Synthesis, Stereochemistry, and Biological Activity of 1α,23,25-Trihydroxy-24-oxovitamin D_3, a Major Natural Metabolite of 1α,25-Dihydroxyvitamin D_3[†]

Nancy E. Lee,[‡] G. Satyanarayana Reddy,*[,§] Alex J. Brown,[∥] and Paul G. Williard[⊥]

Department of Chemistry, Brown University, Providence, Rhode Island 02912, Department of Pediatrics, Women and Infants Hospital of Rhode Island, Brown University School of Medicine, Providence, Rhode Island 02905, Renal Division, Washington University School of Medicine, St. Louis, Missouri 63130, and Department of Chemistry, Simmons College, Boston, Massachusetts 02115

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ABSTRACT: The C(23) epimers of 1α,23,25$(OH)_3$-24-oxovitamin D_3, a major natural metabolite of the secosteroid hormone, 1α,25$(OH)_2D_3$, were chemically synthesized for the first time. The metabolite was synthesized by palladium coupling of the appropriate CD ring analog with an A ring enyne. Various approaches from quinic acid to the A ring precursors were explored, and a new route to the A ring enyne from quinic acid was developed. The C(23) stereochemistry of the natural 1α,23,25$(OH)_3$-24-oxovitamin D_3 produced in neonatal human keratinocytes was determined to be *S* on the basis of the ^{1}H NMR and the HPLC data. The biological activity of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 in primary cultures of bovine parathyroid cells was determined by comparing the potency of this metabolite to that of 1α,25-$(OH)_2D_3$ in suppression of parathyroid hormone (PTH) secretion. The results indicate that 1α,23(*S*),25-$(OH)_3$-24-oxovitamin D_3 potently suppressed PTH secretion even at concentrations as low as 10^{-12} M and is equipotent with 1α,25$(OH)_2D_3$. The high activity of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 cannot be explained on the basis of its affinity for the vitamin D receptor as this metabolite was found to be 10 times less effective than radioinert 1α,25$(OH)_2D_3$ in blocking the uptake and receptor binding of [^{3}H]-1α,25$(OH)_2D_3$ in intact parathyroid cells. Further studies are required to explain the molecular basis for the activity of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 in its ability to suppress PTH secretion. In summary, our present study indicates that the C(23) stereochemistry of the natural 1α,23,25$(OH)_3$-24-oxovitamin D_3 is *S* and this metabolite is equipotent to 1α,25$(OH)_2D_3$ in suppressing PTH secretion.

Vitamin D is known to play an important role in the normal development of the skeleton and maintenance of calcium homeostasis (Norman et al., 1992; Kametani & Furuyama, 1987). In addition to its role in mineral homeostasis, the hormonally active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$],[1] was shown to induce differentiation of leukemia cells (DeLuca et al., 1990; Abe et al., 1981). Other biological findings include its role in immune suppression and treatment of the hyperproliferative skin disorder psoriasis and suppression of parathyroid hormone secretion (Rigby, 1988; Zhou et al., 1991; Tsoukas et al., 1984; Brumbaugh et al., 1975). However, a dose of 1α,25$(OH)_2D_3$ that was required to obtain therapeutically useful results also produced severe hypercalcemia. This has limited the use of 1α,25$(OH)_2D_3$ as a therapeutic agent. Therefore, efforts have been directed toward finding synthetic analogs of 1α,25$(OH)_2D_3$ that can differentiate leukemic cells without producing hypercalcemia. To date, hundreds of synthetic analogs of 1α,25$(OH)_2D_3$ have been synthesized (Wilson & Yasmin, 1992; Dai & Posner, 1994; Bouillion et al., 1995). Most of the analogs are produced mainly through the modification of the side chain of 1α,25$(OH)_2D_3$. Some of these side chain-modified synthetic analogs possess selectively high activity in their ability to inhibit proliferation and induce differentiation of leukemic cells without producing significant hypercalcemia (Bikle, 1992; Bouillon et al., 1995; Norman et al., 1990). Thus, biological activity studies of the synthetic analogs suggested that modification of the side chain of 1α,25$(OH)_2D_3$ can result in the dissociation of its calcemic actions of 1α,25$(OH)_2D_3$ from its recently discovered noncalcemic actions (Perlman et al., 1990).

It is now well-established that the side chain of 1α,25-$(OH)_2D_3$ and its precursor 25$(OH)D_3$ naturally undergo analogous structural modifications in various vitamin D target tissues, resulting in the formation of several intermediary

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[‡] Simmons College.

[§] Brown University School of Medicine.

[∥] Washington University School of Medicine.

[⊥] Brown University.

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[1] Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,-25$(OH)_2$-16-ene-D_3, 1α,25-dihydroxy-16-enevitamin D_3; 1α,25$(OH)_2$-24-oxo-16-ene-D_3, 1α,25-dihydroxy-24-oxo-16-enevitamin D_3; 1α,-23,25$(OH)_3$-24-oxo-D_3, 1α,23,25-trihydroxy-24-oxovitamin D_3; IR, infrared; ^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon-13 nuclear magnetic resonance; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; CH_2Cl_2, methylene chloride; BOM, benzyloxymethyl ether; TBDMS, *tert*-butyldimethylsilyl ether; PPTS, pyridinium *p*-toluenesulfonate; Tf_2NPh, *N*-phenyltriflimide; LHMDS, lithium hexamethyldisilazide; OTf, trifluoromethyl ester; LDA, lithium diisopropylamide; L-Selectride, lithium tri-*sec*-butylborohydride; MsCl, mesyl chloride; Et_3N, triethylamine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; TMS, trimethylsilyl ether; Pd$(PPh_3)_4$, tetrakis(triphenylphosphine)-palladium; TMSCCSnBu_3, tributylstannyl trimethylsilylethyne; MeOH, methanol; Pd$(PPh_3)_2OAc_2$, bis(triphenylphosphine)palladium(II) acetate; Et_2NH, diethylamine; DMF, dimethylformamide.

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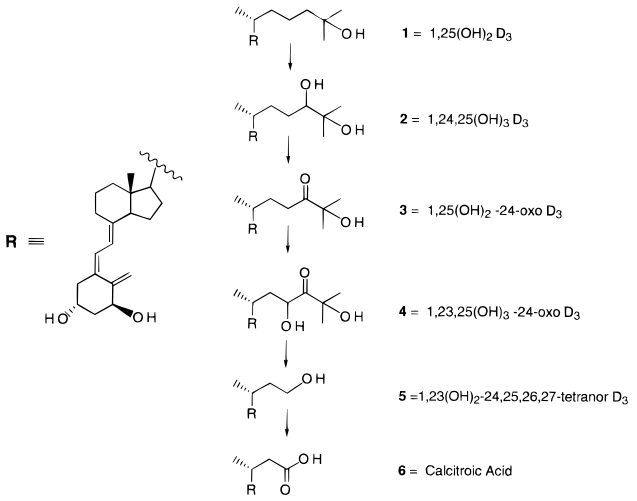


FIGURE 1: Metabolism for 1α,25(OH)$_2$D$_3$ through the C-24 oxidation pathway.

metabolites (Yamada et al., 1983; Bouillion et al., 1995). There exists a significant body of literature with regard to the biological activity of 24(*R*),25(OH)$_2$D$_3$, one of the major intermediary metabolites of 25(OH)D$_3$, and this subject was recently reviewed (Bouillion et al., 1995). One important function of 24(*R*),25(OH)$_2$D$_3$ pertinent to our present study is its role in the inhibition of parathyroid hormone secretion (Canterbury et al., 1978). However, there is a paucity of information with regard to the biological activity of the various natural side chain-modified intermediary metabolites of 1α,25(OH)$_2$D$_3$. This present study was undertaken to investigate the biological activity of 1α,23,25(OH)$_3$-24-oxo-D$_3$, one of the intermediary metabolites of 1α,25(OH)$_2$D$_3$ formed through the C-24 oxidation pathway as shown in Figure 1. This metabolite was previously shown to possess very little calcemic activity when compared to 1α,25(OH)$_2$D$_3$ (Mayer et al., 1983). At present, no information is available with regard to the noncalcemic actions of this major metabolite. Therefore, we evaluated the importance of this major intermediary metabolite in the vitamin D endocrine system with the following aims: (1) to synthesize the C(23) epimers of 1α,23,25(OH)$_3$-24-oxo-D$_3$, (2) to determine the absolute stereochemistry of the C-23 hydroxy group, and (3) to perform preliminary biological activity of the natural epimer of 1α,23,25(OH)$_3$-24-oxo-D$_3$ in its ability to suppress parathyroid hormone secretion in cultured bovine parathyroid cells.

MATERIALS AND METHODS

General

Melting points were determined using a Thomas-Hoover apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 1600 series FTIR spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter in a 1 dm cell. ^{1}H NMR spectra were recorded on either Bruker WM-250 MHz or Bruker AM-400 MHz spectrometers using TMS (0.0 ppm) as an internal standard. ^{13}C NMR spectra were recorded on a Bruker AM-400 MHz spectrometer at 100 MHz using $CDCl_3$ (77 ppm) as an internal standard. Mass spectra were obtained on routine intermediates with a Kratos MS 80RFA mass spectrometer under EI or CI conditions and for the final products on a Hewlett-Packard 5985B mass spectrometer. Thin layer chromatography (TLC) was performed on EM Science precoated silica gel 60F-254 glass-supported plates with 0.25 mm thickness. Spots were visualized by either ultraviolet light, exposure to iodine, or spraying with a 5% solution of phosphomolybdic acid (PMA) in ethanol. Flash chromatography was performed with ICN (70−220 mesh) or Kieselgel (230−400 mesh). Preparative thin layer chromatography was performed on Analtech Silica Gel GF plates (20 cm × 20 cm, 1000 μm thickness). Diethyl ether and THF were distilled from sodium/benzophenone ketyl under nitrogen. All the solvents and reagents used in the experiments were purified and dried according to the methods described by Perrin et al. (1980). All reactions were run under an inert atmosphere of argon. High-performance liquid chromatography (HPLC) was performed with a Waters model 600E chromatograph equipped with a Waters photodiode array detector (model 990) to monitor UV-absorbing material at 254 nm. The 1α,25(OH)$_2$-[26,27-^{3}H]vitamin D$_3$ (specific activity of 160−180 Ci/mmol) was obtained from Amersham. All the natural intermediary metabolites of 1α,-25(OH)$_2$-vitamin D$_3$ shown in Figure 1 were produced using the rat kidney perfusion model as described before (Reddy et al., 1987; Reddy & Tserng, 1989). Unlabeled 1α,25(OH)$_2$-vitamin D$_3$ was a gift from M. Uskokovic of Hoffmann LaRoche.

Chemical Synthesis of the C(23) Epimeric Mixture of 1α,23,25(OH)$_3$-24-oxo-D$_3$

*(3S,5S)-3,5-Bis[(tert-butyldimethylsilyl)oxy]-2-(hydroxymethyl)cyclohexanone (**11**).* LDA was formed by adding *n*-BuLi (2.5 M in hexanes, 0.14 mL, 0.36 mmol) to a solution of diisopropylamine (0.05 mL, 0.36 mmol) in anhydrous THF (1.5 mL) at −78 °C. After the mixture was stirred for 30 min, a solution of the ketone **10** (60 mg, 0.17 mmol) in THF (1.0 mL) was added dropwise over 10 min. The reaction mixture was allowed to stir at this temperature for 3 h. Paraformaldehyde (250 mg) in a two-neck flask (which was under vacuum overnight) was heated in an oil bath at 150 °C with a gentle stream of nitrogen flowing. The anhydrous gaseous formaldehyde was bubbled directly into the reaction mixture with the aid of external nitrogen for 10 min. After being stirred for about 15 min, the reaction mixture was poured into a saturated ammonium chloride solution (10 mL) and extracted with ether (2 × 30 mL). The organic solution was washed with brine and dried ($MgSO_4$). Filtration and concentration *in vacuo* gave a light yellow oil which was chromatographed on silica gel using 25% ethyl acetate in hexanes to give the pure β-hydroxy ketone **11** (51 mg, 78%). R_f = 0.55 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 4.35 (m, 1H-C$_3$), 4.22 (ddd, J = 10.4, 10.3, and 4.4 Hz, 1H-C$_5$), 3.94 (ddd, J = 11.4, 7.1, and 3.4 Hz, 1H-C$_7$), 3.81 (ddd, J = 11.4, 7.1, and 5.9 Hz, 1H-C$_7$), 2.62 (t, J = 7.1 Hz, OH), 2.48 (m, 2H-C$_6$ and C$_2$), 2.40 (dt, J = 13.9, and 3.4 Hz, 1H-C$_2$), 2.19 (m, 1H-C$_4$), 1.84 (ddd, J = 12.9, 10.4, and 2.0 Hz, 1H-C$_4$), 0.89 (s, 9H), 0.84 (s, 9H), 0.079 (s, 6H), 0.045 (s, 3H), 0.035 (s, 3H). IR (neat, cm^{-1}): 3487, 1708. ^{13}C NMR (100 MHz, $CDCl_3$): 210.48, 68.04, 66.56, 60.89, 59.11, 49.25, 42.13, 25.74, 25.55, 17.90, 17.85, −4.42, −4.99, −5.04, −5.12. HRMS (CI, isobutane): calcd for $C_{19}H_{41}O_4Si_2$ (M + H) 389.2543, found 389.2546.

*(3S,5S)-3,5-Bis[(tert-butyldimethylsilyl)oxy]-2-methylenecyclohexanone (**12**).* To a solution of the β-hydroxy

ketone **11** (100 mg, 0.26 mmol) in dry benzene (3 mL) was added triethylamine (0.11 mL, 0.78 mmol). This was followed by an addition of mesyl chloride (0.03 mL, 0.39 mmol). The reaction mixture was allowed to stir for 45 min, at which time TLC indicated that all of the β-hydroxy ketone had been converted to the mesylate with a R_f slightly lower than that of the starting material [mesylate $R_f = 0.45$ (25% ethyl acetate/hexanes)]. DBU (0.04 mL, 0.27 mmol) was added, and the reaction mixture was stirred for another 3 h. The reaction was quenched with water and the mixture extracted with ether (2 × 20 mL). The organic layer was washed with brine and dried ($MgSO_4$). Filtration and concentration *in vacuo* followed by chromatography on a silica gel column using 20% ethyl acetate in hexanes gave the pure α,β-unsaturated ketone **12** (78 mg, 80% for two steps). $R_f = 0.90$ (25% ethyl acetate/hexanes) NOTE: The mesylate is difficult to isolate due to decomposition, and it is better to continue to the next step without isolation. The α,β-unsaturated ketone **12** is also unstable and should be kept in the freezer.

^{1}H NMR (400 MHz, $CDCl_3$): 5.83 (t, $J = 2.0$ Hz, 1H-C_7), 5.43 (t, $J = 2.0$ Hz, 1H-C_7), 4.82 (m, 1H-C_5), 4.39 (m, 1H-C_3), 2.58 (dd, $J = 16.4$ and 4.0 Hz, 1H-C_2), 2.50 (ddd, $J = 16.4$, 4.7, and 1.6 Hz, 1H-C_2), 2.11 (m, 1H-C_4), 1.91 (ddd, $J = 12.2$, 9.6, and 2.6 Hz, 1H-C_4), 0.91 (s, 9H), 0.87 (s, 9H), 0.093 (s, 3H), 0.072 (s, 6H), 0.059 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 200.62, 149.78, 118.75, 68.87, 65.31, 49.03, 41.98, 25.77, 25.67, 18.14, 17.95, −4.84, −4.88, −4.93, −5.01. IR (neat, cm^{-1}): 1703. $[\alpha]^{20}_D$: −8.3° ($c = 0.7$, $CDCl_3$). HRMS (CI, isobutane): calcd for $C_{19}H_{39}O_3Si_2$ (M + H) 371.2437, found 371.2429.

*(3S,5S)-3,5-Bis[(tert-butyldimethylsilyl)oxy]-1-[[(trifluoromethyl)sulfonyl]oxy]-2-methylcyclohex-1-ene (**13**).* To a solution of the α,β-unsaturated ketone **12** (40 mg, 0.108 mmol) in dry THF (3 mL) was added *N*-phenyltrifluoromethanesulfonimide (40 mg, 0.12 mmol). After the resulting solution was cooled to −78 °C, lithium selectride (1 M in THF, 0.11 mL) was added dropwise. The reaction mixture was allowed to warm to −20 °C on its own, and it was kept at −20 °C for another 6 h. When the reaction was complete as monitored by TLC, the mixture was diluted with pentane and washed with brine. Drying ($MgSO_4$), filtration, and concentration *in vacuo* gave a solid oil mixture which was preadsorbed onto silica gel and chromatographed, using 5% ethyl acetate in hexanes to give the triflate **13** (20 mg, 42%). $R_f = 0.80$ (5% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 4.30 (t, $J = 4.1$ Hz, 1H), 4.21 (m, 1H), 2.58 (dd, $J = 16.6$ and 3.7 Hz, 1H), 2.32 (dd, $J = 16.6$ and 5.2 Hz, 1H), 1.78 (br s, 3H), 1.85−1.70 (m, 2H), 0.89 (s, 9H), 0.88 (s, 9H), 0.10 (s, 6H), 0.072 (s, 6H). IR (neat, cm^{-1}): 1413.0, 1141.2. ^{13}C NMR (100 MHz, $CDCl_3$): 142.40, 128,29, 118.36 (CF_3, q, $J = 319.6$ Hz), 68.71, 64.29, 40.53, 37.65, 25.75, 25.71, 17.99, 17.96, 13.84, −4.38, −4.81, −4.89. HRMS (CI, isobutane): calcd for $C_{16}H_{30}O_5SSi_2F_3$ (M − C_4H_9) 447.1305, found 447.1299.

(3S,5R)-3,5-Bis[(tert-butyldimethylsilyl)oxy]-1-[(trimethylsilyl)ethynyl]-2-methylcyclohex-1-ene. Under an argon atmosphere, anhydrous lithium chloride (20 mg, 0.47 mmol) and tetrakis(triphenylphosphine)palladate(0) (160 mg, 0.010 mmol) were dissolved in dry THF (4 mL). When solvation was complete, the triflate **13** (30 mg, 0.67 mmol) in THF (2 mL) and (1-trimethylsilyl)tributyltin acetylene (28 mg, 0.074 mmol) in THF (1 mL) were added *via* cannula. The reaction mixture was gently refluxed using an oil bath at 75 °C over about 12 h (note: the reaction has to be monitored so that the mixture does not dry out before the reaction is complete.) The color of the reaction mixture became a dark yellow to light brown from a light yellow solution. After the reaction mixture was cooled to room temperature, it was diluted with pentane and washed with 10% ammonium hydroxide solution. Washing with brine, followed by drying ($MgSO_4$), filtration, and concentration *in vacuo* gave an orange oil which was loaded onto a preparatory TLC plate. Separation of the intensely UV active enyne was accomplished using 5% ethyl acetate in hexanes (22 mg, 75%). $R_f = 0.90$ (5% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 4.18 (m, 1H-C_5), 4.08 (m, 1H-C_3), 2.40 (dd, $J = 16.6$ and 5.0 Hz, 1H-C_2), 2.07 (dd, $J = 16.6$ and 8.4 Hz, 1H-C_2), 1.92 (br s, 3H-C_7), 1.83 (m, 1H-C_4), 1.65 (ddd, $J = 13.0$, 10.5 and 4.3 Hz, 1H-C_4), 0.89 (s, 9H), 0.88 (s, 9H), 0.19 (s, 9H), 0.094 (s, 3H), 0.090 (s, 3H), 0.061 (s, 6H). IR (neat, cm^{-1}): 2142. ^{13}C NMR (100 MHz, $CDCl_3$): 143.14, 115.11, 105.41, 96.49, 69.87, 64.05, 41.19, 39.36, 25.89, 25.78, 19.15, 18.11, 17.99, 0.10, −4.33, −4.64, −4.71, −4.82. HRMS (CI, isobutane): calcd for $C_{24}H_{45}O_2Si_3$ (M − CH_3) 437.2727, found 437.2725.

*(3S,5R)-3,5-Bis[(tert-butyldimethylsilyl)oxy]-1-ethynyl-2-methylcyclohex-1-ene (**14**).* To a solution of trimethylsilylenyne (20 mg, 0.044 mmol) in methanol (2 mL) was added anhydrous potassium carbonate (20 mg, 0.15 mmol). The reaction mixture was stirred at room temperature for 5.5 h, at which time the reaction was complete as indicated by TLC. Methanol was removed, and the residue was partitioned between brine and pentane. Drying ($MgSO_4$) of the pentane layer, filtration, and concentration *in vacuo* gave **14** as an oil (15 mg, 89%) which was stored in the freezer. $R_f = 0.80$ (5% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 4.20 (t, $J = 3.8$ Hz, 1H), 4.09 (m, 1H), 3.05 (s, 1H), 2.41 (dd, $J = 16.9$ and 4.0 Hz, 1H), 2.08 (dd, $J = 16.9$ and 8.3 Hz, 1H), 1.92 (br s, 3H), 1.83 (m, 1H), 1.69 (ddd, $J = 13.0$, 10.1, and 4.4 Hz, 1H), 0.89 (s, 9H), 0.88 (s, 9H), 0.102 (s, 3H), 0.098 (s, 3H), 0.069 (s, 3H), 0.063 (s, 3H). IR (neat, cm^{-1}): 3311.3, 2092.0, 1256.9. ^{13}C NMR (100 MHz, $CDCl_3$): 143.51, 114.07, 83.83, 79.53, 69.73, 64.09, 41.09, 39.41, 25.87, 25.79, 18.96, 18.11, 18.02, −4.32, −4.67, −4.72, −4.81. HRMS (CI, isobutane): calcd for $C_{17}H_{31}O_2Si_2$ (M − C_4H_9) 323.1863, found 323.1879. $[\alpha]^{20}_D$: −101.14° ($c = 0.88$, $CHCl_3$).

De-A,B-24-oxo-23,25-(isopropylidenedioxy)cholestane 8-(Benzyloxy)methyl Ether. To a solution of the diol **7** (190 mg, 0.43 mmol) in anhydrous DMF (4 mL) was added 2,2-dimethoxypropane (2 mL), followed by pyridinium *p*-toluenesulfonate (30 mg, 0.1 mmol). The reaction mixture was stirred for 2 days and worked up by diluting with water (30 mL) and extracting with ether (3 × 20 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to give an oil which was chromatographed using 10% ethyl acetate/hexanes to give the ketal (160 mg, 78%). $R_f = 0.86$ (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 7.33 (m, 5H), 4.79 (d, $J = 6.9$ Hz, 1H), 4.68 (d, $J = 6.9$ Hz, 1H), 4.61 (s, 2H), 4.31 (m, $1H_{23}$), 3.97 (m, $1H_8$), 1.52 (s, 3H), 1.48 (s, 3H), 1.44 (s, 3H), 1.29 (s, 3H). IR (neat, cm^{-1}): 1737.0. ^{13}C NMR (100 MHz, $CDCl_3$): major isomer, 213.89, 138.13, 128.36, 127.87, 127.55, 100.15, 93.65, 79.39, 74.89, 72.02, 69.17, 57.04, 52.39, 42.26, 40.64, 36.27, 34.36, 31.49, 30.49, 29.39,

27.16, 26.24, 25.69, 25.07, 22.76, 17.95, 13.45. HRMS (EI): calcd for $C_{28}H_{41}O_5$ (M − CH_3) 457.2954, found 457.2942.

*De-A,B-24-oxo-23,25-(isopropylidenedioxy)cholestan-8-ol (**8**).* To a solution of de-*A,B*-24-oxo-23,25-(isopropylidenedioxy)cholestane 8-(benzyloxy)methyl ether (75 mg, 0.16 mmol) in absolute ethanol (2 mL) was added 10% palladium on carbon (20 mg). A balloon was filled with hydrogen gas and was connected to the reaction flask via an adapter. The reaction mixture was flushed with hydrogen gas several times. The balloon was filled again with hydrogen gas, and the reaction mixture was stirred overnight under an atmospheric pressure of hydrogen. When the deprotection was complete, as monitored by TLC, the reaction mixture was filtered through a pad of Celite. Ethanol was removed, and the residue was chromatographed using 20% ethyl acetate/hexanes to give the alcohol **8** (53 mg, 95%). R_f = 0.52 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 4.28 (dd, J = 10.5 and 2.6 Hz, H_{23}-major), 4.25 (dd, J = 7.3 and 4.8 Hz, H_{23}-minor), 4.05 (m, $1H_8$), 1.49 (s, 3H-major), 1.48 (s, 3H-minor), 1.45 (s, 3H-major + minor), 1.42 (s, 3H-minor), 1.40 (s, 3H-major), 1.26 (s, 3H-major + minor), 0.97 (d, J = 6.6 Hz, 3H-minor), 0.94 (s, 3H-major), 0.92 (s, 3H-minor), 0.90 (d, J = 6.4 Hz, 3H-major). IR (neat, cm^{-1}): 3520, 1737.8. ^{13}C NMR (100 MHz, $CDCl_3$): major isomer, 213.86, 100.16, 79.39, 71.95, 69.28, 57.01, 52.59, 42.03, 40.46, 36.22, 33.57, 31.32, 29.38, 27.05, 26.23, 25.69, 25.06, 24.48, 17.81, 17.42, 13.59. HRMS (EI): calcd for $C_{20}H_{33}O_4$ (M − CH_3) 337.2379, found 337.2374.

De-A,B-24-oxo-23,25-(isopropylidenedioxy)cholestan-8-one. Oxalyl chloride (0.025 mL, 0.284 mmol) was dissolved in CH_2Cl_2 (1 mL) and cooled to −78 °C. DMSO (0.037 mL, 0.57 mmol) in CH_2Cl_2 (0.4 mL) was added dropwise, and the reaction mixture was stirred for 45 min. The alcohol **22** (50 mg, 0.142 mmol) in CH_2Cl_2 (1 mL) was added slowly in a dropwise manner over 15 min. The reaction mixture was allowed to warm to −40 °C on its own over 2.5 h and was kept at this temperature for 15 min. After the mixture was recooled to −78 °C, triethylamine (0.16 mL, 1.14 mmol) was added dropwise. The reaction mixture was allowed to warm to −10 °C on its own and was stirred at this temperature for 30 min. Water was added to the reaction and was extracted with CH_2Cl_2 (2 × 20 mL). The organic layer was washed with brine, dried ($MgSO_4$), and filtered, and CH_2Cl_2 was removed to give an oil which was chromatographed with 25% ethyl acetate/hexanes to give the ketone (47 mg, 96%). R_f = 0.54 (25% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 4.29 (m, 1H-C_{23}-major + minor), 2.45 (m, 1H), 2.26 (m, 2H), 1.04 (d, J = 6.6 Hz, 3H-C_{21}-minor), 0.97 (d, J = 6.1 Hz, 3H-C_{21}-major), 0.66 (s, 3H-C_{18}-major), 0.64 (s, 3H-C_{18}-minor) IR (neat, cm^{-1}): 1737.4, 1714.5. ^{13}C NMR (100 MHz, $CDCl_3$): major isomer, 213.64, 211.82, 100.19, 79.40, 71.83, 61.92, 56.99, 49.95, 40.91, 38.99, 36.19, 31.53, 29.33, 27.35, 26.21, 25.67, 25.06, 23.98, 19.05, 17.96, 12.58. HRMS (CI, isobutane): calcd for $C_{21}H_{35}O_4$ (M + H) 351.2535, found 351.2522.

*De-A,B-24-oxo-23,25-(isopropylidenedioxy)cholestan-8-en-8-yl Trifluoromethanesulfonate (**9**).* Solid LiHMDS (41 mg, 0.25 mmol) was dissolved in THF (0.5 mL) and added dropwise *via* cannula to a solution of de-*A,B*-24-oxo-23,25-(isopropylidenedioxy)cholestan-8-one (22 mg, 0.06 mmol) in THF (1.2 mL) at −78 °C. The reaction mixture was stirred at −78 °C for 2 h and then warmed to 0 °C and was kept at this temperature for 7 h. After the mixture was recooled to −78 °C, a solution of *N*-phenyltriflimide (57 mg, 0.16 mmol) in THF (0.5 mL) was added dropwise *via* cannula. The reaction mixture was stirred at −78 °C for 5 min and was then switched to a 0 °C ice bath. After it warmed to room temperature on its own (∼5 h), it was allowed to stir for another 40 h. The reaction was quenched with a saturated NH_4Cl solution and the mixture extracted with ether (2 × 10 mL). The organic layer was washed with a saturated $NaHCO_3$ solution and brine. It was then dried ($MgSO_4$) and filtered, and the ether was removed. The resulting solid/oil mixture was chromatographed with 5% ethyl acetate/hexanes to give the desired triflate **9** (5 mg, 16%) and unreacted starting material which was contaminated by triflimide. R_f = 0.45 (5% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 5.58 (dd, J = 6.8 and 3.4 Hz, $1H_9$), 4.31 (dd, J = 10.5 and 2.9 Hz, $1H_{23}$-major + minor), 2.50 (m, 1H), 2.32 (m, 2H), 2.01 (m, 2H), 1.53 (s, 3H), 1.48 (s, 3H), 1.45 (s, 3H), 1.32 (s, 3H), 1.04 (d, J = 6.5 Hz, H_{21}-minor), 0.97 (d, J = 6.8 Hz, H-C_{21}-major), 0.79 (s, H_{18}-major), 0.78 (s, H_{18}-minor). IR (neat, cm^{-1}): 1738.2, 1415.5, 1144.1, 1206.9. ^{13}C NMR (100 MHz, $CDCl_3$): major isomer, 213.61, 149.76, 118.75 (CF_3, J = 330 Hz), 116.06, 100.23, 79.46, 71.83, 54.57, 50.12, 45.36, 36.22, 34.89, 32.01, 29.36, 28.20, 26.25, 25.71, 25.09, 23.83, 21.43, 17.96, 11.44.

1-[(tert-Butyldimethylsilyl)oxy]-6,7-dehydro-23,25-(isopropylidenedioxy)-24-oxoprevitamin D_3 tert-Butyldimethylsilyl ether (**15**). Bis(triphenyphosphine)palladium(II) acetate (3 mg, 0.004 mmol) and copper(I) iodide (3 mg, 0.016 mmol) were added to an anhydrous DMF solution (0.5 mL) containing the triflate **9** (5 mg, 0.01 mmol), the enyne **14** (5 mg, 0.013 mmol), and diethylamine (0.5 mL). The reaction mixture was stirred at room temperature for 2 h. The color of the reaction changed from yellow-green to brown-green. Ether was added when the reaction was completed and the mixture washed with brine. The organic layer was dried ($MgSO_4$), filtered, and concentrated. The resulting orange oil was chromatographed with 5% ethyl acetate/pentane to give the intensely UV active dienyne **15** (6.8 mg, 93%). R_f = 0.46 (5% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): major isomer, 5.97 (m, 1H-C_9), 4.34 (dd, J = 10.0 and 3.0 Hz, 1H-C_{23}), 4.19 (m, 1H), 4.11 (m, 1H), 0.97 (d, J = 6.3 Hz, 3H-C_{21}), 0.89 (s, 9H, tBu), 0.88 (s, 9H, tBu), 0.73 (s, 3H-C_{18}), 0.09 (s, 6H, $SiMe_2$), 0.06 (s, 6H, $SiMe_2$). IR (neat, cm^{-1}): 1738.2.

1-[(tert-Butyldimethylsilyl)oxy]-23,25-(isopropylidenedioxy)-24-oxoprevitamin D_3 (tert-Butyldimethylsilyl Ether. To a solution of the dienyne **15** (6.8 mg, 0.01 mmol) in dry hexanes (3 mL) were added quinoline (0.1 M in hexanes, 0.1 mL) and Lindlar's Catalyst (16 mg). The reaction mixture was stirred under an atmosphere of hydrogen for 2 h. The reaction was checked by TLC every 15 min in the last hour to avoid overhydrogenation. When most of the starting material was converted to a product with a slightly higher R_f, the reaction mixture was filtered through a pad of Celite. After the reaction mixture was concentrated, the residue was loaded onto a preparatory TLC plate and eluted with 2.5% ethyl acetate/hexanes. Isolation of the triene was accomplished and was subjected to the next step. R_f = 0.48 (5% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): major isomer, 5.89 and 5.72 (d, J = 12.2 Hz, 2H-$C_{6,7}$), 5.55 (m, 1H-C_9), 4.31 (m, 1H-C_{23}), 4.11 (m, 2H-$C_{1,3}$), 0.97 (d, J = 6.0 Hz, 3H-C_{21}), 0.89 (s, 9H, tBu), 0.87 (s, 9H, tBu), 0.72 (s, 3H-C_{18}), 0.09 (s, 6H, $SiMe_2$), 0.06 (s, 6H, $SiMe_2$).

*1α,23,25-Trihydroxy-24-oxovitamin D_3 (**4**).* The previtamin was dissolved in dry isooctane (1 mL) and refluxed in an oil bath at 100−105 °C under an argon atmosphere and in the dark for 4 h. The solvent was removed using a rotary evaporator, and the product was placed under vacuum for a few hours. The protected vitamin was dissolved in methanol (1 mL) and CH_2Cl_2 (0.1 mL) and treated with Dowex 50WX4-400 resin (120 mg). The mixture was stirred vigorously for 14 h in the dark. Filtration and concentration *in vacuo* afforded the metabolite **4** as a white film of solid (2.5 mg, 59% for the last three steps). R_f = 0.2 (75% ethyl acetate/hexanes).

^{1}H NMR (400 MHz, $CDCl_3$): 6.38 and 6.02 (d, J = 11.3 Hz, 2H-$C_{6,7}$), 5.33 (m, 1H-C_{19}), 5.00 (narrow m, 1H-C_{19}), 4.70 (dd, J = 9.9 and 2.6 Hz, 1H$_{23}$-major), 4.64 (dd, J = 7.9 and 4.0 Hz, 1H$_{23}$-minor), 4.43 (dd, J = 7.6 and 4.8 Hz, 1H-C_1), 4.23 (m, 1H-C_3), 2.83 (dd, J = 12.3 and 3.4 Hz, 1H-C_9), 2.60 (dd, J = 13.3 and 3.2 Hz, 1H-C_4), 2.31 (dd, J = 13.3 and 6.4 Hz, 1H-C_4) 1.44 and 1.42 (s, each 3H-$C_{26,27}$), 1.09 (d, J = 6.7 Hz, 3H$_{21}$-minor), 1.06 (d, J = 6.5 Hz, 3H$_{21}$-major), 0.59 (s, 3H$_{18}$-major), 0.56 (s, 3H$_{18}$-minor). IR (neat, cm^{-1}): 3391.4, 2925.3, 1711.7, 1375.1, 1043.7. ^{13}C NMR (100 MHz, $CDCl_3$): major isomer, 217.03, 147.64, 142.87, 133.02, 124.93, 117.19, 111.80, 77.20, 71.01, 70.84, 66.86, 56.90, 56.39, 46.11, 45.27, 42.87, 40.97, 40.50, 33.12, 29.04, 27.77, 27.72, 27.64, 23.53, 22.27, 18.14, 12.08.

In Vitro Production of 1α,23,25-Trihydroxy-24-oxovitamin D_3 in Human Neonatal Keratinocytes

Keratinocytes were prepared as described previously (Kim et al., 1992) from human foreskins. First passage keratinocytes were seeded at 0.5 × 10^6 cells/flask into 75 cm^3 flasks in keratinocyte growth media (KGM, Clonetics, Inc.). The cells were refed every 2−3 days. When the cells were 70−80% confluent (usually after 5 days), the medium was changed to include 1,25$(OH)_2D_3$ (1 μM), 1.5 mM $CaCl_2$, and 0.2% bovine serum albumin. At the end of the experimental period in a 37 °C 5% CO_2 incubator, an equal volume of methanol was added to the cultures. Lipid extraction was performed on both cells and media using the technique of Bligh and Dyer (1959). The lipid extract was subjected to HPLC directly for the separation of various 1α,25$(OH)_2D_3$ metabolites. The metabolite comigrating with the standard 1α,23,25$(OH)_3$-24-oxo-D_3 is purified on two different HPLC systems. The standard 1α,23,25$(OH)_3$-24-oxo-D_3 was generated in rat kidney using the previously published methods from our laboratory (Reddy et al., 1987; Reddy & Tserng, 1989). Half of the keratinocyte metabolite is subjected to mass spectrometry for structure identification, and the remaining half of the metabolite is used for its stereochemistry determination.

Biological Activity Assays

Cell Culture. Bovine parathyroid glands, obtained from a local slaughterhouse and transported to the laboratory in cold PBS, were digested with collagenase as previously described (Brown et al., 1992) and seeded at a density of 80 000 cells/cm^2 in DMEM/Ham's F-12 (1:1) containing 4% heat-inactivated newborn calf serum, 15 mM HEPES, 100 IU/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 2 mM glutamine, and 1% nonessential amino acids. After 24 h, cells were placed in medium containing 0.1% bovine serum albumin and 5 μg/mL transferrin in place of the serum. Cells were grown to confluency (6 days) in this serum-free medium.

Analysis of Parathyroid Hormone Secretion. Parathyroid cell cultures were prepared as described above and grown for 4 days in serum-free medium. The cells were then treated for 3 days with 1α,25$(OH)_2D_3$ or 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 at concentrations ranging from 10 pM to 100 nM with daily changes of the medium. Steady state PTH secretion was determined by washing the cells three times with Dulbecco's PBS and then placing them in serum-free medium for 3 h. The medium was collected, centrifuged at 4 °C, and analyzed for PTH using CH9 antibody as described previously (Brown et al., 1992). The cell monolayers were dissolved in 0.1 N NaOH and assayed for protein by the method of Bradford (1976) using a kit from Bio-Rad Laboratories (Richmond, CA). PTH secretion is expressed as picograms of PTH per milligram of cell protein. A two-tailed *t* test was used to analyze the data.

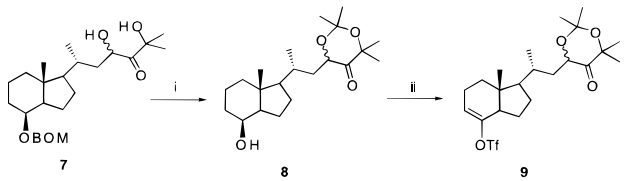
Assay of Vitamin D Receptor in Intact Cells. Confluent parathyroid cells were incubated for 2 h with 0.5 nM 1α,25$(OH)_2$-[26,27-^{3}H]vitamin D_3 and the indicated concentration of radioinert 1α,25$(OH)_2D_3$ or 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 in serum-free medium containing 0.5 mg of BSA/mL. The medium was aspirated, and the cells were washed once with PBS containing 5 mg/mL BSA and then twice more with ice-cold PBS. The cells were sonicated in cold TEDK buffer [10 mM Tris-HCl (pH 7.4), 1.5 mM EDTA, 5 mM dithiothreitol, and 300 mM KCl], and an aliquot was taken to determine the total cell protein. The remaining sonicate was mixed with 200 μL of charcoal/destran, kept on ice for 15 min, and centrifuged at 1000*g* for 15 min. The supernatants were mixed with 4 mL of scintiVerse BD (Fisher Scientific, Pittsburgh, PA) and counted in a Beckman LS-2800 liquid scintillation counter. The data are expressed as femtomoles of [^{3}H]-1,25$(OH)_2D_3$ bound per milligram of cell protein.

RESULTS

*Synthesis and Separation of Two C(23) Epimers of 1α,23,25-Trihydroxy-24-oxovitamin D_3 **4**.* The synthesis of the 23,25-dihydroxy-24-oxo side chain of the CD ring began with Lythgoe's diol. Thus, the required C-22 sulfone was readily prepared from Lythgoe's diol in two steps, and (benzyloxy)methyl ether was chosen as a suitable protecting group for the C-8 alcohol.[2] The 23,25-dihydroxy-24-oxo side chain was introduced using the method reported by Takayama et al. (1989) to give a 2:1 mixture of C-23 epimers. The dihydroxy group at C-23 and C-25 was protected as a ketal,

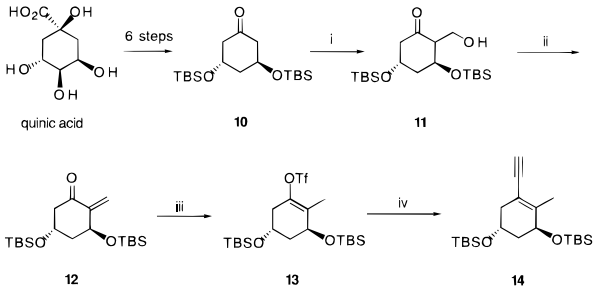
[2] Initially, the TBDMS group was used as the protecting group for the C-8 alcohol. However, the selective deprotection of the TBDMS group in the presence of the ketal group was unsuccessful under the standard conditions. The deprotection required the use of HF which removed all the protecting groups. The BOM group was chosen because the preliminary studies showed that the deprotection proceeded smoothly under normal hydrogenation conditions.

Scheme 1[a]



[a] Reagents: (i) (a) $(CH_3)_2C(OMe)_2$, PPTS, DMF; (b) H_2, 10% Pd/C, EtOH; (ii) (a) CO_2Cl_2, DMSO, Et_3N, CH_2Cl_2; (b) LHMDS, THF, Tf_2NPh.

Scheme 2[a]



[a] Reagents: (i) LDA, THF, CH_2O; (ii) MsCl, Et_3N, benzene, DBU; (iii) L-Selectride, THF, Tf_2NPh; (iv) (a) $Pd(PPh_3)_4$, LiCl, THF, $TMSCCSnBu_3$; (b) K_2CO_3, MeOH.

and the BOM group was removed by hydrogenation to give the alcohol **8** as shown in Scheme 1. The C-8 hydroxy group was oxidized to the ketone using Swern's conditions (Mancuso et al., 1978), followed by conversion to the triflate **9** by treating the ketone with LHMDS and *N*-phenyltriflimide.

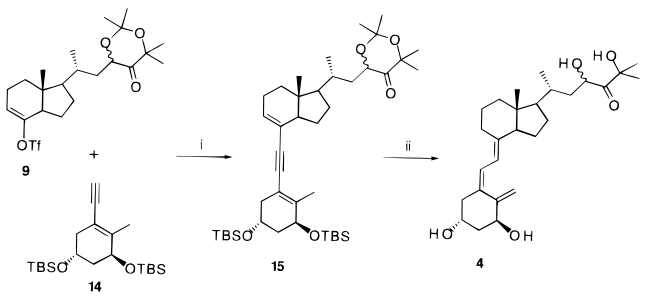
The A ring enyne **14** was synthesized from quinic acid as shown in Scheme 2. Quinic acid was chosen as our starting material for several reasons (Desmaele et al., 1989). Quinic acid is a highly functionalized, commercially available building block, featuring the two hydroxys at C-3 and C-5 having the correct stereochemistry (3*R* and 5*R*) as the desired A ring. However, the entire carbon skeleton of the A ring is not present in quinic acid. A carbon that is destined to be C-19 is missing and thus has the potential for introducing a radiolabeled methylene group or groups other than the methylene group. Therefore, a synthesis of the A ring from quinic acid will allow preparation of A ring analogs as well as the radiolabeled A ring for future mechanism studies.

Quinic acid was converted to the known C_2 symmetrical ketone **10** in six steps (Perlman et al., 1991). Various α-methylenation reactions (Petragnani et al., 1986; Grieco et al., 1975; Stork et al., 1974; Danishefsky et al., 1977; Kallmerten et al., 1985) were attempted on the β-dialkoxy ketone **10**. The best method was using the aldol condensation reaction. Thus, formation of the enolate of ketone **10** was accomplished with LDA, and the enolate was reacted with formaldehyde to give compound **11** in 78% yield. We found that other aldehydes reacted with the enolate of **10** as well.[3] Incorporating various groups and radiolabeling the A ring will be possible using this chemistry.

Elimination of the β-hydroxy group of ketone **11** was accomplished by converting it to a mesylate and then treating it with DBU (Grieco et al., 1980) to give **12**. The preparation of triflate **13** proceeded from **12** by treating the enone with L-Selectride and trapping the enolate with *N*-phenyltriflimide

[3] For example, the addition of benzaldehyde proceeded smoothly to give a mixture of diastereomers in a ratio of 10:1.

Scheme 3[a]



[a] Reagents: (i) $Pd(PPh_3)_2OAc_2$, CuI, Et_2NH, DMF; (ii) (a) H_2, Lindlar's Catalyst, quinoline; (b) isooctane, 100 °C; (c) Dowex 50WX4, MeOH.

(Crisp et al., 1985; McMurry et al., 1983). Subsequent coupling of the triflate **13** with (trimethylsilyl)stannylacetylene, using Stille's standard condition (Scott et al., 1984), followed by deprotection of the trimethylsilyl group gave the enyne A ring **14**. The enyne **14** agreed spectroscopically with the published data (Lythgoe et al., 1981).

The coupling of the A ring enyne **14** with the CD ring triflate **9** was effected using conditions reported by Craig et al. (1992) to give **15** as shown in Scheme 3. Cis hydrogenation of the alkyne followed by the [1,7] sigmatropic hydrogen shift gave the protected metabolite. The protecting groups, the *tert*-butyldimethylsilyl group and ketal, were cleaved by subjecting the protected metabolite to an acidic resin in methanol overnight. After workup and purification, the metabolite **4** was isolated in an overall yield of 55% (from **9**) as a mixture of diastereomers, epimeric at C-23. The ratio of the two diastereomers was approximately 9:1 as determined by the ^{1}H NMR spectrum. The structure of **4** was confirmed by spectroscopic analysis and by comparison with the published spectrum of the metabolite **4** isolated from the natural source (Mayer et al., 1983). The two diastereomers were separated using the HPLC system described in Figure 2. Thus, we obtained each individual diastereomer in pure form for comigration experiments with the natural metabolite.

Determination of Stereochemistry at C-23 of Natural 1α,23,25-Trihydroxy-24-oxovitamin D_3 Produced in Human Neonatal Keratinocytes. Takayama and co-workers have synthesized the two C(23) epimers of 23,25-dihydroxy-24-oxovitamin D_3 (Takayama et al., 1989). They determined the C(23) stereochemistry of the major isomer of synthetic 23,25-dihydroxy-24-oxovitamin D_3 to be *R* on the basis of X-ray analysis and concluded that the minor isomer has the *S* configuration at C(23). We followed Takayama's synthesis in preparing the same side chain which resulted in two C(23) epimers of 1α,23,25-trihydroxy-24-oxovitamin D_3 **4**. The ^{1}H NMR data of the side chain portion of our synthetic mixture of C(23) epimers of 1α,23,24-trihydroxy-24-oxovitamin D_3 are identical to the published ^{1}H NMR data of Takayama's synthetic mixture of C(23) epimers of 23,25-dihydroxy-24-oxovitamin D_3. On the basis of Takayama's assignment of *R* to C(23) by X-ray diffraction analysis of the major isomer, we also assigned our major isomer the *R* configuration and concluded that the minor isomer has the *S* configuration.[4] Direct comparison of the HPLC data of our synthetic mixture of major and minor isomers with the natural metabolite produced in human keratinocytes shows that the natural metabolite behaves in a manner identical to that of our minor synthetic isomer (see Figure 2). Hence,

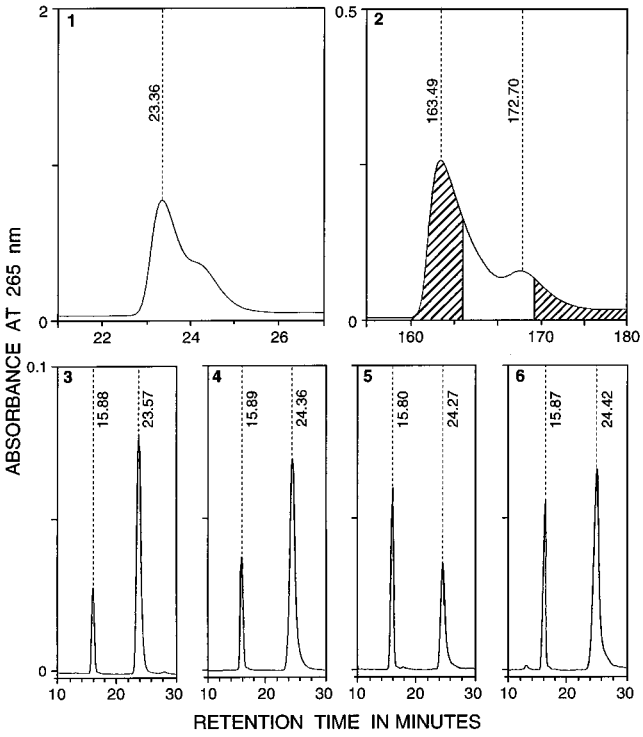


FIGURE 2: HPLC separation of the two C(23) epimers of synthetic 1α,23,25$(OH)_3$-24-oxovitamin D_3 and the comigration of natural 1α,23,25$(OH)_3$-24-oxovitamin D_3 produced in human keratinocytes with one of the two synthetic isomers. HPLC was performed on Zorbax-SIL (9.4 mm × 25 cm) column; the mixture was eluted with a solvent mixture containing hexane and 2-propanol at a flow rate of 2 mL min^{-1}. Profile 1: inadequate separation of C(23) epimers of synthetic 1α,23,25$(OH)_3$-24-oxovitamin D_3 using hexane/2-propanol (90:10 v:v). Profile 2: adequate separation of the two isomers using hexane/2-propanol (95:5 v:v). The first isomer eluting at 163.49 min was collected from minutes 160 to 166, and the second isomer eluting at 172.70 min was collected from minutes 174 to 186. The overlapping peak from minutes 166 to 174 containing the two isomers was recycled utilizing the same HPLC conditions to obtain additional pure material of each individual isomer. Profile 3: coinjection of the first peak along with 1α,25-$(OH)_2D_3$ as a reference standard. Profile 4: coinjection of the second peak along with 1α,25$(OH)_2D_3$ as a reference standard. Profile 5: coinjection of naturally occurring 1α,23,25$(OH)_3$-24-oxovitamin D_3 produced in human keratinocytes along with 1α,25$(OH)_2D_3$ as a reference standard. Profile 6: coinjection of naturally occurring 1α,23,25$(OH)_3$-24-oxovitamin D_3 produced in human keratinocytes along with a purified (by HPLC) sample of the synthetic material corresponding to the minor isomer shown in profile 2 along with 1α,25$(OH)_2D_3$ as a reference standard.

we concluded from these results that the configuration of the natural metabolite of 1α,23,25$(OH)_3$-24-oxovitamin D_3 at C(23) is *S*.

Biological Activity and Vitamin D Receptor Binding Studies of 1α,23(S),25$(OH)_3$-24-oxovitamin D_3. The biological activity of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 in parathyroid cells was determined by comparing the potency of this metabolite to that of 1α,25$(OH)_2D_3$ in suppressing PTH secretion. Parathyroid cells were incubated with various concentrations of 1α,25$(OH)_2D_3$ or 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 for 3 days. The steady state rate of PTH secretion was then measured during a 3 h period. As shown in Figure 3, 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 potently decreased PTH secretion. This suppression was significant ($p < 0.01$) even at 10^{-12} M. While 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 only appeared to be slightly less active than 1α,25$(OH)_2D_3$, the difference was not significant. Whether the suppression of parathyroid hormone secretion is a vitamin D receptor-mediated event, we determined the accessibility of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 to the vitamin D receptor in monolayers of parathyroid cells using a competitive binding assay. Figure 4 shows that 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 was approximately 10 times less effective than radioinert 1α,25$(OH)_2D_3$ in blocking the uptake and receptor binding of [^{3}H]-1α,25$(OH)_2D_3$ in parathyroid cells.

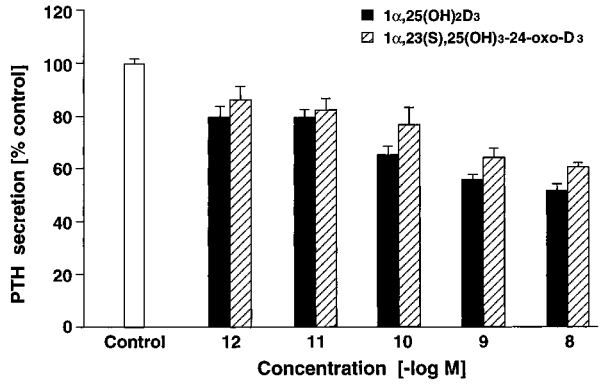


FIGURE 3: Suppression of PTH secretion by 1α,25$(OH)_2D_3$ and 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3. Confluent cultures of bovine parathyroid cells were incubated with the specified concentration of 1α,25$(OH)_2D_3$ (shaded bars) or 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 (hatched bars) for 72 h with media changes every 24 h. The cells were then washed, and steady state PTH secretion was determined during a 3 h incubation. PTH was assessed by radioimmunoassay, and data were corrected for the total cell protein. The data are expressed as mean ± SD and represent combined data from two separate experiments ($n = 12$). All treated samples were significantly less than control ($p < 0.05$).

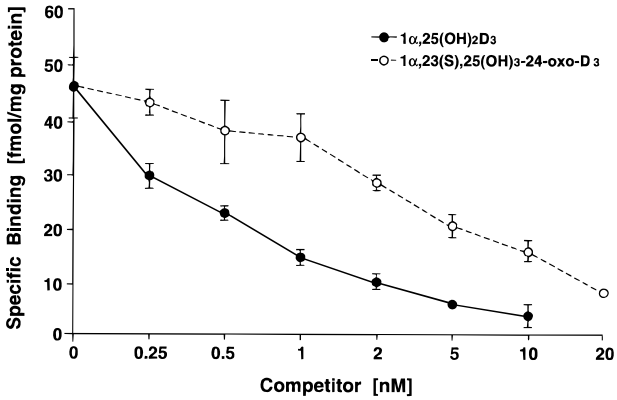


FIGURE 4: Binding of 1α,25$(OH)_2D_3$ and 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 in cultured parathyroid cells. Confluent parathyroid cells were incubated for 2 h with 0.5 nM 1α,25$(OH)_2$-[26,27-^{3}H]vitamin D_3 and the indicated concentration of radioinert 1α,-25$(OH)_2D_3$ (solid line) or 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 (dashed line) in serum-free medium containing 0.5 mg of BSA/mL. Binding to the VDR was determined as described in Materials and Methods. Data are mean ± SD; $n = 3$.

DISCUSSION

Within the past two decades, following the discovery of conversion of vitamin D_3 into the steroid hormone 1α,25-$(OH)_2D_3$, there has been a great interest in isolating and

[4] Takayama reported the ^{1}H chemical shift of the C(23) proton of the major isomer with the *R* configuration to be more downfield at 4.72 ppm compared to that of the minor isomer with the *S* configuration which resonates at 4.66 ppm. The ^{1}H NMR data of compound **4** show two peaks for the C(23) proton: at 4.70 and 4.64 ppm with a ratio of 9:1, respectively. We assigned the more downfield chemical shift at 4.70 ppm of the major isomer the *R* configuration and the minor isomer with the chemical shift at 4.64 ppm the *S* configuration.

identifying the various natural metabolites of both 1α,25-$(OH)_2D_3$ and its precursor, 25(OH)D_3. As a result, to date, more than 30 natural metabolites of vitamin D_3 are known to exist, and they are formed in almost all the target tissues that possess vitamin D receptors and respond to 1α,25-$(OH)_2D_3$ (Bouillon et al., 1995). All the major known natural metabolites of 1α,25$(OH)_2D_3$ are formed as a result of side chain oxidations at C-23, C-24, and C-26. The C-24 oxidation pathway shown in Figure 1 initiated by C-24 hydroxylation leads to the formation of calcitroic acid, which is the major end product of the hormone (Reddy & Tserng, 1989; Makin et al., 1989). This pathway has been extensively studied, and 24-hyroxylase, the key enzyme of this pathway, has been cloned. The regulation of 24-hydroxylase at the molecular level has been studied, and it was also found that the same enzyme catalyzes 24-hydroxylation, 24-oxidation, and subsequent 23-hydroxylation of the C-24 oxo compound (Akiyoshi-Shibata et al., 1994; Okuda et al., 1995). The remaining two pathways initiated by C-23 and C-26 hydroxylations lead to the formation of the final product calcitriol lactone (Ishizuka & Norman, 1987). Very little is known regarding the enzymology of these latter pathways.

At present, it is generally believed that the various intermediary metabolites of 1α,25$(OH)_2D_3$ formed in target tissues have no biological role. This assumption is based on previous studies comparing the calcemic activities of these metabolites with those of 1α,25$(OH)_2D_3$ (Mayer et al., 1983). However, the possibility of the intermediary metabolites possessing biological activities other than calcemic actions has also been considered. In this regard, calcitriol lactone was found to have unique biological activities that are different from its parent 1α,25$(OH)_2D_3$. This natural metabolite has been shown to decrease serum calcium and promote bone formation (Seino & Ishizuka, 1991). Thus, the importance of evaluating the various noncalcemic biological activities of some of the natural intermediary metabolites of 1α,25$(OH)_2D_3$ cannot be underestimated.

In our present study, we initiated a careful evaluation of the biological role of 1α,23,25$(OH)_3$-24-oxovitamin D_3 in vitamin D endocrine system. We first accomplished the successful synthesis of both the C(23) epimers of this major metabolite and determined the C(23) stereochemistry of the natural metabolite to be *S*. A previous study evaluated the calcemic actions (intestinal calcium transport and bone calcium mobilization) and vitamin D receptor binding of 1α,-23(*S*),25$(OH)_3$-24-oxovitamin D_3 (Mayer et al., 1983). This study indicated that 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 possessed minimal calcemic activity. Furthermore, it was also noted that this metabolite exhibited very little binding to the vitamin D receptor. Thus, the calcemic actions of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 correlated well with its binding affinity to the vitamin D receptor. In our present study, we compared 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 with 1α,25$(OH)_2D_3$ in their ability to suppress parathyroid hormone secretion, a well-studied noncalcemic action of 1α,-25$(OH)_2D_3$. The relatively high activity of 1α,23(*S*),25-$(OH)_3$-24-oxovitamin D_3 was unexpected since the affinity of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 for the vitamin D receptor was reported to be 130 times less than that of 1α,-25$(OH)_2D_3$ (Mayer et al., 1983). However, these previous binding studies utilized cell extracts as the source of vitamin D receptor and may not reflect binding to the receptor in intact cells. Therefore, we determined the binding of 1α,-23(*S*),25$(OH)_3$-24-oxovitamin D_3 to the vitamin D receptor in monolayers of bovine parathyroid cells using a competitive binding assay. Even in these studies, 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 was found to be 10 times less potent in its binding to the vitamin D receptor. Thus, the suppressive effect of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 on PTH secretion did not correlate exactly with its binding to the vitamin D receptor.

There may be several explanations for this unexpected high activity of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3. First, on the basis of their structures, it is likely that 1α,23(*S*),25-$(OH)_3$-24-oxovitamin D_3 is more soluble in aqueous solutions than 1α,25$(OH)_2D_3$ and would have greater access to the VDR in the cells. However, in our present experiments, the medium contained 0.1% BSA and no serum. We and others (Dusso et al., 1991; Bikle & Glee, 1989) have previously shown that, under these conditions, all of the 1α,25$(OH)_2D_3$ is in solution and roughly 50% is unbound to protein. Thus, free 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 could be, at most, about 2-fold higher than those of 1α,25$(OH)_2D_3$ if its interaction with BSA is minimal. Another explanation could involve the nongenomic pathways for vitamin D action. These activities are probably mediated by a unique membrane-associated receptor with a ligand specificity that is distinct from the nuclear VDR (Norman et al., 1993a,b; Nemere et al., 1994). In parathyroid cells, 1α,25$(OH)_2D_3$ is known to acutely stimulate phosphoinositide metabolism (Bourdeau et al., 1990) and increase cytosolic calcium (Sugimoto et al., 1988). The role of these acute effects in the suppression of PTH by 1α,25$(OH)_2D_3$ is unclear. The nongenomic actions of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 have not been examined in parathyroid cells. Thus, the possibility of 1α,-23(*S*),25$(OH)_3$-24-oxovitamin D_3 in stimulating nongenomic pathways in the parathyroid cells with a different potency from that of 1α,25$(OH)_2D_3$ cannot be ruled out. In addition, recent studies have indicated that some synthetic analogs of vitamin D, notably the 20-epi vitamin D analogs, bind to the VDR and produce a conformational change that increases their affinity for the vitamin D response elements in target genes and elicit greater transcriptional effects than predicted from their VDR affinity (Peleg et al., 1995, 1996). Therefore, the possibility of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3 producing a conformational change in the nuclear VDR different from that produced by 1α,25$(OH)_2D_3$ still exists.

The potent suppressive action in parathyroid cells along with a lack of calcemic activity in vivo has been noted for several synthetic vitamin D compounds (Brown et al., 1989; Slatopolsky et al., 1995; Fan et al., 1996). In fact, many analogs have been shown to exert selective effects *in vivo* with minimal effects on mineral metabolism. The mechanisms for this selectivity are currently under investigation, but accumulating evidence indicates that multiple factors may be responsible, including altered clearance rates, cell-specific metabolism with the formation of active metabolites, different nongenomic activities, and induction of altered conformations of the nuclear VDR. Further studies are required to explain the apparent selectivity of 1α,23(*S*),25$(OH)_3$-24-oxovitamin D_3. However, our present study for the first time emphasizes the importance of understanding the various as yet unidentified biological roles of the intermediary metabolites of 1α,-25$(OH)_2D_3$ formed in target tissues in the process of its final inactivation.

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REFERENCES

Abe, E., Miyaura, C., & Sakagami, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4990.
Akiyoshi-Shibata, M., Sakaki, Y., Ohyama, Y., Noshiro, M., Okuda, K., & Yabusaki, Y (1994) *Eur. J. Biochem. 224*, 335.
Bikle, D. D. (1992) *Endocr. Rev. 13* (4), 765.
Bikle, D. D., & Gee, E. (1989) *Endocrinology 124*, 649.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.
Bouillon, R., Okamura, W. H., & Norman, A. W. (1995) *Endocr. Rev. 16*, 200.
Bourdeau, A., Almani, F., Grosse, B., & Lieberherr, M. (1990) *Endocrinology 127*, 2738.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Brown, A. J., Ritter, C. S., Finch, J. L., Morrissey, J., Martin, K. J., Murayama, E., Nishii, Y., & Slatopolsky, E. (1989) *J. Clin. Invest. 84*, 728.
Brown, A. J., Berkoben, M., Ritter, C. S., & Slatopolsky, E. (1992) *Endocrinology 130*, 276.
Brumbaugh, P. F., & Haussler, M. R. (1975) *Life Sci. 16*, 353.
Canterbury, J. M., Lerman, S., Claflin, A. J., & Henry, H. (1978) *J. Clin. Invest. 61*, 1375.
Craig, A. S., Norman, A. W., & Okamura, W. H. (1992) *J. Org. Chem. 57*, 4374.
Crisp, G. T., & Scott, W. J. (1985) *Synthesis*, 335.
Dai, H., & Posner, G. H. (1994) *Synthesis 12*, 1383.
Danishefsky, S., Schuda, P. F., Kitahara, T., & Etheredge, S. J. (1977) *J. Am. Chem. Soc. 99*, 6067.
DeLuca, H. F., Krisinger, J., & Darwish, H. (1990) *Kidney Int. 38*, 52.
Desmaele, D., & Tanier, S. (1985) *Tetrahedron Lett. 26*, 4941.
Dusso, A., Negrea, L., Gunawardhana, S., Lopez-Hilker, S., Finch, J., Mori, T., Nishii, Y., Slatopolsky, E., & Brown, A. J. (1991) *Endocrinology 128*, 1687.
Fan, S., Schroeder, N. J., Burrin, J. M., Calverley, M. J., Makin, H. L. J., & Cunningham, J. (1996) *J. Am. Soc. Nephrol. 7*, 1790 (abstract 2715).
Grieco, P. A., Marinovic, N., & Miyashita, M. (1975) *J. Org. Chem. 40*, 1670.
Grieco, P. A., Oguri, T., & Gilman, S. (1980) *J. Am. Chem. Soc. 102*, 5886.
Ishizuka, S., & Norman, A. W. (1987) *J. Biol. Chem. 262*, 7165.
Kallmerten, J., & Gould, T. J. (1985) *J. Chem. Soc. 50*, 1128.
Kametani, T., & Furuyama, H. (1987) *Med. Res. Rev. 7*, 147−171.
Kim, H.-J., Abdelkader, N., Katz, M., & McLane, J. A. (1992) *J. Cell. Physiol. 151*, 579.
Lythgoe, B. (1981) *Chem. Soc. Rev. 10*, 449.
Makin, G., Lohnes, D., Byford, V., Ray, R., & Jones, G. (1989) *Biochem. J. 262*, 173.
Mancuso, A. J., Huang, S. L., & Swern, D. (1978) *J. Org. Chem. 43*, 2480.
Mayer, E., Bishop, J. E., Ohnuma, N., & Norman, A. W. (1983) *Arch. Biochem. Biophys. 224*, 671.
McMurry, J. E., & Scott, W. J. (1983) *Tetrahedron Lett. 24*, 979.
Nemere, I., Dormanen, M. C., Hammond, M. W., Okamura, W. H., & Norman, A. W. (1994) *J. Biol. Chem. 269*, 23750.
Norman, A. W., Zhou, J. Y., Uskokovic, M., & Koeffler, P. H. (1990) *Cancer Res. 50*, 6857.
Norman, A. W., Henry, H. L., Okamura, W. H., Nordin, B. E., Lemire, J. M., deBoland, A. R., Lian, J. B., Kragballe, K., & Suda, T. (1992) *J. Cell. Biochem. 49*, 1−58.
Norman, A. W., Okamura, W. H., Farach-Carson, M. C., Allewart, K., Branisteanu, D., Nemere, I., Muralidharan, K. R., & Bouillon, R. (1993a) *J. Biol. Chem. 268*, 13811.
Norman, A. W., Bouillon, R., Farach-Carson, M. C., Bishop, J. E., Zhou, L. X., Nemoto, I., Zhao, J., Muralidharan, & Okamura, W. H. (1993b) *J. Biol. Chem. 268*, 20022.
Okuda, K.-I., Usui, E., & Ohyama, Y. (1995) *J. Lipid Res. 36*, 1641.
Peleg, S., Sastry, M., Collins, E. D., Bishop, J. E., & Norman, A. W. (1995) *J. Biol. Chem. 270*, 10551.
Peleg, S., Liu, Y. Y., Reddy, S., Horst, R. L., White, M. C., & Posner, G. H. (1996) *J. Cell Biochem. 63*, 149.
Perlman, K., Kutner, A., Prahl, J., Smith, C., Inaba, M., Schnoes, H. K., & DeLuca, H. F. (1990) *Biochemistry 29*, 190.
Perlman, K. L., Swenson, R. E., Paaren, H. E., Schnoes, H. K., & DeLuca, H. F. (1991) *Tetrahedron Lett. 32*, 7663.
Perrin, D. D., Armarego, W. L. F., & Perrin, D. R. (1980) in *Purification of Laboratory Chemicals*, Oxford Pergamon Press, Elmsford, NY.
Petragnani, N., Ferraz, H. M. C., & Silva, G. V. J. (1986) *Synthesis*, 157.
Reddy, G. S., & Tserng, K. Y. (1989) *Biochemistry 28*, 1763.
Reddy, G. S., Tserng, K. Y., Thomas, B. R., Dayal, R., & Norman, A. W. (1987) *Biochemistry 26*, 324.
Rigby, W. (1988) *Immunol. Today 9*, 54.
Scott, W. J., Crisp, G. T., & Stille, J. K. (1984) *J. Am. Chem. Soc. 106*, 4630.
Seino, Y., & Ishizuka, S. (1991) in *Vitamin D gene regulation, structure-function analysis and clinical application* (Norman, A. W., Bouillon, R., & Thomasset, M., Eds.) pp 565−571, de Gruyter, New York.
Slatopolsky, E., Finch, J., Ritter, C., Denda, M., Morrissey, J., Brown, A. J., & DeLuca, H. F. (1995) *Am. J. Kidney Dis. 26*, 852.
Stork, G., & d'Angelo, J. (1974) *J. Am. Chem. Soc. 96*, 7114.
Sugimoto, T., Ritter, C., Ried, I., Morrissey, J., & Slatopolsky, E. (1988) *Kidney Int. 33*, 850.
Takayama, H., Yamada, S., Yamamoto, K., Ino, E., Sakaida, K., Shinki, T., Suda, T., Iitaka, Y., & Itai, A. (1989) *Biochemistry 28*, 4551.
Tsoukas, C., Provvedini, D., & Manolagas, S. (1984) *Science 224*, 1438.
Wilson, S. R., & Yasmin, A. (1992) *Stud. Nat. Prod. Chem. 10*, 43−75.
Yamada, S., Ohmori, M., Takayama, H., Takasaki, Y., & Suda, T. (1983) *J. Biol. Chem. 258*, 457.
Zhou, J. Y., Norman, A. W., Akashi, M., Chen, D. L., Uskokovic, M. R., Aurrecoechea, J. M., Dauben, W. G., Okamura, W. H., & Koeffler, H. P. (1991) *Blood 78*, 75.

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