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 30000g for 10 min at 4 °C. The resulting supernatant was centrifuged again at 225000g 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.

(27) Collins, S. J.; Ruscetti, F. W.; Gallagher, R. E.; Gallo, R. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2458.
(28) Wecksler, W. R.; Norman, A. W. *Anal. Biochem.* **1979**, *92*, 314.

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

Yoshiko Shiina,[†] Chisato Miyaura,[†] Hirofumi Tanaka,[†] Etsuko Abe,[†] Sachiko Yamada,[‡] Keiko Yamamoto,[‡§] Eiichi Ino,[‡] Hiroaki Takayama,[‡] Isao Matsunaga,[⊥] Yasuho Nishii,[⊥] and Tatsuo Suda*[†]

Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan, and New Drug Research Laboratories, Chugai Pharmaceutical Company, Takada, Toshima-ku, Tokyo 171, Japan. *Received November 1, 1984*

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.

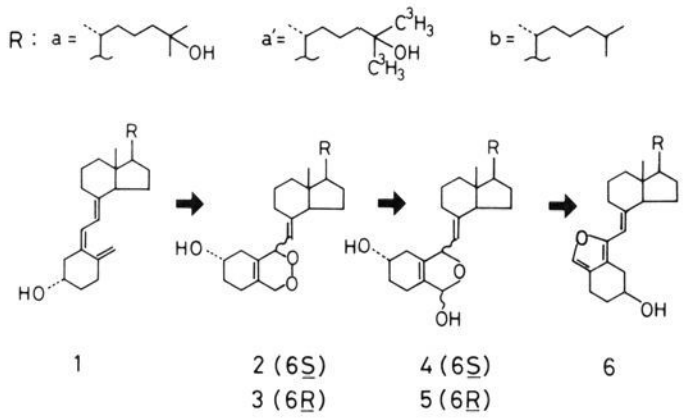
In the preceding paper,[1] we reported the biological activity of vitamin D–singlet oxygen adducts (6,19-dihydro-6,19-epidioxyvitamin D_3, vitamin D endoperoxides)[2] in inducing differentiation of human myeloid leukemia cells (HL-60). The oxygen adducts (**2a** and **3a**) derived from 25-hydroxyvitamin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

[†] Showa University.
[‡] Teikyo University.
[§] Nee Nakayama.
[⊥] Chugai Pharmaceutical Co.

(1) See the preceding paper in this issue.
(2) (a) Yamada, S.; Nakayama, K.; Takayama, H. *Tetrahedron Lett.* **1978**, 4895. (b) Yamada, S.; Nakayama, K.; Takayama, H.; Itai, A.; Iitaka, Y. *Chem. Pharm. Bull.* **1979**, *27*, 1949. (c) *J. Org. Chem.* **1983**, *48*, 3477.

0022-2623/85/1828-1153$01.50/0

Scheme I



as active as the corresponding parent vitamin D compounds, in spite of the absence of the conjugated triene structure typical of vitamin D compounds. However, in contrast to ordinary vitamin D derivatives having the conjugated triene structure,[3] the endoperoxides (**2a** and **3a**) show much less binding affinity to the receptor for 1α,25-dihydroxyvitamin D_3 [1α,25-(OH)$_2D_3$] present in HL-60 cells, relative to the activity in inducing differentiation of the cells. This, together with the finding that the carbocyclic analogues of 25-(OH)D_3 endoperoxides (**2a** and **3a**), (6*S*)- and (6*R*)-25-hydroxy-6,19-dihydro-6,19-ethanovitamin D_3, show only little activity in inducing differentiation of HL-60 cells suggests that the endoperoxides might elicit their activity after being converted into some other compounds.

In the present research, we examined the action of 25-(OH)D_3 endoperoxides (**2a** and **3a**) in detail, employing radioactive 25-(OH)D_3 endoperoxides (**2a**′ and **3a**′), and found how endoperoxide **2a** acts after being transformed into 19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**) and 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**). These two compounds were synthesized chemically and tested for their biological activity. The two new vitamin D derivatives with unique structures showed significant potency in inducing differentiation of HL-60 cells and bone-resorbing activity in vitro. We report the results here in detail.

Results

Incorporation of 25-Hydroxy[^{3}H]vitamin D_3 (1a′) and 25-Hydroxy[^{3}H]vitamin D_3 Endoperoxides (2a′ and 3a′) into HL-60 Cells. HL-60 cells were incubated with 3.2 nM 25-(OH)[^{3}H]D_3 (**1a**′) or 25-(OH)[^{3}H]D_3 endoperoxides (**2a**′ and **3a**′) in the serum-free medium at 37 °C for 1 h under 5% CO_2 in air. 25-(OH)[^{3}H]D_3 was incorporated into the cytosol and chromatin fractions of HL-60 cells, and the radioactivity found in both fractions was markedly decreased by adding a 10 000-fold excess of 25-(OH)D_3. In contrast, when HL-60 cells were incubated with 25-(OH)[^{3}H]D_3 endoperoxides (**2a**′ and **3a**′), no specific incorporation of radioactivity could be detected in either the cytosol or the chromatin fraction (Figure 1).

Transformation of 25-Hydroxyvitamin D_3 Endoperoxides (2a and 3a) during Incubation with or without HL-60 Cells. Since an O–O bond in endoperoxides is known to be chemically unstable,[4] we first examined the possibility that vitamin D endoperoxides (**2** and **3**) may act after being converted to some other compounds during the assay period. To examine this possibility, (6*S*)-25(OH)[^{3}H]D_3 endoperoxide (**2a**′) (3 nM) was incubated with HL-60 cells (1×10^6) in 10 mL of the culture medium for 1–3 days at 37 °C under 5% CO_2 in air. After incubation, the culture medium and cells were collected and lipids were extracted with chloroform. The extracts were chromatographed on a column packed with Sephadex LH-20 (10 g) with a mixed solvent of chloro-

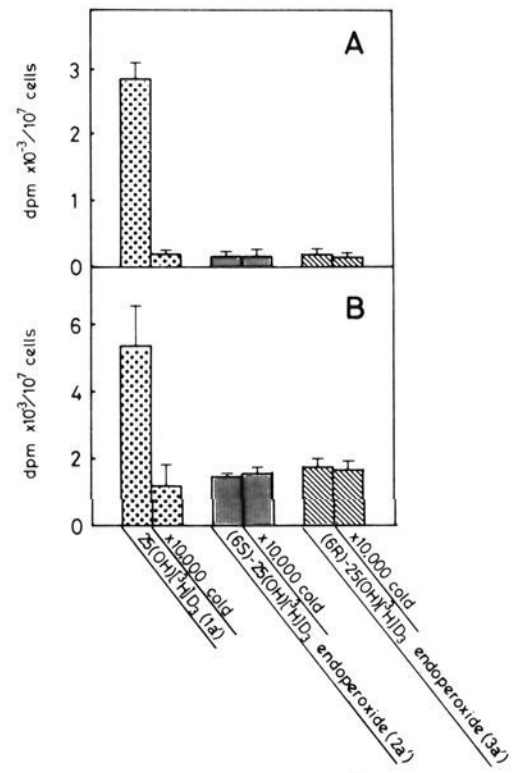


Figure 1. Incorporation of 25-(OH)[^{3}H]D_3 endoperoxides (**2a**′ and **3a**′) and 25-(OH)[^{3}H]D_3 (**1a**′) into the cytosol (A) and chromatin (B) fractions of HL-60 cells. HL-60 cells were incubated with 3.2 nM 25-(OH)[^{3}H]D_3 endoperoxides or 25-(OH)[^{3}H]D_3, in the presence or absence of a 10 000-fold excess of the respective compounds for 1 h at 37 °C under 5% CO_2 in air. After incubation, cells were homogenized and fractionated. The cytosol fraction was treated with hydroxylapatite to separate protein-bound 25-(OH)[^{3}H]D_3 endoperoxides or 25-(OH)[^{3}H]D_3 from the respective free radioisotopes: ▩, 25-(OH)[^{3}H]D_3 (**1a**′); ▥, (6*S*)-25-(OH)[^{3}H]D_3 endoperoxide (**2a**′); ▧, (6*R*)-25-(OH)[^{3}H]D_3 endoperoxide (**3a**′).

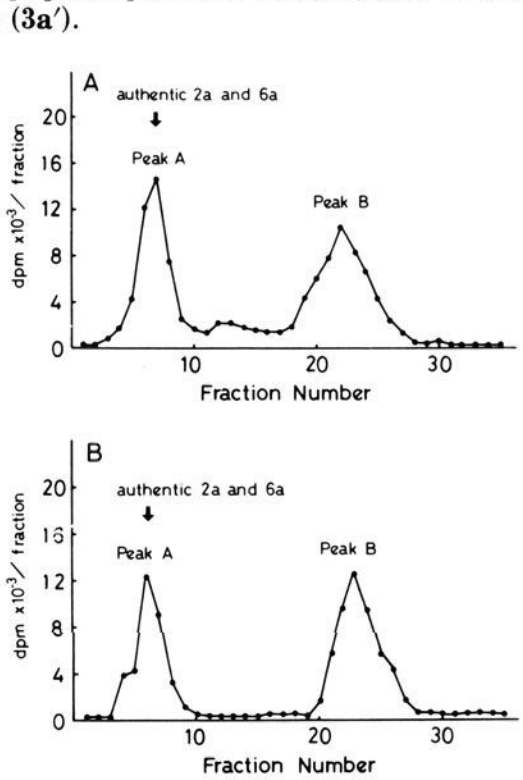


Figure 2. Sephadex LH-20 chromatographic profiles of extracts of culture medium with (A) or without (B) HL-60 cells after incubation with (6*S*)-25-(OH)[^{3}H]D_3 endoperoxide (**2a**′) for 3 days. (6*S*)-25-(OH)[^{3}H]D_3 endoperoxide (3.0 nM) was incubated with culture medium with or without HL-60 cells at 37 °C under 5% CO_2 in air for 3 days. The extract was chromatographed on Sephadex LH-20 column (1.2 × 25 cm) with chloroform–hexane (3:1), and 4-mL fractions were collected. The arrow indicates the elution position of authentic (6*S*)-25-(OH)D_3 endoperoxide (**2a**) and 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**).

(3) Suda, T.; Shiina, Y.; Abe, E.; Miyaura, C. Yamada, S.; Ohmori, M.; Nakayama, K.; Takayama, H.; Matsunaga, I.; Nishii, Y. "Proceedings of the 7th Asia and Oceania Congress of Endocrinology"; Excerpta Medica: Amsterdam-Oxford-Prinston, 1983; p 213.

(4) Denny, R. W.; Nickon, A. *Org. React.* (*N.Y.*) **1973**, *20*, 133. Wasserman, H. H.; Ives, J. L. *Tetrahedron* **1981**, *37*, 1825.

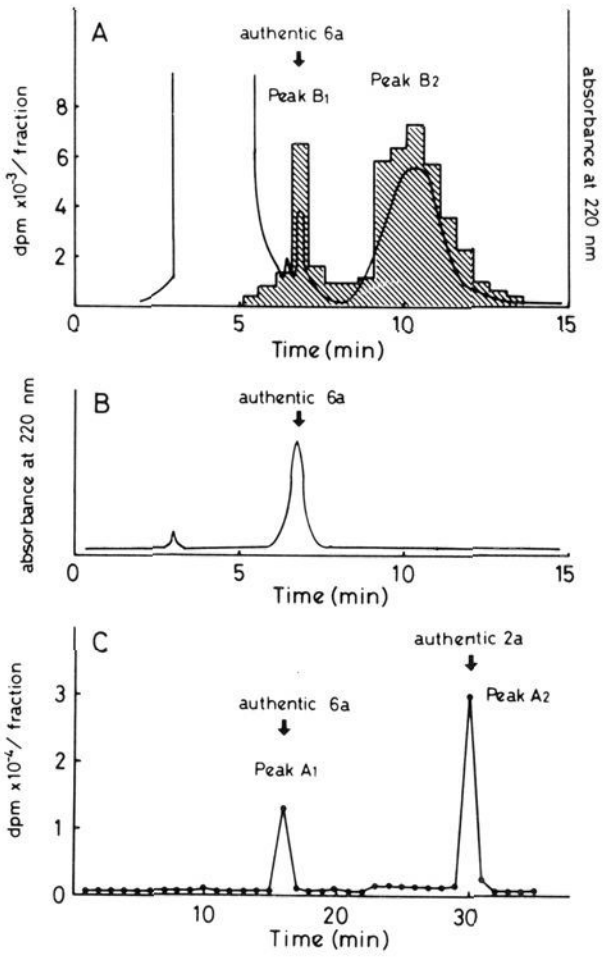


Figure 3. (A) HPLC (Finepak-SIL, 0.46 × 25 cm, 15% 2-propanol in hexane, 1 mL/min) profile of peak B obtained from the Sephadex LH-20 column. The authentic (6*S*)-19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**) was added to peak B before applying to HPLC. The arrow indicates the elution position of authentic 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**). The solid line shows the optical density at 220 nm, and the shaded bars represent the radioactivity in each fraction. (B) HPLC (15% 2-propanol in hexane, 1 mL/min) profile of peak B_2 from the first HPLC column. The arrow indicates the elution position of authentic 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**). (C) HPLC (Finepak SIL 0.46 × 25 cm, 7% 2-propanol in hexane, 1 mL/min) profile of peak A from the Sephadex LH-20 column. Arrows indicate the elution positions of authentic 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**) and (6*S*)-25-(OH)D_3 endoperoxide (**2a**).

form–hexane (3:1). A radioactive peak (peak B) more polar than the endoperoxide (**2a′**) was detected (Figure 2A). About 30, 35, and 45% of the starting material were converted to this polar derivative after 1, 2, and 3 days of incubation, respectively. It should be noted that similar results were obtained when 25-(OH)[^{3}H]D_3 endoperoxide (**2a′**) was incubated in culture medium without the cells (Figure 2B). Peak B from the Sephadex LH-20 column in either case was separated into a major broad peak at 8.5–12 mL (peak B_2) (30% of the starting material after incubation for 3 days) and a minor, less polar peak at 6.8 mL (peak B_1) (6%) on a straight-phase HPLC column (Finepak-SIL, 0.46 × 25 cm, 15% 2-propanol in hexane) (Figure 3A, cross-hatched bars). Peak A from the Sephadex column in either case was also separated into peaks A_1 and A_2 (10 and 23% of the starting endoperoxide, respectively) on the same straight-phase HPLC column eluted with 7% 2-propanol in hexane (Figure 3C).[5] The retention time of peak A_2 was identical with that of the starting endoperoxide (**2a**). The retention time of peak A_1 was identical with that of peak B_1 when it was eluted with 15% 2-propanol in hexane. These results indicate that peak A on the Sephadex LH-20 column originally consists of the starting endoperoxide and peak A_1.

(5) The endoperoxide (**2a**) is stable and is not converted to either **4a** or **6a** under the conditions employed in the isolation procedure.

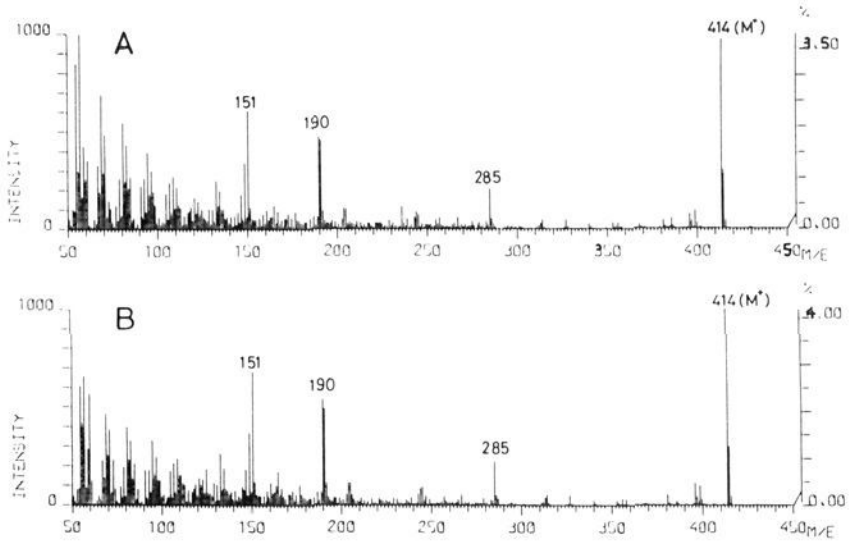


Figure 4. Mass spectra of the isolated (A) and synthetic (B) 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**).

Isolation and Identification of the Transformed Products. (6*S*)-25-(OH)D_3 endoperoxide (**2a**) was incubated in RPMI 1640 medium supplemented with 10% fetal calf serum in the absence of the cells for 3 days at 37 °C,[6] and the methanol–chloroform extract of the incubation mixture was chromatographed on the Sephadex LH-20 column. The more polar peak (B) eluted from the Sephadex LH-20 column was collected and purified on HPLC (Figure 3A). A major broad peak (B_2) was collected and purified on the same HPLC system as the first one. The second HPLC gave a sharp single peak at 6.8 mL, the retention time of which was not identical with that of peak B_2 but was identical with that of peak B_1 on the first HPLC (Figure 3B). This suggests that the initially transformed product, which was eluted as peak B_2, is not stable but is gradually converted to a compound that is eluted at 6.8 mL on HPLC. The compound isolated from the second HPLC column showed absorption maxima at 273, 282, and 294 nm in the UV spectrum and a molecular ion at *m/e* 414 and fragment ions at *m/e* 396, 285, 190, and 151 in the mass spectrum (Figure 4A). The UV spectrum and the mass spectral fragmentation pattern were similar to those of 6,19-epoxyvitamin D_3 (**6b**)[2c] obtained from vitamin D_3 endoperoxides (**2b** and **3b**) by the treatment with a basic reagent followed by dehydration under thermal or acidic conditions, suggesting that the isolated compound (peak B_1) is the corresponding 25-hydroxylated derivative (**6a**). The structure of the isolated compound was confirmed to be 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**) by comparing its UV and mass spectra (Figure 4) and HPLC retention time with those of the authentic compound synthesized chemically from 25-(OH)D_3 endoperoxide (**2a**). It was also assumed that the initially formed product (peak B_2) is 19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**), because it is known[2c] that the vitamin D_3 endoperoxides (**2b** and **3b**) are readily transformed into the cyclic hemiacetals (**4b** and **5b**) when they are treated with a basic reagent or a transition-metal complex. It is also known that the hemiacetals (**4b** and **5b**) are labile and readily dehydrated to the furan (**6b**) by the action of an acidic reagent. Therefore, it is assumed in the present case that the hemiacetal (**4a**) was dehydrated to the furan (**6a**) on the surface of the silica gel HPLC column. The cyclic hemiacetal (**4a**) synthesized chemically showed a similar broad peak on the straight-

(6) Because it is known that the two C-6 epimers of vitamin D_3 endoperoxides (**2b** and **3b**) show little difference in their chemical behavior,[2c] only (6*S*)-25-(OH)D_3 endoperoxide (**2a**) was used as a representative of the two isomers (**2a** and **3a**) to investigate the transformation during incubation.

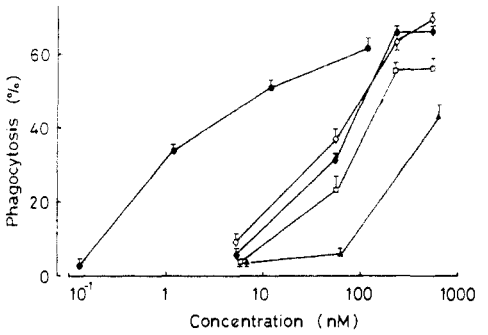


Figure 5. Comparison of the dose–response effects of various derivatives of vitamin D_3 in inducing phagocytic activity in HL-60 cells incubated for 3 days: ●, 1α,25-$(OH)_2D_3$; ◇, (6*S*)-19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**); ♦, 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**); □, (6*S*)-25-$(OH)D_3$ endoperoxide (**2a**); ▲, 25-$(OH)D_3$ (**1a**). The control value was 2.9 ± 0.5%. Points was means ±SEM (represented by the bars) of six replicates.

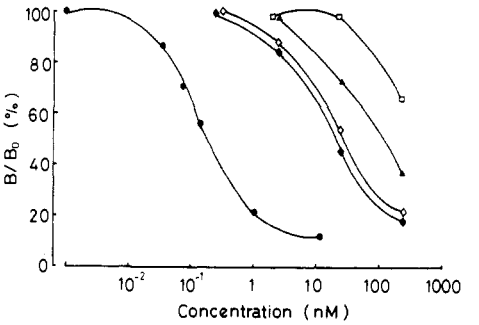


Figure 6. Competition of various derivatives of vitamin D_3 in binding to the specific cytosol receptor for 1α,25-$(OH)_2D_3$ in HL-60 cells. Cytosol (0.4 mg of protein) was incubated with 0.1 nM 1α,25-$(OH)_2[^3H]D_3$ plus graded concentrations of each derivative of vitamin D_3. Data are means of duplicated assays: ●, 1α,25-$(OH)_2D_3$; ◇, (6*S*)-19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**); ♦, 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**); □, (6*S*)-25-$(OH)D_3$ endoperoxide (**2a**); ▲, 25-$(OH)D_3$ (**1a**).

phase HPLC to that of the initially transformed product (peak B_2) (Figure 3A).

Chemical Transformation of (6*S*)-25-Hydroxyvitamin D_3 Endoperoxide (2a) to (6*S*)-19,25-Dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (4a) and 25-Hydroxy-6,19-epoxyvitamin D_3 (6a). (6*S*)-25-$(OH)D_3$ endoperoxide (**2a**) was treated with methanolic KOH solution at room temperature to give the hemiacetal (**4a**) as the major product (70%) and the furan (**6a**) as the minor product (6%). The former (**4a**) was dehydrated to the latter (**6a**) in a quantitative yield by refluxing in $CHCl_3$–$CDCl_3$.[7] The structures of **4a** and **6a** thus synthesized were confirmed by their spectra data. **4a**: mass spectra, *m/e* 432 (M^+), 414, 399, 396, 285, 245; UV (96% EtOH) no absorption maximum above 220 nm. **6a**: mass spectra, *m/e* 414 (M^+), 396, 285, 190, 151; UV (95% EtOH) λ_{max} 273, 282, 294 nm.

Biological Activity of (6*S*)-19,25-Dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (4a) and 25-Hydroxy-6,19-epoxyvitamin D_3 (6a). The dose–response effects of 19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**) and 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**) synthesized on the induction of phagocytic activity of HL-60 cells were examined in comparison with the differentiating action of 25-$(OH)D_3$ endoperoxide (**2a**) (Figure 5). The cells were incubated for 3 days with each derivative. These two new vitamin D_3 derivatives (**4a** and **6a**) were 2-fold more potent than 25-$(OH)D_3$ endoperoxide (**2a**) and 7-fold more potent than 25-$(OH)D_3$ at the concentration to induce half-maximal phagocytic activity. Figure 6 shows the competition of each derivative for specific 1α,25-$(OH)_2[^3H]D_3$ binding sites in the cytosol fraction of HL-60 cells. The binding activity of the hemiacetal (**4a**) and the furan (**6a**) to the cytosol receptor was 5-fold stronger than that of 25-$(OH)D_3$. The potency of the hemiacetal (**4a**) and the furan (**6a**) in inducing differentiation of HL-60 cells was consistent with their efficiency in binding to the cytosol receptor in HL-60 cells.

To examine whether the differentiating activity is in parallel with the in vitro activity in calcium metabolism, the effects of various vitamin D_3 derivatives on in vitro bone-resorbing activity were examined in fetal mouse calvaria prelabeled with ^{45}Ca (Table I). The bones were incubated for 6 days with various concentrations of vitamin D_3 derivatives. Of the vitamin D_3 derivatives tested, 1α,25-$(OH)_2D_3$ was the most potent followed successively by the furan (**6a**), (6*S*)-25-$(OH)D_3$ endoperoxide (**2a**), 25-$(OH)D_3$, and 24(*R*),25-dihydroxyvitamin D_3 [24(*R*),25-$(OH)_2D_3$] in that order. These results suggest that the differentiating activity of vitamin D_3 derivatives in HL-60 cells is in parallel with the bone-resorbing activity, at least in vitro.

Table I. Comparison of the Activity of Various Vitamin D_3 Derivatives in Inducing ^{45}Ca Release from Prelabeled Fetal Mouse Calvaria

compd	concn, nM	^{45}Ca release (T/C)[a]
vehicle		1.02 ± 0.04
	0.12	1.22 ± 0.08
1α,25-$(OH)_2D_3$	1.2	1.92 ± 0.20[b]
	12	2.49 ± 0.15[b]
	1.2	1.00 ± 0.04
25-$(OH)D_3$ (1a)	12	1.33 ± 0.08[c]
	120	1.74 ± 0.12[b]
	1.2	1.06 ± 0.08
(6*S*)-25-$(OH)D_3$ endoperoxide (**2a**)	12	1.61 ± 0.14[b]
	120	2.43 ± 0.28[b]
25-hydroxy-6,19-epoxyvitamin D_3 (**6a**)	1.2	1.05 ± 0.07
	12	1.63 ± 0.05[b]
	120	2.64 ± 0.29[b]
	1.2	0.98 ± 0.05
24(*R*),25-$(OH)_2D_3$	12	1.07 ± 0.11
	120	1.44 ± 0.10[c]

[a] Values are means ±SE of 12–15 bone pairs. [b] Significantly different from the vehicle, $p < 0.001$. [c] Significantly different from the vehicle, $p < 0.01$.

Discussion

In the preceding paper,[1] we showed that the singlet oxygen adducts of various vitamin D derivatives, especially those lacking the 1α-hydroxyl group, are more potent than the corresponding parent vitamin D derivatives in inducing differentiation of HL-60 cells. However, the potency was not correlated with their activity in binding to the cytosol receptor for 1α,25-$(OH)_2D_3$. This is in disagreement with the proposed mechanism of the differentiation of HL-60 cells induced by vitamin D derivatives.[3] This, together with the evidence that the two C-6 epimers of each vitamin D endoperoxide show no significant difference in potency and that the stable carbon analogues of the endoperoxides (6*S*)- and (6*R*)-25-hydroxy-6,19-dihydro-6,19-ethanovitamin D_3 have only little potency in that action, led us to postulate that the vitamin D endoperoxides act after being converted to some other derivatives.

We therefore examined the possibility, using radioactive 25-$(OH)D_3$ endoperoxides (**2a′** and **3a′**). Consistent with

(7) The trace of acids that exists in the solvent system appears to catalyze the dehydration.

the low efficiency in binding to the cytosol receptor for 1α,25-$(OH)_2D_3$, the 25-(OH)[^{3}H]D_3 endoperoxides (**2a′** and **a′**) were scarcely incorporated into either cytosol or chromatin fraction of HL-60 cells during incubation with the cells for 1 h at 37 °C (Figure 1). Tanaka et al.[8] have demonstrated that 1α,25-$(OH)_2$[^{3}H]D_3 is incorporated very rapidly into HL-60 cells and that the incorporation attains a maximum at 30 min. When (6*S*)-25-(OH)[^{3}H]D_3 endoperoxide (**2a′**) was incubated with HL-60 cells for 3 days, about 45% of the material was found to be converted to a more polar compound (peak B_2) and about 10% to a less polar compound (peak A_1). The conversion occurred in the absence of HL-60 cells, simply by incubating with the culture medium. This indicates that the transformation was promoted by a compound(s) present in the culture medium but not by an enzyme present in HL-60 cells.[5] Isolation of the major product (peak B_2) was rather difficult, since the compound was labile and transformed further into the less polar minor product (peak B_1) during the isolation processes. The latter product (peak B_1) was identified to be 25-hydroxy-6,19-epoxyvitamin D_3 (**6a**). Previously we reported that vitamin D_3 endoperoxides (**2b** and **3b**) are transformed into the cyclic hemiacetals (**4b** and **5b**) as the major product and the furan (**6b**) as the major product when they were treated with a nucleophilic (basic) reagent or transition-metal complexes and that the hemiacetals (**4b** and **5b**) were labile and transformed readily into the furan (**6b**) by treating with a trace amount of an acidic reagent or even simply by heating.[2c] On the basis of these experimental results, the structure of the labile initial product (peak B_2) was assumed to be (6*S*)-19,25-dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (**4a**). This was confirmed by comparing the chromatographic behavior of the more polar peak (peak B) from the Sephadex LH-20 column and authentic **4a** synthesized from (6*S*)-25-(OH)D_3 endoperoxide (**2a**) (Figure 3A). Peak A_1 was considered to be identical with peak B_1, since the retention time of peak A_1 on HPLC was identical with that of peak B_1. Since it was confirmed that the authentic hemiacetal (**4a**) was stable and was not transformed into the furan (**6a**) on the Sephadex LH-20 column, it is clear that the furan eluted as peak A_1 was already present in the incubation mixture after incubation for 3 days.

The two new vitamin D derivatives (**4a** and **6a**) with novel structure were chemically synthesized and tested for biological activity. The two compounds (**4a** and **6a**) showed similar potency to induce differentiation of HL-60 cells. They were about 2 times as active as 25-(OH)D_3 endoperoxides (**2a** and **3a**) and 7 times as active as 25-(OH)D_3. The efficiency of the two compounds (**4a** and **6a**) in binding to the cytosol receptor for 1α,25-$(OH)_2D_3$ was nearly parallel to the activity in inducing differentiation of the cells, suggesting that the two compounds induce differentiation by a receptor-mediated mechanism. These results provide supporting evidence for our previous postulation that vitamin D endoperoxides act after being transformed into other derivatives. It was also found that the differentiating activity in HL-60 cells was parallel to the bone-resorbing activity in vitro even in the novel vitamin D derivatives (**2a** and **6a**): they were more potent than 25-(OH)D_3 in resorbing bone from fetal mouse calvaria.

25-Hydroxy-6,19-epoxyvitamin D_3 (**6a**) is a vitamin D derivative with a unique structure where C-6 and C-19 are linked by an oxygen bridge, thus rigidly keeping the 5*E* vitamin D structure. Therefore, the high potency of the furan (**6a**) is easy to understand: namely the 3-hydroxyl group of **6a** is situated at the position of the 1α-hydroxyl group of 1α,25-$(OH)_2D_3$ and it can mimic the function of the biologically important 1α-hydroxyl group. Eisman et al.[9] reported that the 5*E* isomer of 25-(OH)D_3 had higher affinity than 25-(OH)D_3 in binding to the chick intestinal cytosol receptor for 1α,25-$(OH)_2D_3$. However, the binding potency shown by the hemiacetal (**4a**) cannot be explained on the basis of the general structure and activity relationship, because the planar structure of the conjugated triene part of vitamin D is lost in the hemiacetal as is in vitamin D endoperoxides (**2** and **3**). This problem must be taken into account in future investigations.

Experimental Section

General Procedures. Mass spectra were recorded with a JEOL-D300 mass spectrometer. HPLC was performed on a Jasco TWINCLE instrument equipped with a Jasco UVIDEC-610 spectrophotometer. UV spectra were recorded with a Jasco UVIDEC-610 spectrophotometer.

Vitamin D Compounds. 25-(OH)D_3 (**1a**) was purchased from Phillips-Duphar, Amsterdam, The Netherlands. 1α,25-$(OH)_2D_3$ and 24(*R*),25-$(OH)_2D_3$ were the gifts of Dr. I. Matsunaga (Chugai Pharmaceutical, Tokyo). (6*S*)-25-(OH)D_3 endoperoxide (**2a**) was synthesized chemically in our laboratory as previously reported.[1] 1α,25-$(OH)_2$[23,24-^{3}H]D_3 (specific activity 81 Ci/mmol) and 25-(OH)[26,27-^{3}H]D_3 (specific activity 19 Ci/mmol) were obtained from Amersham International, Bucks, UK.

Synthesis of 25-Hydroxy-6,19-dihydro-6,19-epidioxy-[26,27-^{3}H]vitamin D_3 (2a′ and 3a′). A solution of 25-(OH)-[26,27-^{3}H]D_3 (16 μCi, 0.84×10^{-6} mmol) and Rose Bengal (1.5 mg, 1.5 μmol) in ethanol–benzene (1:3, 6 mL) was saturated with oxygen and irradiated externally with a halogen lamp (200 W, Ushio JCV 100-200 GS) at 0 °C for 30 min under oxygen. The solution was diluted with benzene, washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on Sephadex LH-20 (10 g) with $CHCl_3$–hexane (1:1) as the eluent to give the starting material (**1a′**) (5 μCi) and a mixture of two endoperoxides (**2a′** and **3a′**) (8.9 μCi, 56%). The two isomers (**2a′** and **3a′**) were separated on HPLC (Finepak-SIL, 7% 2-propanol in hexane); the retention volumes of **2a′** and **3a′** were 33 and 35 mL, respectively.

Synthesis of (6*S*)-19,25-Dihydroxy-6,19-dihydro-6,19-epoxyvitamin D_3 (4a). (6*S*)-25-(OH)D_3 endoperoxide (**2a**) (1 mg, 2.3 μmol) was dissolved in 5% KOH in methanol and the resultant mixture stirred at room temperature for 25 min under argon. The reaction mixture was diluted with $CHCl_3$, washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on silica gel with ethyl acetate as the eluent to afford **6a** (59 μg, 6%) and **4a** (670 μg, 70%) in this order. The amount of the products was determined by comparing their UV spectra with those of the corresponding compounds (**6b** and **4b**) derived from (6*S*)-vitamin D_3 endoperoxide (**2b**).[2c]

Synthesis of 25-Hydroxy-6,19-epoxyvitamin D_3 (6a). A solution of the hemiacetal (**4a**) (500 μg, 1.2 μmol) in $CHCl_3$–$CDCl_3$ (1:1, 400 μL) was refluxed under argon for 3.5 h. The solvent was evaporated, and the residue was chromatographed on silica gel (1.5 g) with 70% ethyl acetate in hexane as the eluent to give the furan (**6a**) (470 μg, 98%).

Cells and Cell Culture. A human promyelocytic leukemia cell line (HL-60) was provided by Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo. Cells were cultured as described in the preceding paper.[1] Each vitamin D_3 derivative dissolved in ethanol was added to keep a final ethanol concentration of less than 0.1%. Control cultures were given the same volume of ethanol.

Transformation of (6*S*)-25-Hydroxy-6,19-dihydro-6,19-epidioxyvitamin D_3 (2a) by Incubation in Culture Medium. The endoperoxide (**2a**) (500 μg) was similarly incubated in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum in the absence of the cells at 37 °C for 3 days. Methanol–$CHCl_3$

(8) Tanaka, H.; Abe, E.; Miyaura, C.; Kuribayashi, T.; Konno, K.; Nishii, Y.; Suda, T. *Biochem. J.* **1982**, *204*, 713.

(9) Eisman, J. A.; DeLuca, H. F. *Steroids* **1977**, *30*, 245.

(2:1) was added to the incubation mixture, and the whole was extracted with $CHCl_3$. The extracts were evaporated, and the residue was chromatographed on a Sephadex LH-20 column with $CHCl_3$–hexane (3:1) as the eluent. The more polar peak eluted from the column was collected and the solvent was evaporated. The residue was purified on HPLC (Finepak-SIL, 0.46 × 25 cm) with 15% 2-propanol in hexane as the eluent. The major peak eluted between 8.5 and 12 mL was collected and purifed again on the same HPLC system. The major peak eluted between 6.7 and 7.3 mL was collected and subjected to mass spectrometry.

Incorporation of Vitamin D_3 Derivatives into HL-60 Cells. HL-60 cells (1×10^7) were incubated at 37 °C for 1 h in serum-free RPMI 1640 medium with 3.2 nM 25-(OH)[^{3}H]D_3 (**1a'**) or 25-(OH)[^{3}H]D_3 endoperoxides (**2a'** and **3a'**) in the presence or absence of a 10 000-fold excess of 25-(OH)D_3 or 25-(OH)D_3 endoperoxides, respectively. After incubation, cells were washed three times with Ca^{2+}- and Mg^{2+}-free PBS containing 10% heat-inactivated calf serum (Chiba Serum Institute, Chiba, Japan) 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 0.1 M KCl. The cytosol and the chromatin fractions were prepared as previously reported.[8] Radioactivity in the cytosol fraction was determined after treatment with hydroxylapatite to separate protein-bound 25-(OH)[^{3}H]D_3 and 25-(OH)[^{3}H] endoperoxides from the respective free radiosiotope.

Measurement of Phagocytic Activity. Phagocytic activity was measured according to the method of Collins et al.[10] The procedure is described in the preceding paper.[1]

Binding Assay. The procedure of the binding assay is described in the preceding paper.[1]

Measurement of Bone-Resorbing Activity. Sixteen-day pregnant mice, ddY strain (Shizuoka Laboratory Animal Center, Shizuoka, Japan), were injected subcutaneously with 50 μCi of $^{45}CaCl_2$ (New England Nuclear, Boston, MA), and 1 day later, they were sacrificed and the fetuses were isolated. The fetal calvaria were excised and divided into paired halves by dissecting along the midsagittal suture.[11] Each half calvaria was cultured for 24 h at 37 °C under 5% CO_2 in air in 0.5 mL of BGJ_b medium (GIBCO, Grand Island, NY) containing 1 mg/mL of bovine serum albumin (Fraction V, Sigma). After preculture for 24 h, each half calvaria was transferred to fresh medium with (treated) or without (control) vitamin D_3 derivatives and cultured for an additional 6 days. At the end of the culture period, the bones were digested overnight with 0.1 mL of HCl. ^{45}Ca in the medium and bone extracts was counted by a liquid scintillation counter. Results were expressed as the treated/control ratio and calculated by the following formula:

$$^{45}\text{Ca release (\%)} = \frac{^{45}\text{Ca in medium}}{^{45}\text{Ca in medium} + ^{45}\text{Ca in bone}} \times 100$$

$$\text{T/C ratio} = \frac{^{45}\text{Ca release (\%) from the treated calvaria}}{^{45}\text{Ca release (\%) from the control calvaria}}$$

Statistical difference was tested by student's t test.

Registry No. **2a**, 96999-64-3; **2a'**, 97011-22-8; **3a**, 96999-65-4; **3a'**, 96999-66-5; **4a**, 96999-67-6; **6a**, 96999-68-7; 25-(OH)[26,27-^{3}H]D_3, 71595-10-3.

(10) Collins, S. J.; Ruscetti, F. W.; Gallagher, R. E.; Gallo, R. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2458.

(11) Dziak, R.; Chang, Y. W.; Humphries, D.; Lloyd, W.; Wells, H.; Schmid, K.; Nimberg, R. B. *Calcif. Tissue Int.* **1980**, *30*, 191.

New Bidentates as Full Inhibitors of Enkephalin-Degrading Enzymes: Synthesis and Analgesic Properties

Marie-Claude Fournié-Zaluski,† Annie Coulaud,‡ Romaine Bouboutou,† Pierre Chaillet,‡ Jocelyne Devin,† Gilles Waksman,† Jean Costentin,‡ and Bernard P. Roques*†

Département de Chimie Organique, UA 496 CNRS et U 266 INSERM, U.E.R. des Sciences Pharmaceutiques et Biologiques, 75006 Paris, and Laboratoire de Pharmacodynamie, U.E.R. de Médecine et Pharmacie, Université de Rouen, 76800 Saint Etienne du Rouvray, France. *Received October 15, 1984*

New compounds were designed to fully inhibit the in vitro metabolism of enkephalins, ensured by three different metallopeptidases. For this purpose, bidentate ligands as hydroxamate and *N*-hydroxy-*N*-formylamino groups were selected as highly potent metal coordinating agents and introduced on Phe-Gly and Phe-Ala related structures. Compounds corresponding to the general formula $HC(O)N(OH)CH_2CH(CH_2Ph)CONHCH_2COOH$ (compound **7**) and $HN(OH)C(O)CH_2CH(CH_2Ph)CONHCH(R)COOH$ (compound **11**, R = H; compound **13**, R = CH_3) behave as full inhibitors of the three enzymes, with IC_{50}'s in the nanomolar range for enkephalinase, from 0.3 μM to 1 nM for dipeptidylaminopeptidase, and in the micromolar range for a biologically relevant aminopeptidase. Two diastereoisomers of the most active inhibitor **13** were separated by HPLC and their stereochemistry was assigned by ^{1}H NMR spectroscopy. Both isomers were efficient as enkephalinase blockers, but only the *RS* isomer, designated kelatorphan, was able to strongly inhibit aminopeptidase and dipeptidylaminopeptidase. Intracerebroventricular injection in mice of these mixed inhibitors, especially kelatorphan, led to naloxone reversible analgesic responses (hot-plate test) that were slightly better than those produced by a mixture of thiorphan and bestatin, two potent inhibitors of enkephalinase and aminopeptidase, respectively. Kelatorphan was also more efficient in potentiating the analgesia induced by a subanalgesic dose of Met-enkephalin. All these results support a physiological role in pain transmission for enkephalinase and a probably synaptic aminopeptidase M.

Psychic dependence, which is a specific side effect of narcotics, could be related to slow changes in normal homeostasis following an overstimulation of brain receptors involved in behavioral control. Likewise, the respiratory depression caused by high doses of morphine and surrogates is probably due to an overstimulation of opioid receptors located on bulbar respiratory neurons.[1] A large number of opioids have been synthesized with the aim of eliminating these major side effects, but no potent analgesic thus far described has proven to be completely free

† Département de Chimie Organique.
‡ Laboratoire de Pharmacodynamie.

(1) Morin-Surun, M. P.; Gacel, G.; Champagnat, J.; Denavit-Saubié, M.; Roques, B. P. *Eur. J. Pharmacol.* **1984**, *98*, 241.

0022-2623/85/1828-1158$01.50/0