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Articles

Synthesis and Determination of the Stereochemistry of 23,25-Dihydroxy-24-oxovitamin D_3, a Major Metabolite of 24(*R*),25-Dihydroxyvitamin D_3

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ABSTRACT: Two C(23) epimers of 23,25-dihydroxy-24-oxovitamin D_3 (**1**), a major metabolite of 24(*R*),-25-dihydroxyvitamin D_3, were chemically synthesized for the first time and their stereochemistries at C(23) are determined on the basis of X-ray analysis. The C(23) stereochemistry of the natural 23,25-dihydroxy-24-oxovitamin D_3 was determined to be *S* by comparing the spectral properties and HPLC behavior of the two synthetic isomers with those of the natural metabolite.

24(*R*),25-Dihydroxyvitamin D_3 [24*R*,25-$(OH)_2D_3$][1] (DeLuca & Schnoes, 1976, 1983) is a major metabolite of vitamin D_3 circulating at a concentration nearly 100 times higher than the metabolically active form (Shepard et al., 1979), 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$]. However, the biological importance of this metabolite is still controversial. It has been claimed that 24*R*,25-$(OH)_2D_3$ has a number of in vivo actions such as stimulation of skeletal mineralization (Ornoy et al., 1978; Endo et al., 1980; Malluche et al., 1980; Lidor et al., 1987), regulation of secretion of parathyroid

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[1] Abbreviations: 24*R*,25-$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 23,25-$(OH)_2$-24-oxo-D_3, 23,25-dihydroxy-24-oxovitamin D_3; UV, ultraviolet; IR, infrared; ^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon-13 nuclear magnetic resonance; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; 25-OH-24-oxo-D_3, 25-hydroxy-24-oxovitamin D_3; HMPA, hexamethylphosphoramide; MCPBA, *m*-chloroperbenzoic acid; DMSO, dimethyl sulfoxide; 23*S*,25-$(OH)_2D_3$, 23(*S*),25-dihydroxyvitamin D_3; 25*R*-$(OH)D_3$ 26,23*S*-lactone, 25(*R*)-hydroxyvitamin D_3 26,23*S*-lactone; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3.

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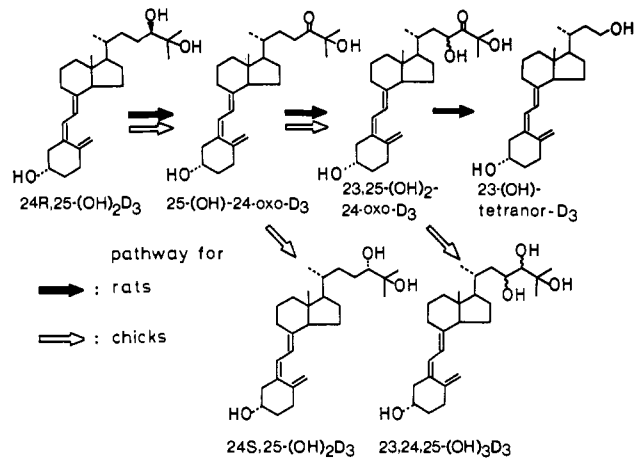
Scheme I: Metabolic Pathway of 24(*R*),25-Dihydroxyvitamin D_3



hormone (Canterbury et al., 1978), promotion of normal hatchability of chicken eggs (Henry & Norman, 1978), stimulation of creatine kinase BB activity (Sömjen et al., 1984), and stimulation of chondroitin sulfate synthesis in cultured bone cells (Corvol et al., 1978). On the other hand, studies using 24,24-difluoro-25-hydroxyvitamin D_3 (Yamada et al., 1979), an analogue unable to be 24-hydroxylated, demonstrated that the compound produced normal growth, reproduction, and skeletal mineralization in rats for two generations (Jarnagin et al., 1983; Brommage et al., 1983). To help clarify the biological role of 24*R*,25-$(OH)_2D_3$, we have been investigating its further metabolism and found the metabolic pathway shown in Scheme I in in vitro studies (Takasaki et al., 1981; Yamada et al., 1983, 1985; Jones et al., 1984; Mayer et al., 1983a). Subsequently, 1α,25-dihydroxyvitamin D_3 was shown to be metabolized at the side chain in a completely analogous manner (Mayer et al., 1983b; Reddy et al., 1987). 23,25-Dihydroxy-24-oxovitamin D_3 [23,25-$(OH)_2$-24-oxo-D_3] is the major metabolite of 24*R*,25-$(OH)_2D_3$ produced by the kidney from both birds (Yamada et al., 1983, 1985) and mammals (Mayer et al., 1983a; Yamada et al., 1985). We synthesized, for the first time, the two C(23) epimers of 23,25-$(OH)_2$-24-oxo-D_3 (**1**), determined their stereochemistry at C(23) on the basis of X-ray analysis, and determined the C(23) stereochemistry of the natural metabolite by directly comparing the two synthetic epimers with the natural metabolite on high-performance liquid chromatography (HPLC). We here report the results in detail.

Materials and Methods

General. Ultraviolet (UV) spectra were recorded on a Hitachi 200-10 double-beam spectrophotometer in 95% ethanol solution. Infrared (IR) spectra were measured on a Hitachi 215 grating infrared spectrometer. Proton and carbon-13 nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were recorded with a JEOL GSX-400 or a Varian XL100 spectrometer in $CDCl_3$; chemical shifts are reported in parts per million downfield from tetramethylsilane, and coupling constants are reported in hertz. Mass spectra (MS) were measured on a JEOL JMS-D300 GC MS instrument, and relative intensities are given in parentheses. High-performance liquid chromoatography (HPLC) was carried out with a Jasco TWINCLE equipped with a Jasco UVIDEC-610 spectrophotometer. All solvents were distilled and/or dried prior to use by using standard methods. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Air-sensitive reaction were run under argon, and reagents were added via oven-dried syringes through septa. 25-Hydroxy-24-oxovitamin D_3 (25-OH-24-oxo-D_3) was synthesized in our laboratory according to our method (Yamada et al., 1981a; Takasaki et al., 1982).

Chemical Synthesis of 23,25-Dihydroxy-24-oxovitamin D_3. 5,7,23-Cholestatriene-3β,24,25-triol Tris(tert-butyldimethylsilyl) Ether (**3**). Lithium diisopropylamide was prepared from diisopropylamine (735 μL, 5.25 mmol) and *n*-BuLi (1.5 M hexane solution, 2.6 mL, 3.9 mmol) in THF (500 μL) at −20 °C, and to the solution was added the ketone **2** (347 mg, 0.66 mmol) in THF (500 μL). After 5 min, *tert*-butyldimethylsilyl chloride (989 mg, 6.6 mmol) in THF (500 μL) and hexamethylphosphoramide (HMPA) (2 mL, 11.5 mmol) were added, and the mixture was stirred for 1 h at −20 °C. The reaction was quenched by adding water and extracted with ethyl acetate; the extracts were washed with brine, dried over Na_2SO_4, and evaporated in vacuo. The residue was chromatographed on silica gel (30 g) with 5% ethyl acetate in hexane as the eluent to give the title compound (**3**) as a colorless glass (412 mg, 83%): ^1H NMR (400 MHz) δ 0.066 and 0.10 (each 6 H, s, $SiCH_3$), 0.164 and 0.168 (each 3 H, s, $SiCH_3$), 0.62 (3 H, s, 18-H), 0.89, 0.90, and 0.96 (each 9 H, s, $SiBu^t$), 3.59 (1 H, m, 3-H), 4.98 (1 H, t, J = 6.7, 23-H), 5.38 (1 H, m, 7-H), 5.55 (1 H, d, J = 5.4, 6-H); ^{13}C NMR (100.64 MHz) δ 38.6 (C-1), 32.0 (C-2), 71.3 (C-3), 41.4 (C-4), 141.2 (C-5), 119.3 (C-6), 116.3 (C-7), 140.7 (C-8), 46.4 (C-9), 37.1 (C-10), 21.2 (C-11), 28.0 (C-12), 43.0 (C-13), 54.5 (C-14), 23.1 (C-15), 39.1 (C-16), 55.3 (C-17), 11.9 (C-18), 16.4 (C-19), 36.3 (C-20), 19.0 (C-21), 32.4 (C-22), 101.4 (C-23), 156.1 (C-24), 75.2 (C-25), 29.4 (C-26), 29.5 (C-27), 25.7, 25.96, 26.04, 26.2, 26.3, and 26.4 [SiC(CH_3)$_3$], 18.3 [SiC(CH_3)$_3$]; MS, m/z 756 (M^+, 40), 699 (23), 625 (19), 609 (21).

4-Phenyl-1,2,4-triazoline-3,5-dione Adduct of 5,7,23-Cholestatriene-3β,24,25-triol Tris(tert-butyldimethylsilyl) Ether (**4**). To a solution of compound **3** (30 mg, 40 μmol) in $CHCl_3$ (5 mL) was added 4-phenyl-1,2,4-triazoline-3,5-dione (7 mg, 40 μmol) at room temperature. After 5 min, the solvent was evaporated and the residue was chromatographed on silica gel (5 g) with 5% ethyl acetate in hexane as the eluent to give the title compound (**4**) as a colorless glass (32 mg, 87%): IR (KBr) 2925, 2855, 1755, 1700 cm^{-1}; ^1H NMR (400 MHz) δ 0.085, 0.096, 0.100, and 0.106 (each 3 H, s, $SiCH_3$), 0.145 [6 H, s, $Si(CH_3)_2$], 0.80 (3 H, s, 18-H), 0.89, 0.90, and 0.94 (each 9 H, s, $SiBu^t$), 1.31 and 1.32 (each 3 H, s, 26- and 27-H), 4.40 (1 H, m, 3-H), 4.93 (1 H, dd, J = 7.5 and 5.7, 23-H), 6.20 (1 H, d, J = 8.3, 7-H), 6.38 (1 H, d, J = 8.3, 6-H), 7.28–7.47 (5 H, m, phenyl); MS, m/z 756 (M^+ − triazolinedione, 29), 699 (21), 625 (18), 609 (21).

5α,8α-([1,2]-4-Phenyl-3,5-dioxo-1,2,4-triazolino)-3β,23ξ,25-trihydroxy-6-cholesten-24-one Tris(tert-butyldimethylsilyl) Ether (**5**). To a solution of **4** (100 mg, 0.11 mmol) in dichloromethane (6 mL) was added 80% *m*-chloroperbenzoic acid (MCPBA) (23 mg, 0.11 mmol) at 0 °C, and the mixture was stirred at that temperature for 1 h. The reaction was diluted with dichloromethane, washed with saturated $NaHCO_3$ solution and water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on silica gel (20 g) with 1.5% ethyl acetate in benzene as the eluent to give **5a** (colorless glass, 22 mg, 22%) and **5b** (colorless glass, 61 mg, 60%) in this order. **5a**: ^1H NMR (100 MHz) δ 0.82 (3 H, s, 18-H), 0.90, 0.92, and 0.94 (each 9 H, s, $SiBu^t$), 1.40 (6 H, s, 26- and 27-H), 4.45 (1 H, m, 3-H), 5.14 (1 H, dd, J = 8.0 and 4.0, 23-H), 6.24 and 6.40 (each 1 H, d, J = 8, 6- and 7-H), 7.30–7.56 (5 H, m, phenyl); MS, m/z 770 (M^+ − triazolinedione − 2, 6), 657 (3), 638 (3), 571 (2), 523 (3),

451 (3), 437 (16). **5b**: ^{1}H NMR (100 MHz) δ 0.81 (3 H, s, 18-H), 0.90, 0.91, and 0.94 (each 9 H, s, SiBu^t), 1.40 and 1.43 (each 3 H, s, 26- and 27-H), 4.43 (1 H, m, 3-H), 5.11 (1 H, t, J = 6.5, 23-H), 6.23 and 6.40 (each 1 H, d, J = 8.0, 6- and 7-H), 7.33–7.52 (5 H, m, phenyl); MS, m/z 770 (M^+ − triazolinedione − 2, 2), 657 (1), 638 (1), 571 (1), 523 (1), 451 (1), 437 (5).

3β,23(S),25-Trihydroxy-5,7-cholestadiene-24-one (**6a**). Anhydrous K_2CO_3 (250 mg, 1.81 mmol) was added to a solution of **5a** (125 mg, 0.13 mmol) in dimethyl sulfoxide (DMSO) (5 mL) and the suspension was stirred at 125 °C under argon for 2.5 h. After cooling, the reaction was diluted with ethyl acetate, washed with water, dried, and evaporated. The residue was chromatographed on silica gel (10 g, 1.5% ethyl acetate in benzene) to give tris(*tert*-butyldimethylsilyl) ether of the provitamin D (**6**). The silyl ether was dissolved in THF (2 mL), and 0.77 M THF solution of *n*-Bu_4NF (405 μL, 0.31 mmol) was added at 0 °C. The mixture was allowed to stir at room temperature for 3.5 h. A saturated ammonium chloride solution was added to the reaction, the mixture was extracted with ethyl acetate, and the extracts were washed with water, dried, and evaporated. The residue was chromatographed on silica gel (7 g) with 5% methanol in dichloromethane as the eluent to give **6a** as a colorless glass (28 mg, 49%): UV λ_{max} 262 (sh), 271, 281.5, 293 nm; IR (KBr) 3350, 2920, 2845, 1700 cm^{-1}; ^{1}H NMR (100 MHz) δ 0.65 (3 H, s, 18-H), 0.96 (3 H, s, 19-H), 1.12 (3 H, d, J = 6, 21-H), 1.42 and 1.44 (each 3 H, s, 26- and 27-H), 3.65 (1 H, m, 3-H), 4.67 (1 H, m, 23-H), 5.42 and 5.59 (each 1 H, m, 6- and 7-H); MS, m/z 430 (M^+, 26), 412 (9), 397 (26), 372 (89), 339 (100), 313 (56).

3β,23(R),25-Trihydroxy-5,7-cholestadien-24-one (**6b**). The major isomer (**5b**) (282 mg, 0.298 mmol) was deprotected following the procedure described above to give **6b** as colorless crystals (69 mg, 54%): mp 194–196 °C (acetone–benzene); UV λ_{max} 262 (sh), 271.5, 282, 293.5 nm; IR (KBr) 3325, 2930, 2865, 1718 cm^{-1}; ^{1}H NMR (100 MHz) δ 0.67 (3 H, s, 18-H), 0.96 (3 H, s, 19-H), 1.09 (3 H, d, J = 6, 21-H), 1.42 and 1.44 (each 3 H, s, 26- and 27-H), 3.65 (1 H, m, 3-H), 4.72 (1 H, m, 23-H), 5.42 and 5.59 (each 1 H, m, 6- and 7-H); MS, m/z 430 (M^+, 3), 412 (4), 397 (3), 372 (98), 339 (100), 313 (70). Anal. Calcd for $C_{27}H_{42}O_4$: C, 75.31; H, 9.83. Found: C, 74.88; H, 9.56.

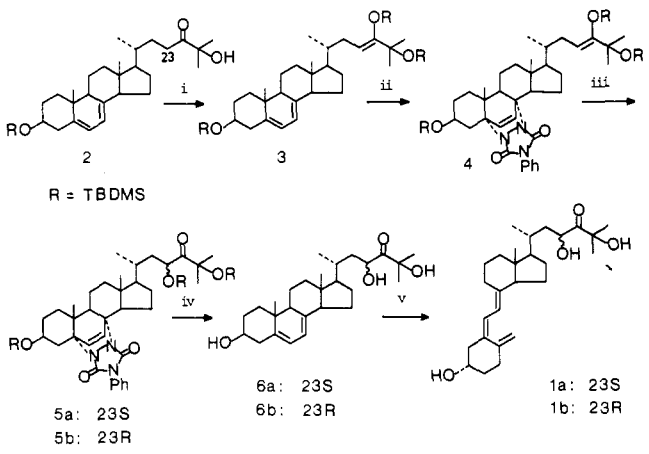
23(S),25-Dihydroxy-24-oxovitamin D_3 (**1a**). A solution of **6a** (9 mg, 21 mol) in ether (200 mL) was purged with argon for 30 min and irradiated with a high-pressure mercury lamp (200 W, Shigemi, Tokyo) in an immersion apparatus equipped with a Vycor filter for 5 min at 0 °C. The solvent was evaporated, and the residue was chromatographed on Sephadex LH-20 (20 g) with $CHCl_3$–hexane (65:35) as the eluent to give the previtamin D (**7a**) (3.6 mg, 40%), 23(*S*),25-dihydroxy-24-oxotachysterol$_3$ (**8a**) (2.8 mg, 31%), and **6a** (1.18 mg, 13%). The previtamin D (**7a**) was dissolved in ethanol (14 mL) and allowed to stand at room temperature in the dark for 14 days. The solvent was evaporated, and the residue was purified on a Sephadex LH-20 column [20 g, $CHCl_3$–hexane (65:35)] to give the title compound (**1a**) (3.2 mg, 89%): UV λ_{max} 265 nm, λ_{min} 228 nm; IR ($CHCl_3$) 3500, 2930, 2860, 1708 cm^{-1}; ^{1}H NMR (100 MHz) δ 0.57 (3 H, s, 18-H), 1.09 (3 H, d, J = 6, 21-H), 1.42 and 1.44 (each 3 H, s, 26- and 27-H), 3.96 (1 H, m, 3-H), 4.66 (1 H, m, 23-H), 4.84 (1 H, bs, 19*E*-H), 5.07 (1 H, b s, 19*Z*-H), 6.04 (1 H, d, J = 11.5, 7-H), 6.24 (1 H, d, J = 11.5, 6-H); MS, m/z 430 (M^+, 13), 412 (6), 397 (6), 372 (47), 339 (29), 253 (22), 136 (100), 118 (86). High-resolution mass calcd for $C_{27}H_{42}O_4$: 430.3082. Found: 430.3086. **8a**: UV λ_{max} 271 (sh), 280, 290 (sh) nm; ^{1}H NMR (100 MHz) δ 0.72 (3 H, s, 18-H), 1.14 (3 H, d, J = 6, 21-H), 1.43 and 1.45 (each 3 H, s, 26- and 27-H), 1.80 (3 H, s, 19-H), 3.99 (1 H, m, 3-H), 4.68 (1 H, m, 23-H), 5.71 (1 H, m, 9-H), 6.02 and 6.70 (each 1 H, d, J = 16, 6- and 7-H); MS, m/z 430 (M^+, 25), 412 (7), 397 (12), 372 (100), 339 (62), 253 (32).

23(R),25-Dihydroxy-24-oxovitamin D_3 (**1b**). The isomeric provitamin D (**6b**) (10.4 mg, 24 μmol) was converted to the corresponding vitamin D (**1b**) as a colorless glass (3.1 mg, 30% overall) by a similar method: UV λ_{max} 265 nm, λ_{min} 228 nm; IR ($CHCl_3$) 3500, 2930, 2860, 1705 cm^{-1}; ^{1}H NMR (100 MHz) δ 0.59 (3 H, s, 18-H), 1.07 (3 H, d, J = 6, 21-H), 1.42 and 1.44 (each 3 H, s, 26- and 27-H), 3.96 (1 H, m, 3-H), 4.72 (1 H, m, 23-H), 4.84 (1 H, b s, 19*E*-H), 5.07 (1 H, b s, 19*Z*-H), 6.04 (1 H, d, J = 11.5, 7-H), 6.24 (1 H, d, J = 11.5, 6-H); MS, m/z 430 (M^+, 5), 412 (1), 397 (3), 372 (13), 339 (12), 253 (12), 136 (78), 118 (100). High-resolution mass calcd for $C_{27}H_{42}O_4$: 430.3082. Found: 430.3079. 23(*R*),25-Dihydroxy-24-oxotachysterol$_3$ (**8b**): UV λ_{max} 271 (sh), 280, 290 (sh) nm; ^{1}H NMR (100 MHz) δ 0.67 (3 H, s, 18-H), 1.04 (3 H, d, J = 6, 21-H), 1.36 and 1.38 (each 3 H, s, 26- and 27-H), 1.72 (3 H, s, 19-H), 3.92 (1 H, m, 3-H), 4.68 (1 H, m, 23-H), 5.67 (1 H, m, 9-H), 5.96 and 6.68 (each 1 H, d, J = 16, 6- and 7-H); MS, m/z 430 (M^+, 57), 412 (10), 397 (22), 372 (100), 339 (75), 253 (31).

Crystal Structure of Compound **6b**. Crystal belongs to a monoclinic space group $P2_1$ with unit cell parameters of a = 12.258(4), b = 31.868(8), c = 6.360(2) Å and β = 92.06(2)° including two molecules in an asymmetric unit. Intensity data were collected on a Philips PW1100 automated four-circle diffractometer using Cu Kα radiation monochromated by a graphite plate. A total of 3784 nonzero, independent reflections within $3 < \theta < 78$ were measured by the θ–2θ scan method. The structure was solved by the direct method using the MULTAN program (Main et al., 1980). Hydrogen atoms were located by several cycles of difference Fourier syntheses. The structure was refined by block-diagonal least-squares calculations assuming anisotropic thermal parameters for non-hydrogen atoms and isotropic ones for hydrogen atoms. The final R factor was 5.6%. The atomic scattering factors for carbon and oxygen were taken from International Tables for X-ray Crystallography, and those for hydrogen atoms were taken from Stewart et al. (1965).

In Vitro Production of 23,25-(OH)$_2$-24-oxo-D_3. (A) Production by Kidney Homogenates from Vitamin D Supplemented Chicks. A total of 15 1-day-old White Leghorn cockerel chicks were maintained for 4 weeks on a vitamin D deficient diet containing 1% calcium and 0.45% phosphorus and dosed orally with 65 nmol of vitamin D_3. Forty-eight hours after the dosage, the animals were killed by decapitation, and the kidneys were quickly removed, rinsed, minced with a garlic press, and homogenized in 4 volumes of 0.2 M sucrose containing 15 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 5 mM sodium succinate. The homogenates (85 mL) were incubated with 81 μg of 25-OH-24-oxo-D_3 at 37 °C for 30 min under 100% oxygen. The reaction was terminated by adding 318 mL of methanol–chloroform (2:1). Extraction was performed as described by Gray et al. (1972). The concentrated lipid extracts were chromatographed on a column (1 × 30 cm) packed with 10 g of Sephadex LH-20. The column was eluted with chloroform–hexane (3:1). The fraction eluted between 35 and 90 mL was pooled and purified on straight-phase HPLC [Finepak SIL, 0.46 × 25 cm, hexane–dichloromethane–methanol (8:1:0.5)]. The fraction eluted between

Scheme II: Synthesis of 23,25-Dihydroxy-24-oxovitamin D_3[a]



[a] Reagents: (i) LDA, THF, −20 °C, then *tert*-butyldimethylsilyl chloride, THF–HMPA, 83%. (ii) 4-phenyl-1,2,4-triazoline-3,5-dione, $CHCl_3$, 83%. (iii) MCPBA, CH_2Cl_2, 82% (total). (iv) (a) K_2CO_3, DMSO, 125 °C; (b) *n*-Bu_4NF, THF, 49–54%. (v) (a) High-pressure Hg lamp, ether; (b) room temperature, EtOH, 14 days, 30–36%.

18 and 19.5 mL from the HPLC column was pooled and purified on reverse-phase HPLC [Finepak SIL C_{18}, 0.46 × 25 cm, H_2O–methanol (1:4)]. The fraction eluted between 10 and 11.5 mL from the reverse-phase column was further purified on straight-phase HPLC [Finepak SIL, 0.46 × 25 cm, 2-propanol–hexane (15:85)] to give a homogeneous metabolite (1.6 μg, determined on the basis of OD at 265 nm in the UV spectrum). The metabolite was identified as 23*S*,25-$(OH)_2$-24-oxo-D_3 by comparing the UV and mass spectra as well as the HPLC retention volume with those of (23*S*)- and (23*R*)-23,25-$(OH)_2$-24-oxo-D_3 synthesized chemically.

(*B*) *Production by Kidney Homogenates from Vitamin D Supplemented Rats.* A total of seven 6-week-old rats (Sprague-Dawley strain) were dosed orally with 100 nmol of vitamin D_3. Forty-eight hours after the dosage, the rats were anesthetized with ether and exsanguinated from the aorta with a syringe. The animals were then perfused with 50 mL of calcium- and magnesium-free phosphate-buffered saline. The color of the kidneys changed from dark violet to pale pink. The kidneys were removed and placed in an ice-cold 15 mM Tris–acetate buffer (pH 7.4) containing 0.19 M sucrose, 2 mM magnesium acetate, and 25 mM sodium succinate. A 10% (w/v) homogenate was prepared in the Tris–acetate buffer. The homogenates (96 mL) were incubated with 40 μg of 25-OH-24-oxo-D_3 at 37 °C for 30 min under 100% oxygen. The reaction was terminated by adding 288 mL of methanol–chloroform (2:1). The metabolites were extracted and purified as described above to give a homogeneous metabolite (4.5 μg), which was identified as 23*S*,25-$(OH)_2$-24-oxo-D_3 on the basis of spectral data (UV and mass) and the behavior on HPLC.

Results

23,25-$(OH)_2$-24-oxo-D_3 (**1**) was synthesized in seven steps starting with 25-hydroxy-24-oxo-7-dehydrocholesterol (**2**) (Scheme II), which was readily prepared from C(22)-steroid sulfone and 3,4-epoxy-2-methyl-2-butanol as previously described (Takayama et al., 1980; Yamada et al., 1981a; Takasaki et al., 1982). The required hydroxyl group at C(23) was introduced via oxidation of the enol silyl ether of the starting ketone (**2**). The ketone (**2**) was treated with lithium diisopropylamide in THF and then with *tert*-butyldimethylsilyl chloride in the presence of hexamethylphosphoramide to give a single silyl enol ether (**3**) in good yield (83%). The sterically less demanded *Z* isomer was assigned to the enolate **3**. The endocyclic diene part of the silyl enol ether (**3**) was protected as a *N*-phenyltriazoline dione adduct, and the adduct (**4**) was oxidized with MCPBA. The oxidation gave a 2:7 mixture of the two 23-hydroxylated epimers (**5a** and **5b**), which were readily separated by simple column chromatography. Deprotection of the diene function followed by removal of the hydroxyl protecting groups gave two C(23) epimers of the desired provitamin D, **6a** and **6b**. Reversal of the deprotections gave unsatisfactory results because of the lability of the α,α′-dihydroxy ketone function under the conditions (K_2CO_3, DMSO, 125 °C) of the removal of the triazoline group.

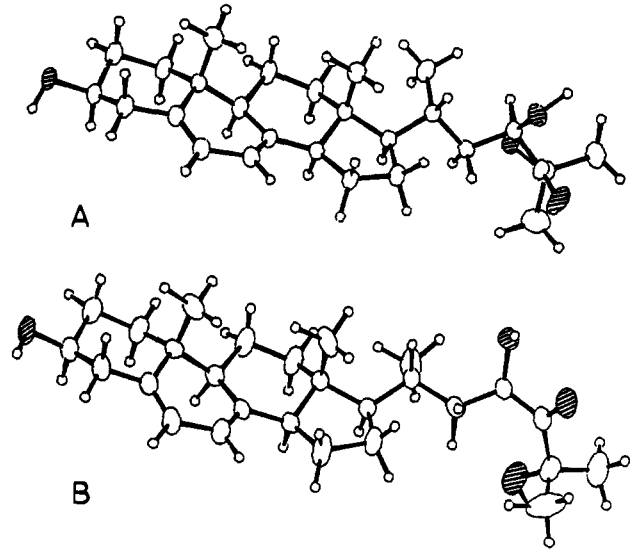


FIGURE 1: Molecular structures of compound **6b**. The two molecules in an asymmetric unit, A and B, are depicted independently by ORTEP drawing to take the same orientation about the steroidal ring skeleton. (Thermal ellipsoids for non-hydrogen atoms are drawn at the 30% probability level, and the spheres for hydrogen atoms are set at an arbitrary small scale.)

Single-crystal X-ray analysis was carried out by using the major isomer (**6b**). The crystal belongs to a monoclinic space group $P2_1$ with unit cell parameters of a = 12.258(4), b = 31.868(8), c = 6.360(2) Å and β = 92.06(2)°, including two molecules, A and B, in an asymmetric unit. The final *R* factor was 5.6%. Figure 1 shows the molecular structures of A and B by an ORTEP drawing (Johnson, 1971). The stereochemistry at C(23) was clearly determined to be *R*. The main structural difference between molecules A and B is in the side-chain conformation. Each molecule in the crystal is linked to the neighboring molecules by three intermolecular hydrogen bonds, which are formed among four oxygenic functional groups in the molecule, hydroxyls at C(3), C(23), and C(25) and carbonyl at C(24), whereas there is one intramolecular hydrogen bond between the hydroxyl groups at C(23) and C(25).

Thus, the stereochemistries at C(23) of **6a** and **6b** were determined to be *S* and *R*, respectively. The two isomeric provitamins, **6a** and **6b**, were converted to the corresponding vitamin D's, (23*S*)- and (23*R*)-23,25-$(OH)_2$-24-oxo-D_3 (**1a** and **1b**), by photolysis followed by thermal isomerization. The spectral properties (UV, MS, and ^1H NMR) of 23*S*,25-$(OH)_2$-24-oxo-D_3 (**1a**) were identical in all respects with those of the natural product (Yamada et al., 1983; Mayer et al., 1983a).

The (23*S*)- and (23*R*)-23,25-$(OH)_2$-24-oxo-D_3 (**1a** and **1b**) thus synthesized were compared directly on HPLC with metabolites produced by incubating 25-OH-24-oxo-D_3 (Takasaki et al., 1982; Yamada et al., 1983, 1985) with kidney homogenates from both chicks and rats. The natural metabolite

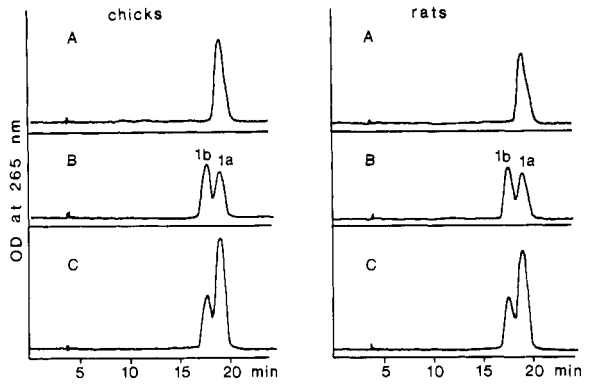


FIGURE 2: Cochromatography on HPLC of (23*S*)- and (23*R*)-23,25-$(OH)_2$-24-oxo-D_3 (**1a** and **1b**) chemically synthesized and the natural metabolites, which were prepared by incubating 25-OH-24-oxo-D_3 with kidney homogenates from vitamin D supplemented chicks and rats. The left panels show the HPLC profiles with chick metabolite, and the right panels show those with rat metabolite. (A) Natural metabolite; (B) two synthetic epimers; (C) mixture of the synthetic epimers and the natural metabolite. HPLC conditions: Finepak SIL C_{18}, 25% water in methanol, 1 mL/min.

obtained from either chicks or rats comigrated with the synthetic 23*S* isomer as shown in Figure 2. Thus, the stereochemistry at C(23) of the natural 23,25-$(OH)_2$-24-oxo-D_3 was determined to be *S* irrespective of the animals producing it.

Discussion

The stereochemistry at C(23) of 23,25-$(OH)_2$-24-oxo-D_3 was proved to be *S*, the same as that of other 23-hydroxylated metabolites such as 23(*S*),25-dihydroxyvitamin D_3 [23*S*,25-$(OH)_2D_3$] (Tanaka et al., 1981; Ikekawa et al., 1981), its further metabolites such as 25(*R*)-hydroxyvitamin D_3 26,23*S*-lactone [25*R*-$(OH)D_3$ 26,23*S*-lactone] (Wichmann et al., 1979; Yamada et al., 1981b, 1984), and their 1α-hydroxylated analogues (Ishizuka et al., 1985). Three hydroxylation pathways are known, 23-, 24-, and 26-hydroxylation, other than the initial 25-hydroxylation in vitamin D side-chain metabolism (DeLuca et al., 1976, 1983). 24-Hydroxylation always takes place at the *pro-R* position, while 26-hydroxylation occurs at both heterotopic methyl groups at C(25). In the biosynthesis of 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$], both *pro-S* and *-R* methyl groups (26- and 27-methyl groups) of 25-$(OH)D_3$ are hydroxylated to give (25*S*)- and (25*R*)-25,26-$(OH)_2D_3$ (Ikekawa et al., 1983), but in the biosynthesis of 25*R*-$(OH)D_3$ 26,23*S*-lactone and its 1α-hydroxylated analogue, 26-hydroxylation occurs only at the *pro-R* methyl group of 23*S*,25-$(OH)_2D_3$. It is now clear that 23-hydroxylation takes place exclusively at the *pro-S* position regardless of the substrate.

Supplementary Material Available

Tables of bond angles, bond lengths, atomic coordinates, temperature factors for non-hydrogen atoms, and F_o–F_c values (16 pages). Ordering information is given on any current masthead page.

Registry No. **1a**, 104758-88-5; **1b**, 104714-41-2; **2**, 119748-19-5; **3**, 119769-54-9; **4**, 119748-20-8; **5a**, 119748-21-9; **5b**, 119816-29-4; **6**, 119748-27-5; **6a**, 119748-22-0; **6b**, 119748-25-3; **7a**, 119748-23-1; **8a**, 119748-24-2; **8b**, 119748-26-4; *tert*-butyldimethylsilyl chloride, 18162-48-6; 4-phenyl-1,2,4-triazoline-3,5-dione, 4233-33-4; 24(*R*),25-dihydroxyvitamin D_3, 55721-11-4.

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Radiation Inactivation Experiments Predict That a Large Aggregate Form of the Insulin Receptor Is a Highly Active Tyrosine-Specific Protein Kinase†

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ABSTRACT: The technique of radiation inactivation has been used on a highly purified insulin receptor in order to determine the functional molecular size responsible for tyrosine-specific protein kinase activity. When both insulin binding and kinase activities were analyzed with the same receptor preparations, the functional size for kinase activity was found to be larger than that for insulin binding activity. The radiation inactivation curve for kinase activity was multiphasic. This indicates that at least two components contribute to total kinase activity. The average minimal functional size for the kinase was 370 000 ± 60 000 daltons ($n = 7$) which corresponds to the $\alpha_2\beta_2$ form of the insulin receptor. The average functional size for larger forms was estimated to be $\sim 4 \times 10^6$ daltons. (To minimize the complexity of the model used in this analysis, we have analyzed the radiation inactivation curves of the insulin receptor kinase activity with a two-component model. However, we believe that the larger component, $>1 \times 10^6$ daltons, is probably not a single molecular weight species but rather represents a continuum of sizes or aggregates of the $\alpha_2\beta_2$ form of the receptor.) These larger forms contributed 93% of the total activity. Mild reduction of the insulin receptor preparation with dithiothreitol (DTT) activated the total kinase activity by 3.5-fold. Under this condition, the minimal functional kinase size was 380 000 ± 30 000 daltons ($n = 6$) while the average functional size for the larger forms was $\sim 3 \times 10^6$ daltons. However, the contribution of the larger forms to the total kinase activity was reduced to 39%. When the 3.5-fold increase in the total kinase activity was taken into account, the activity of the minimal form increased ~30-fold (7% to 213%) whereas that of the larger forms did not change significantly (93% to 137%). These studies suggested that the minimal functional kinase is an $\alpha_2\beta_2$ receptor which can be activated ~30-fold by mild DTT treatment. The minimal kinase size did not change to that of an $\alpha\beta$ form under the condition that reduces disulfide bonds between the two α subunits, indicating that a free $\alpha\beta$ form is not an active kinase. Furthermore, radiation inactivation studies indicated that the kinase forms larger than the $\alpha_2\beta_2$ receptor appear to be extremely active. The presence of high molecular weight kinase was detected as a major ^{32}P peak at the void volume when autophosphorylated receptors were fractionated by Sepharose CL-6B chromatography. This peak was dissociated from major peaks corresponding to either insulin binding or receptor protein. These studies suggested that the larger forms of the insulin receptor, $(\alpha_2\beta_2)_{8-10}$, which are minor components in the receptor population, contribute the majority of the kinase activity. This conclusion derived from the present studies may be relevant to the notion that receptor aggregation with a concomitant increase in kinase activity plays an important role in the signal transduction of insulin.

The insulin receptor is a tyrosine-specific protein kinase. The kinase which resides in the cytoplasmic domain of the receptor β subunit is activated immediately after the binding of insulin to the extracellular domain (α subunit). This activation process appears to be essential for insulin signal transduction (Ullrich et al., 1985; Ebina et al., 1985; Rosen, 1987).

Structural requirements for the insulin receptor kinase activation have been studied in a variety of ways: (i) Binding of insulin to the outer surface domain activates the cytoplasmic kinase, which in turn autophosphorylates. This autophosphorylation process further activates the receptor kinase (Tornquist & Avruch, 1988). (ii) Mild dithiothreitol (DTT)[1]

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[1] Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mrads, megarads; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; WGA, wheat germ agglutinin; EGF, epidermal growth factor; DTT, dithiothreitol.

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