Found: 442.3441.

Δ^{22}-24,24,24-Trihomo-1α,25-dihydroxyvitamin D_3 (**4**). **4** was obtained from 2-methyl-7-(phenylsulfonyl)-2-[(triethylsilyl)oxy]heptane (**7**) and the protected aldehyde **10** by the method described for **3** through the intermediate hydroxy sulfone **11** (n = 3) to give the (22*E*)-dehydrotriol **4**. UV (EtOH) λ_{max} 264 nm, λ_{min} 228 nm, A_{264}/A_{228} = 1.81; ^1H NMR ($CDCl_3$) δ 0.56 (3 H, s, 18-CH_3), 1.00 (3 H, d, J = 6.6 Hz, 21-CH_3), 1.23 (6 H, s, 26,27-CH_3), 4.23 (1 H, m, 3-H), 4.43 (1 H, m, 1-H), 5.00 (1 H, br s, 19*Z*-H), 5.32 (1 H, br s, 19*E*-H), 5.29 (2 H, m, 22-H and 2o-H), 6.01 (1 H, d, J = 11.3 Hz, 7-H); MS, m/z (rel intensity) 456 (M^+) (11), 438 (50), 420 (30), 402 (8), 287 (10), 269 (23), 251 (23), 152 (35), 134 (100). Exact mass calcd for $C_{30}H_{48}O_3$: 456.3602. Found: 456.3610.

Ultraviolet absorption spectra were recorded with a Hitachi Model 60-100 UV–vis spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded in the solvent noted at 270 or 400 MHz with a Bruker WH-270 or AM-400 FT spectrometer. Chemical shifts (δ) are reported downfield from internal Me_4Si (δ 0.00) or $CHCl_3$ (δ 7.24). Low- and high-resolution mass spectra were recorded at 70 eV (unless otherwise stated) on a Kratos MS-50 TC instrument equipped with a Kratos DS-55 data system. High-resolution data were obtained by peak matching. Samples were introduced into the ion source maintained at 120–250 °C via a direct insertion probe.

Silica gel 60 (Merck, 70–230 or 230–400 mesh) was used for column chromatography. Thin-layer chromatography (TLC) was performed by using precoated aluminum silica gel sheets with UV indicator from EM Science (Gibbstown, NJ). Solvent systems used: hexane–ethyl acetate 1:1 and hexane–ethyl acetate 3:1. High-performance liquid chromatography (HPLC) was performed on a Waters Associates liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 6 UK universal injector, and a Model 450 variable-wavelength detector. Zorbax silica (Phenomenex) columns (6.2 mm × 20 cm and 10 mm × 25 cm) were used. Solvent systems: A, 3% 2-propanol in hexane; B, 2% 2-propanol in hexane; C, 6% 2-propanol in hexane; D, 10% 2-propanol in hexane; E, 20% 2-propanol in hexane; F, 2% ethyl acetate in hexane. Silica gel SepPak (Waters Associates) cartridges were used for the prefiltration of HPLC samples.

THF was distilled from sodium benzophenone ketyl. Other solvents were purified by standard methods. *n*-Butyllithium in hexanes (Aldrich) was titrated with 1-propanol in the presence of 1,10-phenanthroline in THF under argon.

HL-60 Differentiation Assays. Human leukemia HL-60 cells were obtained through Professor William Mellon (College of Pharmacy, University of Wisconsin—Madison) from American Type Tissue Culture. The cells were maintained in continuous suspension culture in RPMI 1640 medium (GIBCO), supplemented with 10% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere at 5% CO_2 in air. The doubling time of HL-60 cells is 30 h under these conditions. HL-60 cells were seeded at 1 × 10^5 cells/mL in 10 mL of medium in culture dishes. The cells were treated in parallel with vitamin D derivatives dissolved in ethanol, keeping the final ethanol concentration below 0.2%. Control dishes received only the appropriate level of ethanol. The cells were incubated for 96 h, collected by centrifugation, washed twice with serum-free medium, and counted with a hemacytometer. Cell viability was confirmed by trypan blue exclusion. The harvested cell suspension in RPMI 1640 was used to determine the percentage of cells that exhibited functional and enzymatic markers characteristic of monocytes as determined by three different assays.

Morphological determination of cell differentiation was carried out on an aliquot of cell suspension smeared on a slide, stained with Wright stain, and the percent differentiated cells was recorded.

For the phagocytosis assay, dried *Saccharomyces cerevisiae* had been boiled in phosphate-buffered saline for 1 h, collected by centrifugation, and hand stained for 15 min with trypan blue dye. These cells were then washed twice with PBS and resuspended in RPMI 1640 at a concentration of 2 × 10^8 cells/mL. Treated HL-60 cells were suspended in RPMI 1640 containing 20% human type AB serum (GIBCO) and 20% fetal calf serum at a final concentration of 2 × 10^6 cells/mL. A 1:1 (v/v) mixture of yeast and HL-60 cell suspension was incubated for 1 h at 37 °C, and the percentage of cells that had ingested the blue-stained yeast cells was determined.

For nonspecific acid esterase activity, the cytochemical assays using α-naphthyl acetate and naphthol AS-D chloroacetate esterase were done with a commercially available kit (Sigma 90).

Nitroblue tetrazolium (NBT) reduction assay is used to measure the ability of the treated cells to produce superoxide when challenged with phorbol ester. Cell suspension (200 μL) (3×10^5 cells) was mixed with 200 μL of freshly prepared phorbol ester–NBT solution (a PBS solution containing 100 ng of phorbol ester/mL and 1 mg of NBT/mL and incubated for 30 min at 37 °C). The number of cells containing intracellular black-blue formazan deposits was determined by light microscopy using a hemacytometer. The results were plotted on semilog paper, and ED_{50}'s were determined by the concentration of compound that caused 50% of the cells to be differentiated according to the assay.

Calcium Mobilization Assays. Male weanling rats were obtained from the Sprague Dawley Co. (Madison, WI) and were fed a low-calcium, vitamin D deficient diet and water supplied ad libitum for 3 weeks (Suda et al., 1970). The animals were then divided into appropriate groups and were given the appropriate concentrations by intraperitoneal injection of compound dissolved in 0.1 mL of propylene glycol. In experiment I, the propylene glycol solution was delivered by osmotic minipump, Model 2002, by the Alzet Co. (Palo Alto, CA). In this case, the concentration of compound was increased such that 50 μL would deliver the daily dose. All doses were provided for 7 days. At the end of the 7-day period, the animals were killed and used to determine intestinal calcium transport and serum calcium concentration. Intestinal calcium transport was carried out by modification of the method of Martin and DeLuca (1969), whereas bone calcium mobilization was estimated by the rise in serrum calcium of vitamin D deficient rats on a low-calcium diet (Blunt et al., 1968).

Receptor Displacement Studies. Preparation of HL-60 cell extract was carried out at 0–4 °C. HL-60 cells were processed as described (Ostrem et al., 1987). HL-60 cells were washed 3 times with Ca^{2+} and Mg^{+}-free PBS. Cell pellets were suspended in $TEDK_{300}$ buffer (Tris-HCl 50 mmol, EDTA 1.5 mmol, dithiothreitol 5 mmol, and KCl 300 mmol) with 5 mmol of diisopropyl fluorophosphate and sonicated with three 20-s cycles interrupted by 60-s pauses. The sonicate was centrifuged at 105000*g* for 60 min to yield the receptor preparation. The receptor preparation was frozen in liquid nitrogen and stored at −80 °C until used. Protein concentration was determined by the method of Bradford (1974) using bovine serum albumin as the standard. Rat intestinal receptor was prepared as previously described (Pierce et al., 1988).

Displacement by 24-Homologues of 1,25-(OH)$_2$-[26,27-^{3}H]D_3 from HL-60 or Rat Intestinal Receptor. The displacement experiment was performed essentially as described (Inaba et al., 1989). Briefly, graded amounts of either 1,25-$(OH)_2D_3$ or analogues were dissolved in 12 μL of ethanol. 1,25-Dihydroxy-[26,27-^{3}H]vitamin D_3, dissolved in 8 μL of ethanol, was mixed with 180 μL of HL-60 cell extract to make a final volume of 200 μL. The protein concentration of HL-60 cell extracts was adjusted to 1.0 mg/mL. The mixture was incubated for 60 min at 25 °C. Bound 1,25-$(OH)_2$-[26,27-^{3}H]D_3 was determined by using the hydroxylapatite assay (Dame et al., 1985).

Results

Our previous work had demonstrated that 24-homo-1,25-$(OH)_2D_3$ had 10 times greater activity than 1,25-$(OH)_2D_3$ in causing differentiation of HL-60 cells while it had $^1/_{10}$ the activity of 1,25-$(OH)_2D_3$ in calcium mobilizing activity (Ostrem et al., 1987). The results of that study are reproduced here for comparison with the new synthetic compounds prepared for the present study. An obvious question is, therefore, by increasing the side-chain length at the 24-position, can one continue to increase differentiative activity in HL-60 cells while diminishing calcium mobilizing activity? We therefore carried out chemical synthesis of 24,24-dihomo-1,25-$(OH)_2D_3$, its Δ^{22}-analogue, and Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$. The chemical synthesis is an extension of our synthetic strategy involving the production of side-chain pieces to be condensed with the C-22 aldehyde **10** (Kutner et al., 1988). This synthetic approach was successful in providing the required compounds.

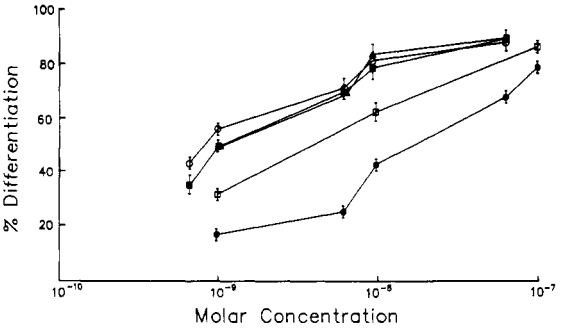


FIGURE 1: Differentiation activity of the analogues of 1,25-$(OH)_2D_3$. Percent differentiation is computed on the basis of cells showing nonspecific esterase. (□) 1,25-$(OH)_2D_3$; (●) Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$; (○) 24-homo-1,25-$(OH)_2D_3$; (■) 24,24-dihomo-1,25-$(OH)_2D_3$; (△) Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$.

Table I: HL-60 Differentiating Activity of 24-Homologues of 1,25-$(OH)_2D_3$[a]

compound	concn (M)	NSE (%)	NBT (%)	phagocytosis (%)
1,25-$(OH)_2D_3$	1×10^{-7}	87 ± 2	85 ± 3	88 ± 3
	1×10^{-8}	61 ± 3	60 ± 2	62 ± 4
	1×10^{-9}	34 ± 2	38 ± 3	34 ± 3
24-homo-1,25-$(OH)_2D_3$	5×10^{-8}	89 ± 2	89 ± 3	90 ± 3
	1×10^{-8}	80 ± 2	82 ± 4	78 ± 2
	5×10^{-9}	68 ± 4	69 ± 3	70 ± 3
	1×10^{-9}	56 ± 3	53 ± 5	49 ± 3
	5×10^{-10}	43 ± 2	40 ± 4	39 ± 2
24,24-dihomo-1,25-$(OH)_2D_3$	5×10^{-8}	90 ± 3	89 ± 5	88 ± 3
	1×10^{-8}	83 ± 3	82 ± 3	80 ± 3
	5×10^{-9}	67 ± 2	65 ± 5	66 ± 2
	1×10^{-9}	49 ± 2	51 ± 4	51 ± 4
Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$	5×10^{-8}	92 ± 2	93 ± 3	90 ± 3
	1×10^{-8}	78 ± 3	81 ± 2	80 ± 4
	5×10^{-9}	67 ± 2	68 ± 3	65 ± 3
	1×10^{-9}	49 ± 2	50 ± 4	51 ± 3
	5×10^{-10}	36 ± 4	38 ± 4	37 ± 4
Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$	1×10^{-7}	79 ± 2	76 ± 2	77 ± 2
	5×10^{-8}	68 ± 2	68 ± 4	54 ± 3
	1×10^{-8}	41 ± 4	36 ± 1	40 ± 2
	5×10^{-9}	25 ± 3	25 ± 2	23 ± 2
	1×10^{-9}	16 ± 2	13 ± 2	16 ± 4

[a] Results are expressed as percent of total cells counted that have differentiated.

As shown in Figure 1, 24,24-dihomo-1,25-$(OH)_2D_3$ and 24-homo-1,25-$(OH)_2D_3$ are approximately 10 times more active than the native hormone in causing differentiation of HL-60 cells. Thus, the addition of more than one carbon does not increase differentiative activity further. The addition of an additional carbon as in Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$ results in differentiative activity half that of the native hormone. Thus, the optimal increase in chain length at the 24-position for differentiation activity is between one and two carbons. Table I illustrates that other measurements of differentiation activity gave the same result.

Of great interest is the calcium mobilizing activity provided by the 24-homologues (Table II). There is not doubt that 1,25-$(OH)_2D_3$ is the superior compound in terms of mobilizing

Table II: Calcium Mobilizing Activity of 24-Homologated 1,25-Dihydroxyvitamin D Compounds[a]

expt	compound	dose (pmol/day)	Ca transport (SEM)	serum Ca (mg/100 mL)
I	-control	0	2.6 ± 0.4^{a}	3.9 ± 0.3^{a}
	+1,25-$(OH)_2D_3$	6.5	$4.4 \pm 0.6^{b^1}$	
		32.5	$4.8 \pm 0.2^{b^2}$	$4.3 \pm 0.3^{b^2}$
		65	$7.3 \pm 1.9^{b^3}$	$5.1 \pm 0.9^{b^3}$
	24-homo-1,25-$(OH)_2D_3$	6.5	$3.2 \pm 0.4^{c^1}$	
		32.5	$4.6 \pm 0.8^{c^2}$	
		65	$6.4 \pm 1.3^{c^3}$	$4.0 \pm 0.31^{c^3}$
II	control	0	4.8 ± 0.26^{a}	4.1 ± 0.11^{a}
	1,25-$(OH)_2D_3$	32.5	$11.2 \pm 0.58^{b^1}$	$4.9 \pm 0.2^{b^1}$
		65	$13.4 \pm 1.1^{b^2}$	$4.9 \pm 0.2^{b^2}$
	24,24-dihomo-1,25-$(OH)_2D_3$	285	9.4 ± 0.77^{c}	
		570		$4.2 \pm 0.2^{c^2}$
		1140		$3.6 \pm 0.19^{c^3}$
		2280		$3.8 \pm 0.2^{c^4}$
		0		$3.6 \pm 0.02^{a^1}$
	Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$	285	6.8 ± 0.5^{d}	$4.1 \pm 0.1^{d^2}$
		570		$4.1 \pm 0.1^{d^3}$
		1140		$3.8 \pm 0.2^{d^4}$
		2280		$3.6 \pm 0.1^{d^5}$
III	control		5.2 ± 0.23^{a}	4.0 ± 0.1^{a}
	1,25-$(OH)_2D_3$	600	12.0 ± 1.5^{b}	5.5 ± 0.1^{b}
	Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$	55		$4.1 \pm 0.3^{c^1}$
		275	$7.4 \pm 0.3^{c^2}$	$3.8 \pm 0.1^{c^2}$
		550	$6.0 \pm 0.4^{c^3}$	$4.0 \pm 0.2^{c^3}$
		1096	$5.9 \pm 0.3^{c^4}$	$4.0 \pm 0.2^{c^4}$

[a] Vitamin D deficient rats were fed a low-calcium diet and given the indicated daily dose of compound in propylene glycol intraperitoneally or by Alzet minipump (experiment I) for 7 days. Controls received the vehicle. At 7 days the rats were killed for the determinations. There were at least six rats per group. Statistical analysis was done by Student's *t* test. Experiment I, Ca transport: b^1, b^2, b^3 from a, $p < 0.025$; c^1, c^2 from a, NS; c^3 from a, $p = 0.025$; c^1c^2, c^3 from b^1, b^2, b^3, NS; b^3 from b^1, b^2, NS. Experiment I, serum Ca: b^3 from a, $p < 0.01$; b^2 from a, NS. Experiment II, Ca transport: b^1, b^2 from a, $p < 0.001$; c from a, $p < 0.001$; d from a, $p < 0.001$; c from b^1, $p < 0.05$; c from b^2, $p = 0.01$; d from b^1, b^2, $p < 0.001$; d from c, $p = 0.01$; b^1 from b^2, NS. Experiment II, serum Ca: b^1, b^2 from a, $p < 0.005$; c^{2-4}, d^{1-5} from a, NS; b^1, b^2 from c^{2-4}, $p < 0.001$; b^1, b^2 from d^{1-5}, $p < 0.001$; d^{1-5} from a^1 and a, NS. Experiment III, Ca transport: b from a, $p = 0.001$; c^2 from a, $p < 0.05$; c^3 and c^4 from a, NS; c^{2-4} from b, $p < 0.01$. Experiment III, serum Ca: b from a, $p < 0.001$; c^{1-4} from a, NS; b from c^{1-4}, $p < 0.001$.

calcium from the skeleton. 24-Homo-1,25-$(OH)_2D_3$ showed no calcium mobilizing activity from the skeleton when provided at 65 pmol/day, whereas 1,25-$(OH)_2D_3$ elicited calcium mobilizing activity at 65 pmol/day. When provided at as much as 2280 pmol/day, neither of the dihomo compounds elicited a bone calcium mobilization response, whereas significant bone mobilizing response was found with 1,25-$(OH)_2D_3$ provided at 32 pmol/day. These results suggest that the dihomo compounds are approximately 1000 times less active in mobilizing skeletal calcium than is 1,25-$(OH)_2D_3$. Not surprisingly, therefore, no bone calcium mobilization was found with Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$.

In the case of intestinal calcium transport, 6.5–32.5 pmol/day of 1,25-$(OH)_2D_3$ appears to saturate this system. The 24-homo-1,25-$(OH)_2D_3$ compound is less active than 1,25-$(OH)_2D_3$ in this test. However, the dihomo compounds do not saturate even when provided at 285 pmol and thus are at least 10 times less active than 1,25-$(OH)_2D_3$. The trihomo compound shows little or no activity at even 1096 pmol/day. Although exact estimates of activity in this system are not possible from the data available, it is clear that the dihomo and trihomo compounds are at least 10 times less active in intestinal calcium transport than is 1,25-$(OH)_2D_3$.

These striking results immediately raise the question of how these substances interact with the receptor obtained from the

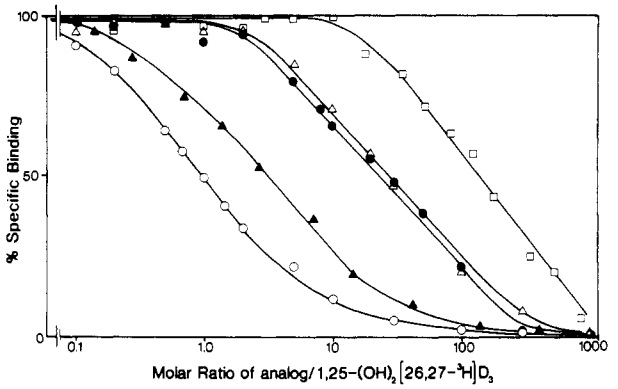


FIGURE 2: Displacement of 1,25-$(OH)_2$-[26,27-^{3}H]D_3 from specific binding sites in HL-60 cell extracts by 1,25-$(OH)_2D_3$ and 24-homo analogues. The data represent at least two separate experiments, and each value is representative of two determinations. (○) 1,25-$(OH)_2D_3$; (▲) 24-homo-1,25-$(OH)_2D_3$; (●) 24,24-dihomo-1,25-$(OH)_2D_3$; (△) Δ^{22}-24,24-dihomo-1,25-$(OH)_2D_3$; (□) Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$.

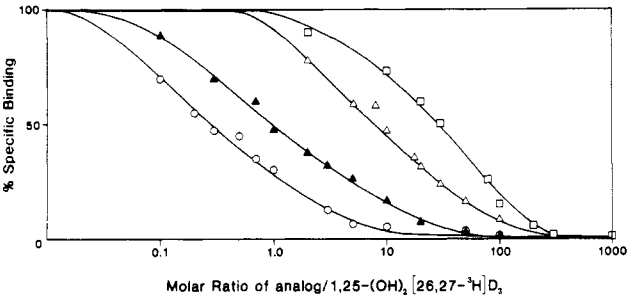


FIGURE 3: Displacement of 1,25-$(OH)_2$-[26,27-^{3}H]D_3 from specific binding sites in rat intestinal extracts by 1,25-$(OH)_2D_3$ and the 24-homo analogues. Legend and description as in Figure 2.

HL-60 cells. Displacement curves shown in Figures 2 and 3 illustrate that the dihomo compounds are approximately 30 times less active in binding to the HL-60 1,25-$(OH)_2D_3$ receptor, whereas the trihomo compound is approximately 130 times less active in binding to the receptor. In the case of the trihomo compound, although it is a very poor binder relative to 1,25-$(OH)_2D_3$, its activity in causing differentiation approximates that of the native hormone. In the case of the dihomo compound, it is clear that these compounds are 30 times less able to bind to the receptor but they are 10 times more active in causing differentiation. Furthermore, the affinities for the homologues is identical for rat intestinal and HL-60 receptor preparations, demonstrating that receptor affinities cannot account for the selective activities observed.

Discussion

This paper provides clear evidence that the biological activities of the active form of vitamin D can be separated by using specific analogues. It is now evident that Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$ retains almost full activity [i.e., half that of 1,25-$(OH)_2D_3$] in causing differentiation of HL-60 cells into monocytes, whereas it has lost most of its calcium mobilizing activity. Because some intestinal calcium transport activity is noted at high doses of the dihomo compounds, these compounds should increase serum calcium slightly when calcium is present in the intestine. The 24,24-dihomo-1,25-$(OH)_2D_3$ compounds, whether saturated in the 22-position or unsaturated, have 10-fold higher HL-60 differentiative activity than 1,25-$(OH)_2D_3$ but have markedly diminished calcium mobilizing activity. The 24-homo-1,25-$(OH)_2D_3$ reported earlier shows a 10-fold increase in the HL-60 activity and a 5–10-fold decrease in calcium mobilizing

activity (Ostrem et al., 1987). If the differentiative activity is of therapeutic importance in the treatment of leukemia or other neoplastic disease (Eisman et al., 1987), then the 24-homologated 1,25-$(OH)_2D_3$ compounds may be of considerable interest.

Of great importance is that Δ^{22}-24,24,24-trihomo-1,25-$(OH)_2D_3$ expresses only one of the three activities of 1,25-$(OH)_2D_3$ tested, yet it interacts with the 1,25-$(OH)_2D_3$ receptor. So far, cloning evidence suggests that there is only one 1,25-$(OH)_2D_3$ receptor (Baker et al., 1988; Burmester et al., 1988). The activity induced by homologation suggests that the compound interacts in such a way with the receptor as to cause expression of genes related to the differentiative process, while the genes related to the calcium mobilizing process are poorly expressed. How this change in hormonal structure can bring about such discrimination in activities remains to be deduced. Likely, the conformational changes exerted on the receptor by the ligands must be different, which may cause selective binding of the receptor to only certain recognition sites on the genome. Thus, pursuit of which genes are induced by the trihomo compound will be of considerable interest. Furthermore, how this analogue interacts with the receptor and what changes it induces in the receptor as compared to 1,25-$(OH)_2D_3$ will be of considerable interest. By displacement studies it appears that the trihomo-1,25-$(OH)_2D_3$ compound binds less well to the HL-60 receptor than does 1,25-$(OH)_2D_3$ (Figure 2). However, its binding to the receptor is not sufficiently low as to account for the lack of calcium mobilization. There is also the possibility that 1,25-$(OH)_2D_3$ functions in some instances by a cell membrane mechanism. If that is the case, structural changes such as homologation on C-24 might make the hormone unacceptable for one of these functions. However, there is little evidence for the nonnuclear mechanisms suggested.

If the differentiative activity of 1,25-$(OH)_2D_3$ can be used as a therapeutic means of controlling promyelocyte malignant leukemia cells, the 24-homologated series could be of considerable therapeutic importance. However, it is not known whether this strategy will provide a means of controlling leukemia or not. Attempted use of 1α-OH-D_3 for the control of the malignant state has not met with great success except in conjunction with cytotoxic agents (Hollstrom et al., 1988). Very likely, hypercalcemia would result from the use of 1α-OH-D_3 or 1,25-$(OH)_2D_3$ before concentrations could be achieved that could bring about differentiative control of malignant cells. The present compounds would provide a sufficient activity preference as to make it possible to examine the question of whether differentiative control will be an effective means of controlling malignant growth.

It is of some interest that the insertion of a trans double bond at the 22-carbon of 24,24-dihomo-1,25-$(OH)_2D_3$ did not significantly affect the activities tested here. This is not surprising since trans Δ^{22} is a common modification of vitamin D compounds that does not appear to alter biological activity in most mammals (DeLuca & Schnoes, 1983). In this instance it was more convenient to prepare the trans Δ^{22} analogues than the saturated analogues.

The biological testing followed standard protocols. However, the calcium mobilizing activity is measured in vivo, whereas the differentiative activity is measured in vitro. The in vitro method provides important answers but does not take into account pharmacokinetics and turnover of the compounds in vivo. Furthermore, it is not known whether these compounds can be delivered adequately in vivo. Until an in vivo differentiative activity test is devised, little more can be done than to test differentiative activity in vitro. Another possible problem is that the differentiation study was carried out in human cells, while the calcium tests were carried out in the rat. However, the ligand binding studies show that the affinities for the analogues were identical for the receptor from the human cells and rat intestines (see Figures 2 and 3). It is of some interest that 22-oxa-1,25-(OH)2D_3 also shows preferential activity on differentiation (Murayama et al., 1986). Whether this is the result of a change in side-chain length or the oxygen bridge cannot be deduced at the present time. Compounds modified in the side chain that show preferential activity in HL-60 differentiation over calcium mobilization have been prepared (Baggiolini et al., 1989; Calverley, 1987), but comprehensive data on biological activities are as yet unavailable. The results of the present study and the 22-oxa compounds are nevertheless very clear that compounds have now been prepared that can facilitate one function of vitamin D while having little or no action on another.

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Tryptophan Luminescence from Liver Alcohol Dehydrogenase in Its Complexes with Coenzyme. A Comparative Study of Protein Conformation in Solution

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ABSTRACT: The extent of fluorescence quenching and that of phosphorescence quenching of Trp-15 and Trp-314 in alcohol dehydrogenase from horse liver as well as the intrinsic phosphorescence lifetime of Trp-314 in fluid solution have been utilized as structural probes of the macromolecule in binary and ternary complexes formed with coenzyme, analogous, and various substrate/inhibitors. Luminescence quenching by the coenzyme reveals that (1) while the reduced form quenches Trp emission exclusively from the fluorescent state, the oxidized form is very effective on the phosphorescent state as well and that (2) among the series of NADH binary and ternary complexes known by crystallographic studies to attain the closed form, distinct nicotinamide/indole geometrical arrangements are inferred from a variable degree of fluorescence quenching. Information of the dynamic structure of the coenzyme-binding domain derived from the phosphorescence lifetime of Trp-314 points out that within the series of closed NADH complexes there is considerable conformational heterogeneity. In solution, the variability in dynamical structure among the various protein complexes emphasizes that the closed/open forms identified by crystallographic studies are not two well-defined macrostates of the enzyme.

Alcohol dehydrogenase from horse liver (LADH)[1] is one of the best-characterized enzymes (Eklund & Bränden, 1983, 1985; Eklund et al., 1986). The abundance of information available on kinetic, structural, and physicochemical properties makes it a suitable model system for deeper insight into the chemistry and physics of enzyme catalysis. Refined crystallographic structures for the apo- and holoenzyme show that in general coenzyme binding induces a change in conformation in the macromolecule from an open to a closed form (Eklund, 1986; Eklund & Bränden, 1985; Eklund et al., 1984). The latter is characterized by a rigid body rotation of the catalytic domain relative to the coenzyme-binding domain with closure of the cleft separating the domains. Following these findings, the compulsory binding order mechanism proposed by Theorell and Chance (1951) to describe the kinetics of alcohol oxidation could also be rationalized on a structural basis.

Recent crystallographic investigations with a number of substrate/inhibitor and coenzyme/analogue combinations have pointed out, however, that there is not a single holostructure for LADH, and depending on the ligand combination, ternary complexes do form in which the conformation is either open (similar to that of the apoenzyme) or intermediate between open and closed forms (Cedergren-Zeppezaur, 1986). Evidence that a single holoenzyme structure is inadequate to represent LADH in its complexes with oxidized coenzyme comes also from spectroscopic studies in solution with Co-substituted LADH (Maret & Zeppezauer, 1986; Zeppezauer, 1986).

In this work, through the fluorescence and phosphorescence emission of the two Trp residues, we inquire on the conformation of the LADH molecule in solution in an assortment of binary and ternary complexes for which X-ray data are available. Emission data can provide structural information in two ways: (1) From the extent of fluorescence and phosphorescence quenching emission data can test for possible changes in the geometrical arrangement between tryptophans and the nicotinamide ring of the coenzyme. (2) From the rate of decay of the triplet state of Trp-314, emission data can monitor the dynamical structure of the coenzyme-binding domain (Strambini & Gonnelli, 1985; Strambini & Gabellieri, 1987). The latter approach displays a remarkable sensitivity to changes in protein structure and has been instrumental in highlighting the effects induced, for example, by mild concentrations of guanidine hydrochloride (Strambini & Gonnelli, 1986) or incorporation of the enzyme in reverse micelles

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[1] Abbreviations: LADH, liver alcohol dehydrogenase from horse; NADH, reduced nicotinamide adenine dinucleotide; NAD^+, oxidized nicotinamide adenine dinucleotide; MPD, 2-methyl-2,4-pentanediol; DMSO, dimethyl sulfoxide; IBA, isobutyramide; ADPR, adenosine-(5′)diphospho(5)-β-D-ribose; PYR, pyrazole; IMID, imidazole; H_2NADH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; Trp, tryptophan; EtOH, ethanol; TFE, trifluoroethanol.

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