Articles

Syntheses and Differentiating Action of Vitamin D Endoperoxides. Singlet Oxygen Adducts of Vitamin D Derivatives in Human Myeloid Leukemia Cells (HL-60)

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Singlet oxygen adducts of various vitamin D derivatives, 6,19-dihydro-6,19-epidioxyvitamin D (vitamin D endoperoxides, **2** and **2'**), were chemically synthesized, and their biological activity in inducing differentiation of a human myeloid leukemia cell line (HL-60 cells) was examined. The potency of the endoperoxides derived from vitamin D derivatives possessing the 1α-hydroxyl group such as 1α,25-dihydroxyvitamin D_3 endoperoxides (**2b** and **2b'**) was markedly (10^{-2}) diminished relative to the respective parent vitamin D compounds. In contrast, 25-hydroxyvitamin D_3 endoperoxides [25-(OH)D_3 endoperoxides, **2a** and **2a'**] and their analogues fluorinated at the 24- or 26- and 27-positions were 2.5–10 times more potent than 25-hydroxyvitamin D_3 (**1a**) in spite of the absence of the conjugated triene structure typical of vitamin D compounds. The potency of these vitamin D endoperoxides (**2** and **2'**), especially those lacking the 1α-hydroxyl group, in inducing differentiation of HL-60 cells was not correlated with their activity in binding to the cytosol receptor for 1α,25-dihydroxyvitamin D_3 (**1b**). The binding efficiency to the receptor was relatively lower than the differentiating activity. To examine the action of vitamin D endoperoxides, carbon analogues of 25-(OH)D_3 endoperoxides, two C-6 epimers of 25-hydroxy-6,19-dihydro-6,19-ethanovitamin D_3 (**6** and **6'**), were synthesized. The carbon analogues (**6** and **6'**) had no potential to induce differentiation of HL-60 cells. These results suggest that vitamin D endoperoxides (**2** and **2'**) express their biological activity probably after being converted to some other compounds.

It has been established that 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$ (**1b**)],[1] a metabolite of vitamin D_3 active in inducing intestinal calcium transport and bone mineral mobilization, functions at the target tissues by a receptor-mediated mechanism as other steroid hormones do.[1] Recently, the receptor protein for 1α,25-$(OH)_2D_3$ (**1b**) has been found in a variety of tissues such as pituitary,[2] placenta,[3] ovary,[4] thymus,[5] human myeloid leukemia cells,[6] and rat osteogenic sarcoma cells[7] beside its well-known target tissues, intestine and bone. Furthermore, new actions of the active metabolite other than the role in calcium metabolism have been found in recent years. In particular, much attention has been focused on the action of 1α,25-$(OH)_2D_3$ (**1b**) in inhibiting proliferation of tumor cells.[8-10] Abe et al.[9] found that 1α,25-$(OH)_2D_3$ (**1b**) not only suppresses cell growth but also induces differentiation of a murine myeloid leukemia cell line (M1) into monocyte macrophages. Subsequently, Miyaura et al.[10] reported that 1α,25-$(OH)_2D_3$ (**1b**) is also capable of inducing differentiation of a human myeloid leukemia cell line (HL-60). 1α,25-$(OH)_2D_3$ (**1b**) appears to induce those effects by binding to a specific cytosol receptor protein found in HL-60 cells.[6] The specificity of various derivatives of vitamin D_3 in inducing differentiation was well correlated with that of their association with the cytosol receptor. From these results, Tanaka et al.[6] proposed that the active metabolite of vitamin D_3 induces differentiation of human myeloid leukemia cells by a mechanism similar to that presented in the classical concept of steroid hormone action.

This system provides a biologically relevant and technically simple method to assay vitamin D compounds. We have been investigating the relationship between the molecular structure and biological activity of vitamin D compounds by this new assay system. 1α,25-$(OH)_2D_3$ (**1b**) is the most potent of all derivatives examined except for its 24,24-difluorinated analogue.[11] Both hydroxyl groups at the 1α- and the 25-position were found equally important in inducing differentiation of HL-60 cells.[12] The potential of 1α,25-$(OH)_2D_3$ (**1b**) is significantly reduced by structural modifications such as introducing additional

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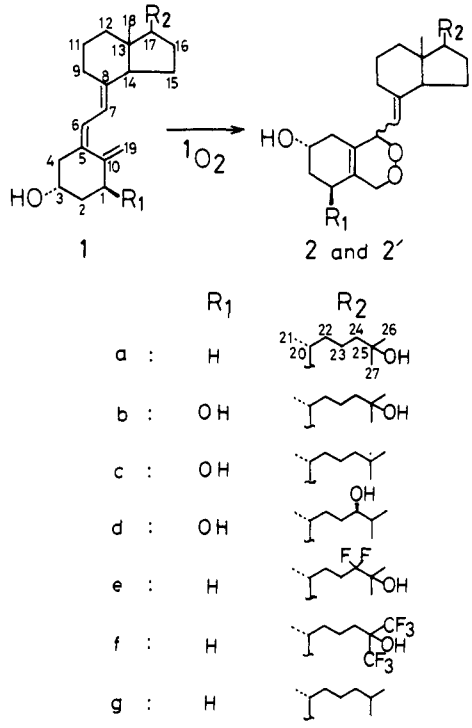
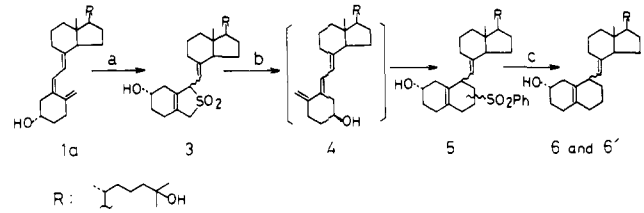
Scheme I



hydroxyl group to the side chain, replacing one of the hydroxyl groups by hydrogen, introducing other functional groups, and so on.[12] This structure–biological activity relationship is closely related to that found in the classical actions in calcium metabolism.[13,14]

We have been interested in the oxidation of the conjugated triene part of vitamin D in conjunction with biological oxidation and have reported the oxidation of vitamin D with singlet oxygen,[15] peracid,[16] and alkyl hydroperoxide in the presence of transition-metal catalysts.[16] Such oxidation produces vitamin D derivatives oxygenated at the conjugated triene function. While investigating the differentiating actions of these oxygenated vitamin D derivatives in HL-60 cells, we found an interesting biological potential in the singlet oxygen adducts of vitamin D derivatives, 6,19-dihydro-6,19-epidioxyvitamin D (vitamin D endoperoxides, **2** and **2′**). We also synthesized 6,19-dihydro-6,19-ethanovitamin D derivatives (**6** and **6′**), carbocyclic analogues of vitamin D endoperoxides (**2** and **2′**), and tested their biological activity in comparison with the endoperoxides (**2** and **2′**). Here, we report the results in detail.[17]

Scheme II[a]



[a] Key: (a) SO_2, −10 °C; (b) phenyl vinyl sulfone, $NaHCO_3$, toluene, 100 °C; (c) Na-Hg, Na_2HPO_4, MeOH, room temperature.

Results

Vitamin D Endoperoxides (2 and 2′). Oxidation of vitamin D derivatives (**1a–g**) with singlet oxygen, generated in situ from ground-state oxygen by a dye-sensitized photochemical reaction, gave two C-6 epimers (**2** and **2′**) of the corresponding oxygen adducts at the s-cis diene part, (6*R*)- and (6*S*)-6,19-dihydro-6,19-epidioxyvitamin D (vitamin D endoperoxides), in about 1:1 ratio in 50–60% total yields (Scheme I).[15] Of the two C-6 epimers, the one (**2**) that migrated fast on a straight-phase HPLC was designated the less polar isomer, and the other (**2′**), the more polar isomer. The structure of the endoperoxides (**2** and **2′**) was confirmed by comparing their spectral data with those of vitamin D_3 endoperoxides (**2g** and **2g′**).[15] The stereochemistry at C-6 of the endoperoxides (**2a** and **2a′**) derived from 25-hydroxyvitamin D_3 [25-(OH)D_3 (**1a**)] was assigned on the basis of the CD spectra; the less polar isomer (**2a**), which showed negative CD, was assigned to the 6*R* isomer and the more polar (**2a′**), which showed positive CD, to the 6*S* isomer.[15b,c] The stereochemistry at C-6 of the other endoperoxides could not been determined because of the lack of sufficient data.

25-Hydroxy-6,19-dihydro-6,19-ethanovitamin D_3 (6 and 6′). Two C-6 epimers of 25-hydroxy-16,19-dihydro-6,19-ethanovitamin D_3 (**6** and **6′**) were synthesized starting with 25-(OH)D_3 (**1a**) via the 1,4-cycloadducts (**5**) with phenyl vinyl sulfone in 60% total overall yield (Scheme II). The adducts (**5**) were obtained in good yield (83% as a mixture of regio- and stereoisomers) by the reaction of 5(*E*)-25-(OH)D_3 (**4**) with phenyl vinyl sulfone; the reaction of the *Z* isomer (**1a**) with the same dienophile afforded a mixture of pyro- and isopyrovitamin D derivatives[18] as a result of thermal sigmatropic 1,7-rearrangement and subsequent electrocyclic B-ring closure of the starting vitamin D under the reaction conditions (100 °C, toluene). The *E* isomer (**4**) was produced in situ from the sulfur dioxide adduct (**3**) of 25-(OH)D_3.[19] The adducts (**5**) were subjected to reductive desulfonylation to yield the desired 6,19-ethanovitamin D as a mixture of the two C-6 epimers (**6** and **6′**, 1:1 ratio, 79% total yield) which were separated by using HPLC.

Biological Activities of Vitamin D Endoperoxides (2 and 2′) and 25-Hydroxy-6,19-dihydro-6,19-ethanovitamin D_3 (6 and 6′). The dose–response effects of various vitamin D endoperoxides (**2** and **2′**) in inducing phagocytic activity of HL-60 cells, a typical marker of the differentiation, were examined in comparison with the parent vitamin D compounds (**1**). The cells were incubated for 3 days with each compound, at which time the dif-

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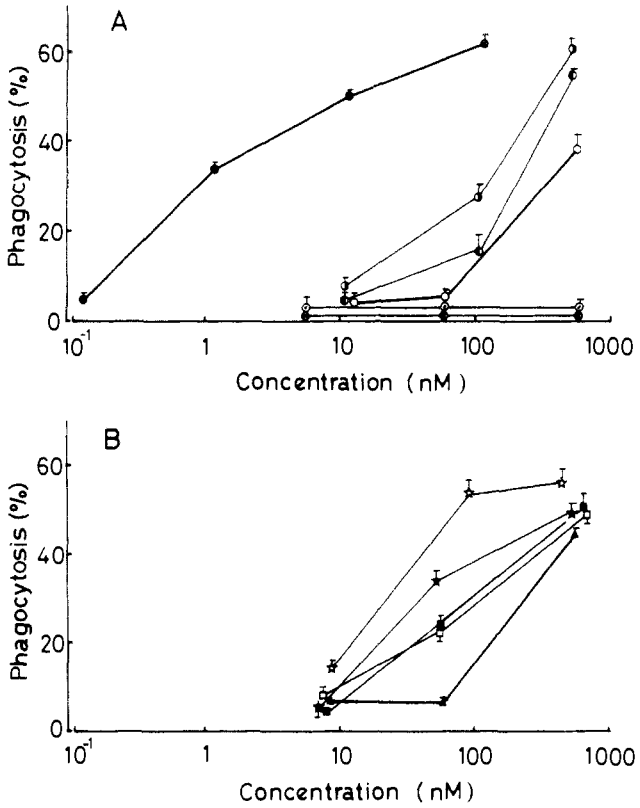

Figure 1. Comparison of the effects of vitamin D endoperoxides (2 and 2′) and their corresponding vitamin D compounds (1) in inducing phagocytic activity of HL-60 cells. The cells were incubated for 3 days with each derivative of vitamin D_3. (A) Vitamin D_3 derivatives possessing the 1α-hydroxyl group: ●, 1α,25-$(OH)_2D_3$ (**1b**); ◑, 1α,25-$(OH)_2D_3$ endoperoxide less polar (**2b**); ◐, 1α,25-$(OH)_2D_3$ endoperoxide more polar (**2b′**); ○, 1α-(OH)D_3 (**1c**); ⊗, 1α-(OH)D_3 endoperoxide less polar (**2c**); ⊙, 1α-(OH)D_3 endoperoxide more polar (**2c′**). (B) Vitamin D_3 derivatives lacking the 1α-hydroxyl group: ▲, 25-(OH)D_3 (**1a**); ■, 25-(OH)D_3 endoperoxide less polar (**2a**); □, 25-(OH)D_3 endoperoxide more polar (**2a′**); ★, 24-F_2-25-(OH)D_3 endoperoxides (**2e** + **2e′**); ☆, 26,27-F_6-25-(OH)D_3 endoperoxides (**2f** + **2f′**). The control value was 2.3 ± 0.4. Points are means ±SEM (represented by the bars) of three to six replicates.

ferentiating activity of 1α,25-$(OH)_2D_3$ (**1b**) attained a maximum. The endoperoxides (**2** and **2′**) were found to be divided into two groups on the basis of their potentiality in inducing phagocytic activity of HL-60 cells (Figure 1). The first group includes the endoperoxides derived from vitamin D_3 derivatives with a 1α-hydroxyl group: 1α,25-dihydroxyvitamin D_3 endoperoxides [1α,25-$(OH)_2D_3$ endoperoxides, **2b** and **2b′**], 1α-hydroxyvitamin D_3 endoperoxides [1α-(OH)D_3 endoperoxides, **2c** and **2c′**], 1α,24-(*R*)-dihydroxyvitamin D_3 endoperoxides [1α,24(*R*)-$(OH)_2D_3$ endoperoxides, **2d** and **2d′**]. The members of the first group showed significantly reduced potentials relative to the parent vitamin D compounds (1) (Figure 1A). They were a hundredth or less active than the corresponding vitamin D. In contrast, the members of the second group, which include the endoperoxides derived from vitamin D_3 derivatives lacking the 1α-hydroxyl group, 25-(OH)D_3 endoperoxides (**2a** and **2a′**) and their fluorinated analogues (**2e** and **2e′**; **2f** and **2f′**), showed enhanced (2.5–6.5 times) potentials (Figure 1B) compared with the corresponding vitamin D compounds. Fluorine substitution enhanced differentiating activity.[12] Thus, 24,24-difluoro-25-hydroxyvitamin D_3 endoperoxides [24-F_2-25-(OH)D_3 endoperoxides, **2e** and **2e′**] and 26,26,26,27,27,27-hexafluoro-25-hydroxyvitamin D_3 endoperoxides [26,27-F_6-25-(OH)D_3 endoperoxides, **2f** and **2f′**] were about 3 times

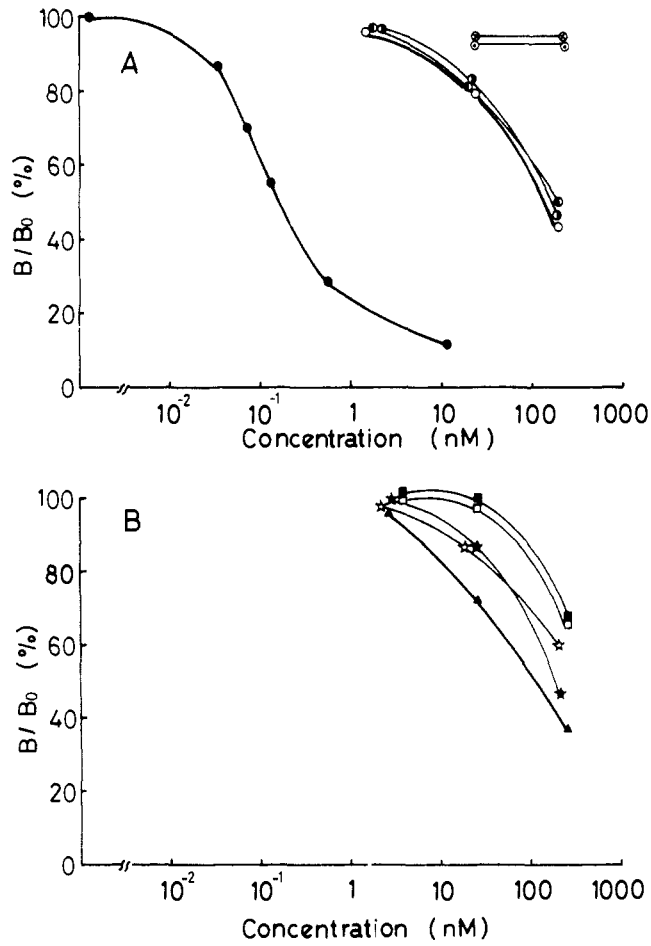

Figure 2. Competition of vitamin D endoperoxides and their corresponding vitamin D compounds for the specific 1α,25-$(OH)_2[^3H]D_3$ binding sites in the cytosol fraction of HL-60 cells. Cytosol (0.4 mg of protein) was incubated with 0.1 nM 1α,25-$(OH)_2[^3H]D_3$ plus graded concentrations of each derivative of vitamin D_3. Data are means of duplicate assays. (A) Vitamin D_3 derivatives possessing the 1α-hydroxyl group: ●, 1α,25-$(OH)_2D_3$ (**1b**); ◑, 1α,25-$(OH)_2D_3$ endoperoxide less polar (**2b**); ◐, 1α,25-$(OH)_2D_3$ endoperoxide more polar (**2b′**); ○, 1α-(OH)D_3 (**1c**); ⊗, 1α-(OH)D_3 endoperoxide less polar (**2c**); ⊙, 1α-(OH)D_3 endoperoxide more polar (**2c′**). (B) Vitamin D_3 derivatives lacking the 1α-hydroxyl group: ▲, 25-(OH)D_3 (**1a**); ■, 25-(OH)D_3 endoperoxide less polar (**2a**); □, 25-(OH)D_3 endoperoxide more polar (**2a′**); ★, 24-F_2-25-(OH)D_3 endoperoxides (**2e** + **2e′**); ☆, 26,27-F_6-25-(OH)D_3 endoperoxides (**2f** + **2f′**).

as active as 25-(OH)D_3 endoperoxides (**2a** and **2a′**) and about 10 times as active as 25-(OH)D_3 (**1a**). It is noteworthy that no significant difference was observed between the potentialities of the two C-6 epimers of vitamin D endoperoxides (**2** and **2′**).

To clarify the mechanism of the action of vitamin D endoperoxides (**2** and **2′**) in inducing differentiation of HL-60 cells, their affinity for the specific binding sites of 1α,25-$(OH)_2D_3$ (**1b**) in the cytosol fraction of the cells was examined. Figure 2 shows the competition of the endoperoxides (**2** and **2′**) for the specific binding sites, and Table I summarizes the relative activity of various vitamin D derivatives and their corresponding endoperoxides in inducing phagocytosis and in binding to the cytosol receptor of HL-60 cells. The binding efficiency of the endoperoxides of the first group was significantly lowered (10^{-3}) as expected from their low activity in inducing differentiation of HL-60 cells (Figure 2A). The binding efficiency of the endoperoxides of the second group, however, did not change in parallel with their potentials in inducing phagocytic activity (Figure 2B). The relative

Table I. Relative Activity of Vitamin D Endoperoxides (**2** and **2′**) and Their Corresponding Vitamin D Compounds (**1**) and 25-Hydroxy-6,19-dihydro-6,19-ethanovitamin D_3 (**6** and **6′**) in Inducing Phagocytosis and Binding to the Cytosol Receptor of HL-60 Cells

compd	phagocytosis[a]	binding act.[b]
Vitamin D Derivatives Possessing the 1α-Hydroxyl Group		
1α,25-$(OH)_2D_3$ (**1b**)	1	1
1α,25-$(OH)_2D_3$ endoperoxide less polar (**2b**)	130	800
1α,25-$(OH)_2D_3$ endoperoxide more polar (**2b′**)	200	800
1α,24(*R*)-$(OH)_2D_3$ (**1d**)	1	1
1α,24(*R*)-$(OH)_2D_3$ endoperoxide less polar (**2d**)	150	800
1α,24(*R*)-$(OH)_2D_3$ endoperoxide more polar (**2d′**)	200	800
1α-$(OH)D_3$ (**1c**)	320	800
1α-$(OH)D_3$ endoperoxide less polar (**2c**)	>1000	>10000
1α-$(OH)D_3$ endoperoxide more polar (**2c′**)	>1000	>10000
Vitamin D Derivatives Lacking the 1α-Hydroxyl Group		
25-$(OH)D_3$ (**1a**)	320	600
25-$(OH)D_3$ endoperoxide less polar (**2a**)	120	3000
25-$(OH)D_3$ endoperoxide more polar (**2a′**)	130	3000
24-F_2-25-$(OH)D_3$ (**1e**)	200	1000
24-F_2-25-$(OH)D_3$ endoperoxides (**2e** + **2e′**)	45	1000
26,27-F_6-25-$(OH)D_3$ (**1f**)	200	50
26,27-F_6-25-$(OH)D_3$ endoperoxides (**2f** + **2f′**)	30	1500
25-(OH)-6,19-ethano-D_3 less polar (**6**)	>1000	>10000
25-(OH)-6,19-ethano-D_3 more polar (**6′**)	>1000	>10000

[a] To compare the potency in inducing differentiation, the molar ratio of vitamin D derivatives to 1α,25-$(OH)_2D_3$ (**1b**) in inducing half-maximal phagocytic activity was calculated. [b] For comparison of the binding activity, 1α,25-$(OH)_2$[^{3}H]D_3 in approximately saturating amounts and varying amounts of unlabeled derivatives were added to the cytosol fraction of HL-60 cells. The molar ratio of unlabeled vitamin D derivatives to the labeled 1α,25-$(OH)_2D_3$ required for 50% displacement of the label from the receptor was calculated.

binding efficiency of the endoperoxides became weaker, whereas the relative potency in inducing phagocytosis became stronger, compared with their parent vitamin D compounds. Thus, while 25-$(OH)D_3$ endoperoxides (**2a** and **2a′**) were 2.5 times as active as 25-$(OH)D_3$ (**1a**) in the differentiating action, their affinity for the receptor protein was about one-fifth that of 25-$(OH)D_3$. Each pair of C-6 epimers showed no significant difference in the efficiency to bind to the receptor.

The biological activities of the two C-6 epimers of 25-hydroxy-6,19-ethanovitamin D_3 (**6** and **6′**) in inducing phagocytosis of HL-60 cells and in binding to the cytosol receptor for 1α,25-$(OH)_2D_3$ (**1b**) were examined. Both epimers (**6** and **6′**) showed no appreciable potentials in either action (Table I).

Discussion

We examined the biological activity of various vitamin D endoperoxides (**2** and **2′**), singlet oxygen adducts of vitamin D derivatives at the s-cis diene part, in inducing differentiation of HL-60 cells.

Oxygen adduct formation significantly reduced the potential in inducing phagocytic activity of HL-60 cells in the vitamin D derivatives with the 1α-hydroxyl group. This is quite reasonable because the oxygen adduct formation destroys the conjugated triene structure typical of vitamin D compounds and significantly modifies the stereochemical structure of the parent vitamin D molecule. The carbon at the 6-position is changed from a planar-trigonal (sp^2) atom in vitamin D (**1**) to a nonplanar tetrahedral (sp^3) atom in the endoperoxides (**2** and **2′**). In this regard, it is noteworthy that the oxygen adducts of vitamin D derivatives lacking the 1α-hydroxyl group such as 25-$(OH)D_3$ endoperoxides (**2a** and **2a′**), 24-F_2-25-$(OH)D_3$ endoperoxides (**2e** and **2e′**), and 26-27-F_6-25-$(OH)D_3$ endoperoxides (**2f** and **2f′**) were several times more active than the corresponding vitamin D compounds in inducing differentiation of HL-60 cells.

Tanaka et al.[6] suggested that 1α,25-$(OH)_2D_3$ (**1b**) induces differentiation of HL-60 cells by a receptor-mediated mechanism. In accord with that postulation, the binding efficiency of most vitamin D_3 derivatives to the specific cytosol receptor of HL-60 cells was fairly well correlated with their potency in inducing phagocytosis of the cells.[12] If vitamin D endoperoxides (**2** and **2′**) induce differentiation of HL-60 cells by a receptor-mediated mechanism, their affinity for the receptor protein should be correlated with their activity in inducing phagocytosis. But, the two activities were not parallel in the endoperoxides (**2** and **2′**). This was especially apparent in the endoperoxides of the second group, those lacking the 1α-hydroxyl group, where the differentiating activity was enhanced but the affinity for the cytosol receptor was reduced relative to the parent vitamin D compounds.

The following possibilities can be considered for the explanation of the difference of the two activities. First, it may be that vitamin D endoperoxides (**2** and **2′**) might act without the mediation of the cytosol receptor. However, this is inconsistent with the following findings: (i) In most vitamin D derivatives examined so far the two activities were closely related, supporting the mechanism of the differentiation mediated by the cytosol receptor.[12] (ii) 1α,25-$(OH)_2D_3$ (**1b**) cannot induce differentiation of the mutant cells of HL-60 which have reduced amounts of the cytosol receptor for 1α,25-$(OH)_2D_3$.[20] Second, the difference may be ascribed to the different assay time when the two activities were measured. Induction of the phagocytic activity was measured 3 days after the addition of the substrate, whereas the binding efficiency to the cytosol receptor was determined 1 h after the addition of the substrate. As one consequence of the time difference, it may be considered that vitamin D endoperoxides (**2** and **2′**) induce phagocytosis after being converted to some other compounds, since an O–O bond in endoperoxides has been known to be chemically unstable.[21] We have shown that vitamin D_3 endoperoxides (**2g** and **2g′**) are sensitive to acids, bases, and transition-metal complexes,[15c] although they are stable in crystals or in neutral organic solvents for several days at ambient temperature or for several years at freezing temperature.

To help clarify the problem, we examined the biological activities of two C-6 epimers of 25-hydroxy-6,19-dihydro-6,19-ethanovitamin D_3 (**6** and **6′**), stable carbocyclic analogues of 25-$(OH)D_3$ endoperoxides (**2a** and **2a′**), whose stereochemical structure is similar to that of the endoperoxides. It is known that in the family of prostaglandins carbon analogues of prostacyclin such as carbacyclin show activity similar to prostacyclin.[22] Therefore, it is expected that if the endoperoxides (**2** and **2′**) elicit their activity by themselves, the ethanovitamin D's (**6** and **6′**) would show a potential similar to that of 25-$(OH)D_3$ endoperoxides (**2a** and **2a′**). The fact that both epimers of the ethanovitamin

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D (6 and 6′) were far less active than 25-(OH)D_3 endoperoxides (2a and 2a′) in inducing differentiation of HL-60 cells seems to support the possibility that the endoperoxides may act after being converted to some other compounds. The fact that a pair of epimeric endoperoxides (2 and 2′) show no significant difference in inducing differentiation of HL-60 cells seems to support the postulation; the endoperoxides might act after being converted to a compound in which the chirality at C-6 has been lost.

In summary, the singlet oxygen adducts of vitamin D derivatives, vitamin D endoperoxides (2 and 2′), have peculiar differentiating activity in HL-60 cells in spite of the absence of the conjugated triene structure. Lines of evidence suggest the possibility that the endoperoxides might act after being converted to some other compounds.

Experimental Section

General Methods. Mass spectra were obtained by using a JEOL JMS-D300 mass spectrometer with an interfaced computer. All spectra were run at 70 eV with a source temperature programmed in the range of 50–400 °C at 200 °C/min. The proton magnetic resonance (^{1}H NMR) spectra were taken in $CDCl_3$ on a Varian XL-100 spectrometer, and the chemical shift values were reported in parts per million downfield from internal tetramethylsilane. Circular dichroism (CD) spectra were recorded with a Jasco J-20A spectropolarimeter in methanol or hexane. Ultraviolet (UV) spectra were recorded with a Hitachi 200-10 double-beam spectrophotometer in a 95% ethanol solution.

Vitamin D Compounds. 25-(OH)D_3 (1a) was purchased from Philips-Duphar, Amsterdam, The Netherlands. 1α,25-(OH)$_2D_3$ (1b) and 1α-hydroxyvitamin D_3 [1α-(OH)D_3 (1c)] were synthesized chemically in our laboratory according to the method of Kaneko et al.[23] 24,24-Difluoro-25-hydroxyvitamin D_3 [24-F_2-25-(OH)D_3 (1e)] was chemically synthesized in our laboratory according to the method of Yamada et al.[24] 26,26,26,27,27,27-Hexafluoro-25-hydroxyvitamin D_3 [26,27-F_6-25-(OH)D_3 (1f)] was chemically synthesized in our laboratory according to the method of Kobayashi et al.[25] 1α,24(*R*)-Dihydroxyvitamin D_3 [1α,24(*R*)-(OH)$_2D_3$ (1d)] was synthesized in our laboratory according to the method of Ochi et al.[26] 1α,25-(OH)$_2$[23,24-^{3}H]D_3 (specific activity 80 Ci/mmol) was obtained from Amersham International, Bucks, UK.

(6*R*)- and (6*S*)-25-Hydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 (2a and 2a′). A solution of 25-(OH)D_3 (1a) (22 mg 55 μmol) and Rose Bengal (80 mg, 82 μmol) in 95% ethanol (80 mL) was placed in an immersion vessel, purged with oxygen, and irradiated by a water-cooled 200-W halogen lamp (Ushio JCV 100-200GS). Oxygen was kept bubbling through the solution during the irradiation, and the outside of the vessel was cooled with ice. The reaction was monitored by TLC (silica gel, 2:8 ethyl acetate–hexane) and terminated when almost all of the starting material was consumed. The solvent was evaporated in vacuo; the residue was dissolved in ethyl acetate, washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on Sephadex LH-20 (15 g) with hexane–chloroform (1:1) as the eluent to afford a mixture of 2a and 2a′ (14 mg). The mixture was separated by using HPLC (μ-Porasil, 0.79 × 30 cm, 1:9 2-propanol–hexane) to afford less polar 2a (6.6 mg, 28%) and more polar 2a′ (7.4 mg, 31%). 2a: mp 137–138 °C (from 2-propanol–hexane); mass spectrum *m/e* 432 (M^+), 414, 396, 151; ^{1}H NMR ($CDCl_3$) δ 0.56 (3 H, s, H-18), 0.94 (3 H, d, *J* = 6 Hz, H-21), 1.22 (6 H, s, H-26 and H-27), 4.10 (1 H, m, H-3), 4.36 (1 H, d, *J* = 15 Hz, H-19), 4.44. (1 H, d, *J* = 15 Hz, H-19), 4.92 (1 H, d, *J* = 10 Hz, H-6), 5.20 (1 H, d, *J* = 10 Hz, H-7); CD (methanol) 210 nm (Δε −39.4). 2a′: mp 148–150 °C (from 2-propanol–hexane); ^{1}H NMR ($CDCl_3$) δ 0.56 (3 H, s, H-18), 0.94 (3 H, d, *J* = 6 Hz, H-21), 1.22 (6 H, s, H-26 and H-27), 3.96 (1 H, m, H-3), 4.20 (1 H, d, *J* = 16 Hz, H-19), 4.60 (1 H, d, *J* = 16 Hz, H-19), 4.80 (1 H, d, *J* = 9 Hz, H-6 or H-7), 5.28 (1 H, d, *J* = 9 Hz, H-7 or H-6); CD (methanol) 213 nm (Δε +9.9).

1α,25-Dihydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 (2b and 2b′). In a similar manner, a solution of 1α,25-(OH)$_2D_3$ (1b) (840 μg, 2 μmol) and Rose Bengal (20 mg, 20 μmol) in 95% ethanol (15 mL) was irradiated under oxygen for 1.5 h. After a similar workup, the products were chromatographed on Sephadex LH-20 (6 g) with hexane–chloroform–methanol (30:70:2) as the eluent to afford a mixture of 2b and 2b′. The mixture was further chromatographed on silica gel (3 g) with ethyl acetate–dichloromethane (9:1) as the eluent to afford less polar 2b (75 μg, 8%) [based on the OD at 220 nm (ε 7500) in the UV spectrum], a mixture of 2b and 2b′ (150 μg, 17%), and more polar 2b′ (150 μg, 17%). 2b: mass spectrum *m/e* 430 (M^+–H_2O), 412, 394, 379. 2b′: mass spectrum *m/e* 430 ($M^+ - H_2O$), 412, 394, 379.

1α-Hydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 (2c and 2c′). A solution of 1α-(OH)D_3 (1c) (1.7 mg, 4 μmol) and Rose Bengal (20 mg, 20 μmol) in 95% ethanol (20 mL) was similarly irradiated under oxygen. After a similar workup, the products were chromatographed on Sephadex LH-20 (6 g) with hexane–chloroform (35:65) as the eluent to afford less polar 2c (0.53 mg, 29%) and more polar 2c′ (0.68 mg, 37%). 2c: mass spectrum *m/e* 414 ($M^+ - H_2O$), 301, 283, 167; ^{1}H NMR ($CDCl_3$) δ 0.58 (3 H, s, H-18), 0.89 (6 H, d, *J* = 6 Hz, H-26 and H-27), 0.93 (3 H, d, *J* = 6 Hz, H-21), 4.24 (1 H, m, H-3), 4.41 (1 H, m, H-1), 4.52 (1 H, d, *J* = 8 Hz, H-19), 4.82 (1 H, d, *J* = 18 Hz, H-19), 5.03 (1 H, d, *J* = 9 Hz, H-6 or H-7), 5.19 (1 H, d, *J* = 9 Hz, H-7 or H-6); CD (methanol) 208 nm (Δε −50.1). 2c′: mp 135–136 °C (from methanol); mass spectrum *m/e* 414 ($M^+ - H_2O$), 301, 206, 167; ^{1}H NMR ($CDCl_3$) δ 0.56 (3 H, s, H-18), 0.86 (6 H, d, *J* = 6 Hz, H-26 and H-27), 0.91 (3 H, d, *J* = 6 Hz, H-21), 4.12 (1 H, m, H-3), 4.25 (1 H, m, H-1), 4.67 (2 H, s, H-19), 4.74 (1 H, d, *J* = 9 Hz, H-6 or H-7), 5.33 (1 H, d, *J* = 9 Hz, H-7or H-6); CD (methanol) 212 nm (Δε +13.4).

1α,24(*R*)-Dihydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 (2d and 2d′). A solution of 1α,24(*R*)-(OH)$_2D_3$ (1d) (660 μg, 1.6 μmol) and Rose Bengal (20 mg, 20 μmol) in 95% ethanol (15 mL) was similarly irradiated under oxygen for 1 h. After a similar workup, the products were chromatographed on Sephadex LH-20 (6 g) with hexane–chloroform–methanol (30:70:3) as the eluent to give a mixture of 2d and 2d′ (358 μg). The mixture was separated by using HPLC (Lichrosorb, 2-propanol–hexane, 2:8) to afford less polar 2d (78 μg, 11%) and more polar 2d′ (132 μg, 19%). 2d: mass spectrum *m/e* 430 ($M^+ - H_2O$), 412, 394, 379. 2d′: mass spectrum *m/e* 430 ($M^+ - H_2O$), 412, 394, 379.

24,24-Difluoro-25-hydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 (2e and 2e′). A solution of 24-F_2-25-(OH)D_3 (1e) (2 mg, 4.6 μmol) and Rose Bengal (10 mg, 10 μmol) in benzene–ethanol (31.5:3.5) (35 mL) was irradiated under oxygen in a similar manner as described above. After a similar workup, the products were chromatographed on silica gel (3 g) with hexane–ethyl acetate (3:2) as the eluent to give 2e and 2e′ as a mixture (670 μg, 31%): mass spectrum *m/e* 468 (M^+), 450, 330, 312, 299, 285, 258.

26,26,26,27,27,27-Hexafluoro-25-hydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 (2f and 2f′). A solution of 26,27-F_6-25-(OH)D_3 (1f) (2.0 mg, 4 μmol) and Rose Bengal (10 mg, 10 μmol) in benzene–ethanol (5:1) (24 mL) was irradiated under oxygen for 15 min as described above. After a similar workup, the products were chromatographed on silica gel (2.5 g) with hexane–ethyl acetate (3:2) as the eluent to afford 2f and 2f′, as a mixture (846 μg, 40%): mass spectrum *m/e* 540 (M^+), 522, 508, 504, 402, 384, 371, 330, 285.

Adducts of 25-Hydroxyvitamin D_3 with Vinyl Phenyl Sulfone (5). 25-(OH)D_3 (1a) (25 mg, 0.06 mmol) was dissolved in liquid sulfur dioxide (8 mL), and the solution was stirred at the boiling temperature of sulfur dioxide for 10 min. The sulfur dioxide was removed, and the residue was dissolved in toluene (200 μL). Phenyl vinyl sulfone (21 mg, 0.125 mmol) and sodium bicarbonate (20 mg, 0.24 mmol) were added to the solution, and the mixture was heated at 100 °C for 14 h under argon. The solvent was evaporated, and the residue was chromatographed

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on silica gel (5 g) with ethyl acetate–hexane (1:2) as the eluent to give a mixture of the adducts (**5**): 29.7 mg (83%); mass spectrum *m/e* 568 (M^+), 550, 426; ^1H NMR ($CDCl_3$) δ 0.52 and 0.76 (3 H, s, H-18), 0.96 (3 H, d, J = 6 Hz, H-21), 1.22 (6 H, s, H-26 and H-27), 3.95 (1 H, m, H-3), 5.01 (1 H, d, J = 10 Hz, H-7), 7.40–8.04 (5 H, m, aromatic H).

25-Hydroxy-6,19-dihydro-6,19-ethanovitamin D_3 (6 and 6′). To a solution of the adducts (**5**) (29.7 mg, 0.052 mmol) in methanol (4 mL) was added 5% sodium amalgam (238 mg, 0.52 mmol) and disodium hydrogen phosphate (73.4 mg, 0.52 mmol), and the mixture was stirred at room temperature for 2 h under argon. The reaction mixture was filtered, and the solvent was evaporated. The residue was chromatographed on silica gel (5 g) with ethyl acetate–hexane (1:2) as the eluent to yield a mixture (ca. 1:1) of the two C-6 epimers of the 6,19-ethanovitamin D (**6** and **6′**) (17.7 mg, 79%). The mixture was separated by HPLC on μ-Porasil (0.78 × 30 cm) with 7% 2-propanol in hexane as the eluent to afford the less polar **6** and more polar **6′**. **6**: mass spectrum *m/e* 428 (M^+), 410, 164, 146; ^1H NMR ($CDCl_3$) δ 0.56 (3 H, s, H-18), 0.97 (3 H, d, J = 5 Hz, H-21), 1.22 (6 H, s, H-26 and H-27), 3.96 (1 H, m, H-3), 4.82 (1 H, d, J = 10 Hz, H-7); CD (hexane) 209 nm ($\Delta\epsilon$ −123.8), **6′**: mass spectrum *m/e* 428 (M^+), 410, 164, 146; ^1H NMR ($CDCl_3$) δ 0.54 (3 H, s, H-18), 0.97 (3 H, d, J = 5 Hz, H-21), 1.22 (6 H, s, H-26 and H-27), 3.92 (1 H, m, H-3), 4.80 (1 H, d, J = 10 Hz, H-7); CD (hexane) 205 nm ($\Delta\epsilon$ +33.1).

Cells and Cell Culture. The human promyelocytic leukemia cells (HL-60) were provided by Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo. Cells were cultured at 37 °C in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO_2 in air. Under these conditions the doubling time of HL-60 cells was 24 h. The cells (5×10^5) were innoculated in 5 mL of the medium in a 60-mm petri dish, and each vitamin D_3 derivative dissolved in ethanol was added to keep a final ethanol concentration of less than 0.1%. The control culture was given the same colume of ethanol.

Measurement of Phagocytic Activity. Phagocytic activity was measured according to the method of Collins et al.[27] Cells were suspended at a concentration of 1×10^6 cells/mL of RPMI 1640 medium supplemented with 10% fetal calf serum and 10% human AB serum. *Candida albicans* was washed with saline and added to the cell suspension at a final concentration of 4×10^6/mL. The suspension was incubated at 37 °C for 30 min, and the percentage of cells that had phagocytozed at least one fungus was determined with a hemocytometer.

Binding Assay. HL-60 cells were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and sonicated with an ultrasonic disruptor in a solution containing 10 mM Tris/HCl, pH. 7.4, 2 mM EDTA, 0.5 mM dithiothreitol, and 0.3 M KCl. The homogenates were centrifuged at 30000*g* for 10 min at 4 °C. The resulting supernatant was centrifuged again at 225000*g* for 60 min at 4 °C in a Hitachi 65P-7 ultracentrifuge to yield a cytosol fraction. Of the cytosol fraction, 0.5 mL (0.4 mg of protein) was incubated with 0.1 nM 1α,25-$(OH)_2[^3H]D_3$ and graded amounts of authentic derivatives of vitamin D_3 for 60 min at 25 °C. The bound and free 1α,25-$(OH)_2[^3H]D_3$ were separated by adding hydroxylapatite.[28]

Registry No. **1a**, 19356-17-3; **1b**, 32222-06-3; **1c**, 41294-56-8; **1d**, 57333-96-7; 1e, 71603-41-3; **1f**, 75303-43-4; **2a**, 73306-90-8; **2a′**, 73249-00-0; **2b** (isomer 1), 87977-67-1; **2b** (isomer 2), 87977-68-2; **2c** (isomer 1), 73248-99-4; **2c** (isomer 2), 73285-51-5; **2d** (isomer 1), 87977-65-9; **2d** (isomer 2), 87977-66-0; **2e** (isomer 1), 96647-93-7; **2e** (isomer 2), 96647-94-8; **2f** (isomer 1), 96616-68-1; **2f** (isomer 2), 96616-69-2; **5**, 96616-71-6; **6** (isomer 1), 96616-70-5; **6** (isomer 2), 96647-95-9; O_2, 7782-44-7; SO_2, 7446-09-5; $PhSO_2CH{=}CH_2$, 5535-48-8.

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Mechanism of the Differentiating Action of 25-Hydroxyvitamin D_3 Endoperoxides in Human Myeloid Leukemia Cells (HL-60)

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The action of 25-hydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 [25-$(OH)D_3$ endoperoxides, **2a** and **3a**] in inducing differentiation of human myeloid leukemia cells (HL-60) was studied by using their radioactive derivatives (**2a′** and **3a′**). When HL-60 cells were incubated with the labeled endoperoxides (**2a′** and **3a′**) in serum-free RPMI 1640 medium, no radioactivity was incorporated into either the cytosol or the chromatin fraction of the cells. When the radioactive endoperoxide (**2a′**) was incubated in the culture medium for 3 days, with or without HL-60 cells, about 45% of the compound was similarly converted to 19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**) and about 10% to 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**). These two new vitamin D derivatives were synthesized chemically and tested for their biological activities. Both compounds (**4a** and **6a**) were about 2 times as active as 25-$(OH)D_3$ endoperoxides (**2a** and **3a**) and about 7 times as active as 25-hydroxyvitamin D_3 (**1a**) in inducing differentiation of HL-60 cells. The differentiating activity of these compounds was well correlated with their activity in binding to the cytosol receptor for 1α,25-dihydroxyvitamin D_3 in HL-60 cells. The in vitro bone-resorbing activity of 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**) and 25-$(OH)D_3$ endoperoxide (**2a**) was higher than that of 25-hydroxyvitamin D_3 (**1a**), indicating that the differentiating activity also paralleled the bone-resorbing activity in these vitamin D derivatives. These results suggest that 25-$(OH)D_3$ endoperoxides (**2a** and **3a**) induce differentiation of HL-60 cells and bone resorption after being converted to these two compounds.

In the preceding paper,[1] we reported the biological activity of vitamin D–singlet oxygen adducts (6,19-dihydro-6,19-epidioxyvitamin D_3, vitamin D endoperoxides)[2] in inducing differentiation of human myeloid leukemia cells (HL-60). The oxygen adducts (**2a** and **3a**) derived from 25-hydroxyvitamin D_3 [25-$(OH)D_3$] and their derivatives fluorinated at the side-chain part are several times

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