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Chemical Synthesis of (24*R*)-24,25-Dihydroxy[26,27-^{3}H]vitamin D_3 of High Specific Activity†

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ABSTRACT: Chemical synthesis of (24*R*)-24,25-dihydroxy-[26,27-^{3}H]vitamin D_3 and its 24-epimer has been devised that allows introduction of ^{3}H at the terminal step of the synthesis. The epimeric mixture is derivatized as the tris(trimethylsilyl) ethers and resolved by high-performance liquid chromatography. The product has a specific activity of 178 Ci/mmol and is fully active in binding to the rat plasma vitamin D binding protein and in the elevation of serum calcium levels of vitamin D deficient rats. The synthesis begins with the readily available 3β-hydroxy-5-cholenic acid methyl ester and involves a Pummerer rearrangement, introduction of the Δ7, irradiation, and isolation of the 26,27-dinor-25-carboxylic acid methyl ester of vitamin D_3. This compound is then treated with a Grignard reagent containing ^{3}H (80 ± 10 Ci/mmol).

The availability of radioactive vitamin D derivatives, labeled at known sites to high specific activity, has been an important factor in recent successful efforts directed at the elucidation of vitamin D metabolism to its tissue-active hormonal form (Suda et al., 1971; Neville & DeLuca, 1966; Holick et al., 1976; Jones et al., 1975; Tohira et al., 1977; Bell et al., 1973; DeLuca et al., 1968; Yamada et al., 1978). A number of tritiated vitamin D derivatives of high specific activity have been synthesized, and with these compounds many important aspects of vitamin D metabolism, its regulation, and the mechanism of action had been investigated under physiological conditions. One of the major metabolites of vitamin D is (24*R*)-24,25-dihydroxyvitamin D_3 [(24*R*)-24,25-$(OH)_2D_3$][1] (Suda et al., 1970a,b; Holick et al., 1972). Though a number of important roles have been suggested for this metabolite, its function is still not understood. An important tool in such an investigation is radiolabeled (24*R*)-24,25-$(OH)_2D_3$ of high specific activity. Several 24,25-$(OH)_2D_3$ syntheses have been reported, including some very elegant stereoselective methods; however, none of these appeared suitable for the synthesis of the title compound, mainly because the 26,27-dimethyl groups were introduced early in the synthesis, making radioactive labeling impractical (Lam et al., 1973; Seki et al., 1973; Eyley et al., 976; Partridge et al., 1976; Takayama et al., 1980; N. Koizumi, M. Ishiguro, M. Yasuda, and N. Ikekawa, unpublished results). It is the purpose of this paper to report the chemical synthesis of (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 and

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[1] Abbreviations: 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 24,25-$(OH)_2$[26,27-^{3}H]D_3, 24,25-dihydroxy[26,27-^{3}H]vitamin D_3; (+)-MTPA, (+)-α-methoxy-α-(trifluoromethyl)phenylacetate or the corresponding acetyl radical; UV, ultraviolet; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

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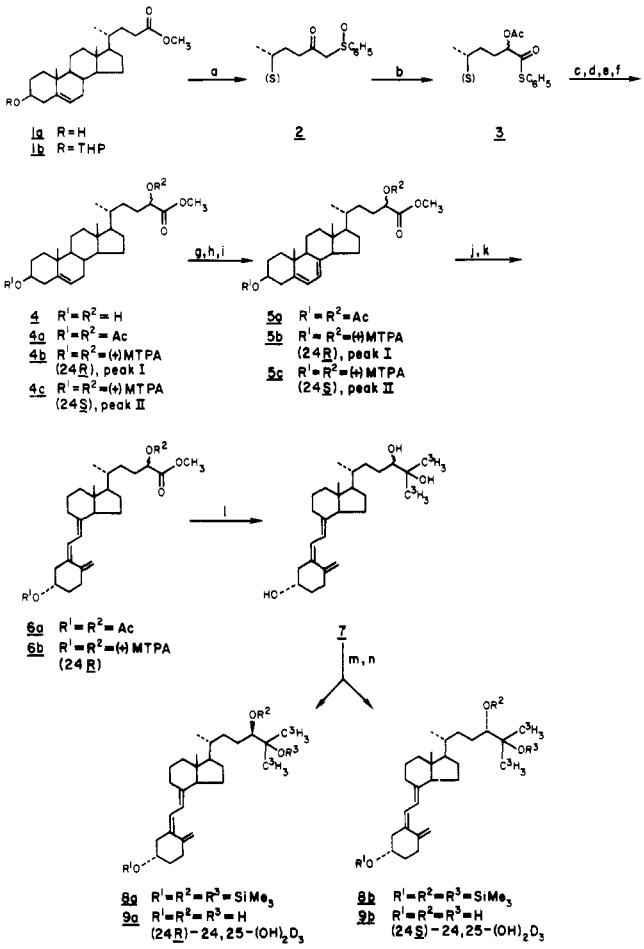

FIGURE 1: Preparation of (24*R*)- and (24*S*)-24,25-dihydroxy-[26,27-^{3}H]vitamin D_3. Reagents and reaction conditions: (a) phenyl methyl sulfoxide/*n*-butyllithium/diisopropylamine/THF, −78 °C; (b) Ac_2O/pyridine/toluene, Δ; (c) Et_3N/benzene; (d) KOH/MeOH/THF; (e) CH_2N_2/ether; (f) MeOH/CH_2Cl_2, pTsOH; (g) dibromantin/$NaHCO_3$/hexane; (h) collidine/toluene; (i) pTsOH/dioxane; (j) *hν*/ether/benzene; (k) EtOH, Δ; l) C^3H_3MgBr (or CH_3MgBr for preparation of unlabeled material); (m) (trimethylsilyl)imidazole, HPLC separation; (n) MeOH/HCl. (S) in part structures **2** and **3** represents the steroid nucleus as in structure **1b**.

(24*S*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 with high specific activity (Figure 1).

Materials and Methods

(*A*) *General.* Ultraviolet (UV) absorbance spectra were taken in 95% ethanol with a Hitachi Model 100-60 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were taken in $CDCl_3$ with a Bruker WH-270 FT spectrometer. Mass spectra were obtained at 115 °C above ambient temperature with an AEI MS-9 spectrometer coupled with a DS-50 data system. High-performance liquid chromatography (HPLC) was done on a microparticulate silica gel column (Zorbax-SIL, Du Pont, 0.94 × 25 cm) with a Waters Associate Model ALC/GPC-204 liquid chromatograph. Thin-layer chromatography (TLC) was done one silica gel 60 F-254 aluminum-backed, precoated sheets with a thickness of 0.25 mm. Sephadex LH-20 was purchased from Pharmacia Chemicals (Piscataway, NJ). Radioactivity was measured with a Packard Model 3255 liquid scintillation counter.

Column chromatography was done with Macherey, Nagel MN silica gel 60 (0.05–0.2 mm/70–270 mesh, ASTM), Brinkmann Instruments, Inc. (Westbury, NY). 3β-Hydroxy-5-cholenic acid methyl ester and 3β-hydroxy-5-cholenic acid were purchased from Steraloids (Wilton, NH), *N*-(trimethylsilyl)imidazole (TMSI) was from Pierce Chemical Co. (Rockford, IL), and methyl phenyl sulfoxide was from Fairfield Chemical Co. (Blythewood, SC). 25-OH-[26,27-^{3}H]D_3 (160 Ci/mmol) was synthesized by a previously described method (Napoli et al., 1979). 25-OH-D_3 was a gift from Upjohn Co. (Kalamazoo, MI), while (24*R*)-24,25-$(OH)_2D_3$ was a gift from the Hoffmann-La Roche Co. (Nutley, NJ).

(*B*) *Biological Activity of* (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3. Female weanling rats were obtained from Holzman Co. (Madison, WI) and fed a vitamin D deficient diet containing 0.47% Ca and 0.3% P (Suda et al., 1970a,b). They were divided into three groups of four to five rats each. One group received 0.05 mL of 95% ethanol intrajugularly, another received 125 ng of crystalline (24*R*)-24,25-$(OH)_2D_3$ and another received 125 ng of the synthetic (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 dissolved in 0.05 mL of ethanol intrajugularly. Twenty-four hours later, all rats were killed, and their serum, diluted with 0.1% $LaCl_3$, was analyzed for calcium with a Perkin-Elmer atomic absorption spectrophotometer, Model 403.

(*C*) *Displacement of 25-OH-*[26,27-^{3}H]D_3 *or* (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 *from the Rat Plasma Protein.* Graded amounts of 25-OH-D_3 or (24*R*)-24,25-$(OH)_2D_3$ were dissolved in 50 μL of 95% ethanol in test tubes. Triplicates of each sample were assayed for displacement of 25-OH-[26,27-^{3}H]D_3 or (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 from the rat plasma binding protein by unlabeled compounds as described by Shepard et al. (1979).

(*D*) *Synthesis of Radiolabeled* (24*R*)-24,25-$(OH)_2D_3$. (*1*) *Cholenic Acid Methyl Ester Tetrahydropyranyl Ether* (**1b**). 3β-Hydroxy-5-cholenic acid methyl ester (6.5 g) was dissolved in dichloromethane (50 mL) to which a catalytic amount of *p*-toluenesulfonic acid (pTsOH) (60 mg) and freshly distilled 2,3-dihydropyran (2.03 g, 2.20 mL) was added. The mixture was stirred for 4 h. The reaction mixture was then washed successively with dilute $NaHCO_3$, water, and brine, dried over anhydrous $MgSO_4$, and filtered, and the solvent was evaporated under reduced pressure. The crude crystalline residue was purified by silica gel column chromatography and eluted with 20% ethyl acetate/hexane mixture to give **1b** (6.3 g, 83%): NMR ($CDCl_3$) δ 0.67 (s, 18-CH_3) 0.96 (d, J = 6.2 Hz, 21-CH_3), 1.04 (s, 19-CH_3) 3.64 (s, $COOCH_3$), 3.75 (m, 3-H), 4.72 (acetal H), 5.34 (m, 6-H). Anal. Calcd for $C_{30}H_{48}O_4$: C, 76.23; H, 10.24. Found: C, 76.18; H, 10.20.

(*2*) *26,27-Dinor-25-*(*phenylsulfinyl*)*cholest-5-en-24-one 3β-Tetrahydropyranyl Ether* (**2**). To an anhydrous solution of diisopropylamine (3.1 g, 4.5 mL) in anhydrous tetrahydrofuran (30 mL) under argon atmosphere was added *n*-butyllithium (1.6 M solution, 19.0 mL) at −78 °C, distilled phenyl methyl sulfoxide (3.9 g) dissolved in anhydrous tetrahydrofuran (20 mL) was added, and the mixture was stirred for an additional 15 min at 0 °C. **1b** (6.0 g) was dissolved in anhydrous tetrahydrofuran (30 mL) and added to the above solution. The mixture was stirred at 0 °C for 45 min, poured into a saturated NH_4Cl solution, and extracted with ether. The combined organic phase was washed with brine, dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure to give a semicrystalline residue which was purified by column chromatography on a silica gel MN (70–250 mesh, 4 ×40 cm) column, eluted with a 1:1 ethyl acetate/hexane mixture to give pure **2** (6.2 g, 84%). NMR ($CDCl_3$) δ 0.68 (s, 18-CH_3), 0.86 (d, J = 6.1 Hz, 21-CH_3), 1.00 (s, 19-CH_3), 3.58 (m, 3-H), 3.80, 3.86 (two doublets, due to (*RS*)-sulfoxides, 25-CH_2), 4.72 (m, H-acetal), 5.34 (m, 6-H), 7.44–7.76 (m, aromatic); IR ($CHCl_3$) 1710, 1130, 1115, 1025, 710, 690

cm^{-1}; mass spectrum, *m/e* 496 (M^+ – dihydropyran), 480, 462, 447. Anal. Calcd for $C_{36}H_{52}O_4S$: C, 74.44; H, 9.37; S, 5.51. Found: C, 74.11; H, 9.18; S, 5.49.

(3) 3β,24ξ-Dihydroxy-25-homo-5-cholenic Acid Methyl Ester (**4**). *Pummerer Rearrangement of β-Keto Sulfoxide* **2**. To a solution of sulfoxide **2** (6.0 g), in 6.0 mL of toluene, was added 30 mL of acetic anhydride, and a catalytic amount of pyridine (0.4 mL), and the solution was heated with stirring under nitrogen atmosphere at 125–130 °C for 4 h followed by reflux for an additional 2 h. The solvent was evaporated under reduced pressure, and the residue (intermediate **3**) was redissolved in benzene (30 mL) to which triethylamine (8 mL) was added. The mixture was stirred under nitrogen atmosphere at room temperature for 16 h and then refluxed for 2 h. The solvent was evaporated under reduced pressure and the residue hydrolyzed under a nitrogen atmosphere with a mixture of KOH (5 g) in water (20 mL), methanol (100 mL), and tetrahydrofuran (30 mL) by stirring at room temperature for 3 h and then refluxing for 2 h. The mixture was then cooled, and acidified with 1 N HCl, and extracted with chloroform (800 mL). The organic phase was washed with water, dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was dissolved in chloroform (100 mL) and a freshly prepared ethereal diazomethane solution (from 50 mmol of Diazald) added, until gas evolution ceased. After the solvents were evaporated under reduced pressure, the partially remaining tetrahydropyranyl protecting group was removed by stirring the mixture in dichloromethane (40 mL) with methanol (50 mL) and a catalytic amount of *p*-toluenesulfonic acid (100 mg) for 16 h at room temperature. The organic phase was washed with a solution of 10% $NaHCO_3$ and water, dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The crude residues were purified by column chromatography on silica gel (130 g) (4 × 20 cm, MN silica gel, 70–270 mesh) using a solvent system of 50% ethyl acetate/hexane (or benzene/ether, 4:1) to give after recrystallization from benzene 2.07 g of **4** with an additional 0.41 g from the mother liquor, with an overall yield of 58% from sulfoxide **2**: mp 143–145 °C; NMR ($CDCl_3$) δ 0.68 (s, 18-CH_3), 0.87 (d, J = 6.0 Hz, 21-CH_3), 1.05 (s, 10-CH_3), 3.52 (m, 3α-H), 3.77 (s, $COOCH_3$), 4.18 (m, 24-H)), 5.36 (m, 6-H); IR (KBr) 3400, 1730 cm^{-1}; mass spectrum, *m/e* 418 (M^+), 403, 400, 385, 333. Anal. Calcd for $C_{26}H_{42}O_4$: C, 74.60; H, 10.11. Found: C, 74.49; H, 10.02.

(4) 3β,24ξ-Diacetoxy-25-homo-5-cholenic Acid Methyl Ester (**4a**). A solution of the dihydroxy ester **4** (300 mg) and acetic anhydride (1.5 mL) in pyridine (6.0 mL) was stirred at room temperature for 3 h. To the reaction mixture was added ethyl acetate (25 mL), and the organic phase was washed with 1 N HCl, 10% $NaHCO_3$, and water, dried over anhydrous $MgSO_4$, filtered, and evaporated. The white crystalline residue was purified on a silica gel column (30 g of MN silica gel, 70–270 mesh) and the product eluted with a 20% ethyl acetate/hexane mixture to give the pure diacetoxy derivative (**4a**) (300–310 mg): NMR ($CDCl_3$) δ 0.68 (s, 18-CH_3), 0.93 (d, J = 6.2 Hz, 21-CH_3), 1.01 (s, 19-CH_3), 2.03 (s, 3β-$COCH_3$), 2.12 (s, 24-$COCH_3$), 3.74 (s, $COOCH_3$), 4.60 (m, 3α-H), 4.95 (m, 24-H), 5.48 (m, 6-H). Anal. Calcd for $C_{30}H_{46}O_6$: C, 71.68; H, 9.22. Found: C, 71.70; H, 9.19.

(5) 3β,24ξ-Diacetoxy-25-homo-5,7-choladienic Acid Methyl Ester (**5a**). The 3β,24-diacetoxy ester (**4a**) (300 mg), $NaHCO_3$ (280 mg), and 1,3-dibromo-5,5-dimethylhydantoin (120 mg) in hexane (30 mL) were refluxed under nitrogen for 30 min. The reaction mixture was filtered while hot and washed with hexane, and the combined hexane solutions were evaporated under reduced pressure. The residue obtained was dissolved in a solution of 2,4,6-trimethylpyridine (0.24 mL) and xylene (6 mL) and refluxed under nitrogen for 1.5 h. The reaction mixture was cooled, diluted with ether (25 mL), washed with $NaHCO_3$ solution and water, dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by an HPLC microparticulate column (Zorbax-SIL, Du Pont, 0.94 × 25 cm) and eluted with 2% 2-propanol in hexane to give pure **5a** (90 mg): NMR ($CDCl_3$) δ 0.54 (s, 18-CH_3), 0.94 (d, J = 6.0 Hz, 21-CH_3), 2.01 (s, 3β-$OCOCH_3$), 2.12 (s, 24-$OCOCH_3$), 3.68 (s, $COOCH_3$), 4.12 (m, 3α-H), 4.95 (m, 24-H), 5.35, 5.56 (m, 6 and 7-H); mass spectrum (high-resolution *m/e* found 500.3139, calcd 500.313767) *m/e* 500 (M^+), 440 (M^+ – AcOH), 425 (M^+ – AcOH – CH_3), 313 (M^+ – side chain), 253 (M^+ – AcOH – side chain); UV max (EtOH) 292 (7050), 282 (12.300), 272 (11.600), 264 (shoulder) nm. Anal. Calcd for $C_{30}H_{44}O_6$: C, 71.97; H, 8.86. Found: C, 71.91; H, 8.71.

(6) 3β,24ξ-Diacetoxy-26,27-dinor-9,10-secocholesta-5,7,10(19)-triene-25-carboxylic Acid Methyl Ester (**6a**). A solution of **5a** (17 mg) in 20% benzene/ether mixture (150 mL) under argon was cooled in an ice bath and irradiated with a medium pressure mercury Hanovia lamp (608A-36 UV lamp; Canrad-Hanovia, Newark, NJ) through a Vycor filter (220 nm) for 5 min. The solvents were evaporated under reduced pressure, and the residue was dissolved in ethanol (5 mL) and refluxed under argon for 2 h. The ethanol was evaporated and the residue purified by HPLC microparticulate silica gel chromatography (Zorbax-SIL, 0.94 × 25 cm column) and eluted with 0.7% 2-propanol in hexane to give the vitamin derivative **6a** (17 mg). UV λ_{max} in EtOH 262 nm; UV λ_{min} 225 nm; NMR ($CDCl_3$) δ 0.54 (s, 19-CH_3), 0.94 (d, J = 6.0 Hz, 21-CH_3), 2.01 (s, 3β-$OCOCH_3$), 2.12 (s, 24-$OCOCH_3$), 6.68 ($COOCH_3$), 4.82, 5.05 (m, 19-H_2), 4.95 (m, 24-H), 6.03, 6.23 (6- and 7-H). Mass spectrum (exact mass calcd 500.3138, found 500.3130), *m/e* 500 (M^+), 440 (M^+ – AcOH), 425 (M^+ – AcOH – CH_3), 313 (M^+ – side chain), 253 (M^+ – AcOH – side chain).

(7) 24ξ,25-Dihydroxyvitamin D_3 (**7**). The vitamin D methyl ester (**6a**) (0.5 mg) was dissolved in anhydrous ether (2.0 mL) and a 2.85 M solution of CH_3MgBr (0.2 mL) added under argon and kept for 2 h at room temperature. The reaction mixture was decomposed by the careful addition of ice-cold saturated solution of NH_4Cl and extracted with ether, the ether solution was washed with brine, dried over anhydrous $MgSO_4$, and filtered, and the solvent was removed. The residue was purified by an HPLC microparticulate column (Zorbax-SIL, Du Pont, 0.94 × 25 cm) eluted with 10% 2-propanol in hexane. The product comigrated with authentic (24*R*)-24,25-$(OH)_2D_3$. Although it was an epimeric mixture of the (24*R*,*S*)-24,25-$(OH)_2D_3$, the NMR and mass spectrum were identical with those of the natural product.

(E) Separation of the 24-Hydroxy Epimers. Synthetic 24ξ,25-dihydroxyvitamin D_3 (48 μg) was silylated in hexane (100 μL) with (trimethylsilyl)imidazole (TMSI) at 70 °C for 20 min. The solvent was evaporated with nitrogen gas, the residue redissolved in hexane (500 μL), and water added (300 μL). The water was removed and the hexane phase dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness with nitrogen. The residue was dissolved in hexane, and the two epimers were separated by HPLC [Zorbax-SIL microparticulate silica gel column, Du Pont (0.94 × 25 cm)] using 0.2% ethyl acetate in hexane. The more hydrophilic (second) compound was identical with the silylated derivative of the

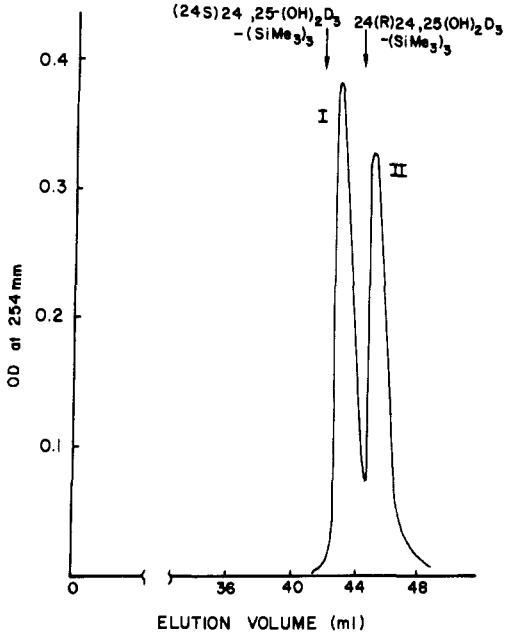

FIGURE 2: HPLC separation of (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3-$(SiMe_3)_3$ derivative from the (24*S*)-24,25-$(OH)_2$[26,27-^{3}H]D_3-tris-(Me_3Si) derivative. The microparticulate silica gel column (Zorbax-Sil, Du Pont, 0.94 × 25 cm) was developed with 0.2% ethyl acetate in hexane, flow rate 2 mL/min. The first peak is (24*S*)-24,25-$(OH)_2$[26,27-^{3}H]D_3-tris(Me_3Si), and the second peak is the corresponding 24*R* derivative. The arrows indicate the peak positions for unlabeled identical derivatives. Note the significant isotopic shifts.

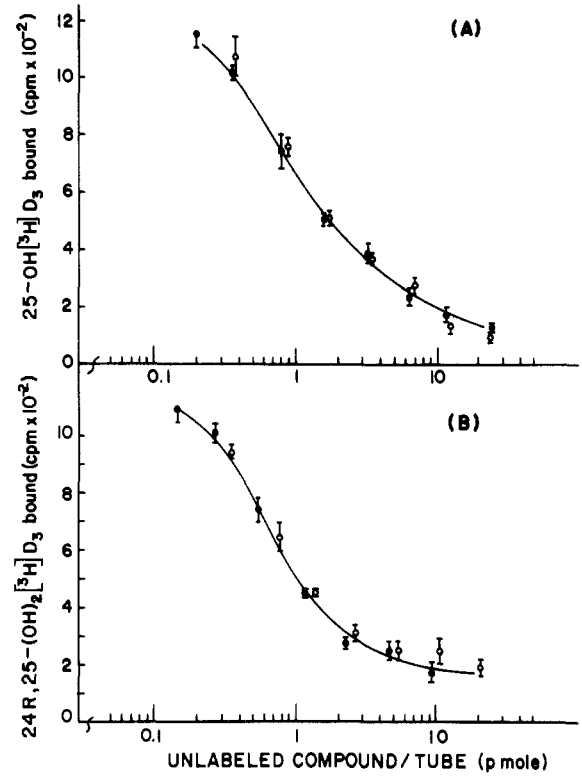

FIGURE 3: Displacement of 25-OH-[26,27-^{3}H]D_3 (A) or (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 (B) from the rat plasma binding protein by unlabeled 25-OH-D_3 (closed circles) or unlabeled (24*R*)-24,25-$(OH)_2D_3$ (open circles). Triplicate determinations for each concentration of nonradioactive compounds were carried out as described in the text. Each point represents mean value with standard deviation.

natural (24*R*)-24,25-$(OH)_2D_3$ (**8a** unlabeled form), and the more hydrophobic (first) peak was the expected (24*S*)-24,25-$(OH)_2D_3$ $(Me_3Si)_3$ derivative **8b** (unlabeled form).

(1) (24R)-24,25-(OH)$_2$D$_3$ and (24S)-24,25-(OH)$_2$D$_3$ (**9a** *and* **9b**). To purified (24*R*)-24,25-$(OH)_2D_3$ tris(trimethylsilyl) derivative **8a** was added methanol (500 μL) containing 1 N HCl (50 μL). The mixture was kept at room temperature for 1 h. The methanol was evaporated with nitrogen and the residue taken up in ethyl acetate. It was washed with a 10% $NaHCO_3$ solution, dried over anhydrous $MgSO_4$, filtered, and reduced to dryness with nitrogen. The residue was purified by HPLC (Zorbax-SIL, Du Pont, 0.94 × 25 cm) and eluted with 10% 2-propanol in hexane to give pure (24*R*)-24,25-$(OH)_2D_3$ (**9a** unlabeled). There were no other peaks on HPLC, and the synthetic **9a** comigrated with authentic (24*R*)-24,25-$(OH)_2D_3$. (The overall yield for the silylation and desilylation is 60–65%.) It had the same mass spectrum as authentic (24*R*)-24,25-$(OH)_2D_3$. (24*S*)-24,25-$(OH)_2D_3$ (**9b**, unlabeled) was prepared and purified in the same manner.

(2) (24R)- and (24S)-24,25-Dihydroxy[26,27-^{3}H]D$_3$. Methyl ester **6a** (1.0 mg, 2.2 mol) in 2 mL of dry ether was treated with 0.5 mmol of C^3H_3MgBr in 0.5 mL of dry ether in the laboratories of New England Nuclear (Boston, MA). After workup [the actual Grignard reaction and its workup were performed in the laboratories of New England Nuclear, Boston, MA; purification of (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 and all other reactions and experiments were conducted in our laboratories] the radiolabeled material (500 mCi) was purified by chromatography on a Sephadex LH-20 column (2.2 × 30 cm) developed with hexane/chloroform/methanol, 9:1:1, to give 100 mCi of **7** which eluted from 175 to 375 mL. [Though the 24,25-$(OH)_2D_3$ elutes from the LH-20 in a much smaller fraction, as a precaution a large "batch type" fraction was collected.] After evaporation it was further purified by HPLC on a 0.45 × 25 cm microparticulate silica gel column (Zorbax-SIL, Du Pont) eluted with 4% 2-propanol in hexane. Standard (24*R*)-24,25-$(OH)_2D_3$ elutes at 18–20 mL in this system. The material eluting at 18–22 mL was collected. A total of 38 mCi of 24,25-$(OH)_2$[26,27-^{3}H]D_3 was obtained. Silylation and desilylation was carried out as described for the unlabeled compound to obtain 9.8 mCi of (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 (compound **9a**) and 7.8 mCi of (24*S*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 (compound **9b**) with a specific activity of 178 mCi/μmol. The separation is shown in Figure 2.

It should be noted that isotopically labeled (24*R*)-24,25-$(OH)_2D_3$ separates from unlabeled (24*R*)-24,25-$(OH)_2D_3$ as the tris(trimethylsilyl) ethers on HPLC (Figure 2). We have previously noted a similar separation of 1,25-$(OH)_2$[26,27-^{3}H]D_3 from unlabeled 1,25-$(OH)_2D_3$ on HPLC (Napoli et al., 1980).

We tested the activity of the radiolabeled (24*R*)-24,25-$(OH)_2D_3$ in binding to the rat plasma vitamin D transport protein (Figure 3A,B). Displacement of radiolabeled (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 (Figure 3A) or 25-OH-[26,27-^{3}H]D_3 by unlabeled (24*R*)-24,25-$(OH)_2D_3$ or 25-OH-D_3 was determined. The displacement was identical in each case, showing that labeled (24*R*)-24,25-$(OH)_2D_3$ is indistinguishable from 25-OH-D_3 and (24*R*)-24,25-$(OH)_2D_3$ as reported previously for the unlabeled compounds.

(3) 3β,24ξ-Dihydroxy-25-homo-5-cholenic Acid Methyl Ester 3,24-Di-MTPA (**4b,c**). The dihydroxy ester (**4**) (92 mg) was stirred with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (192 mg) in pyridine (3.0 mL) for 18 h. The 3β,24-di-MTPA (**4b,c**) was extracted with ethyl acetate, and the organic phase was washed with 1 N HCl, water, 10% $NaHCO_3$ solution, and brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. The 3β,24-di-MTPA (**4b,c**) was separated by HPLC on a semipreparative, microparticulate

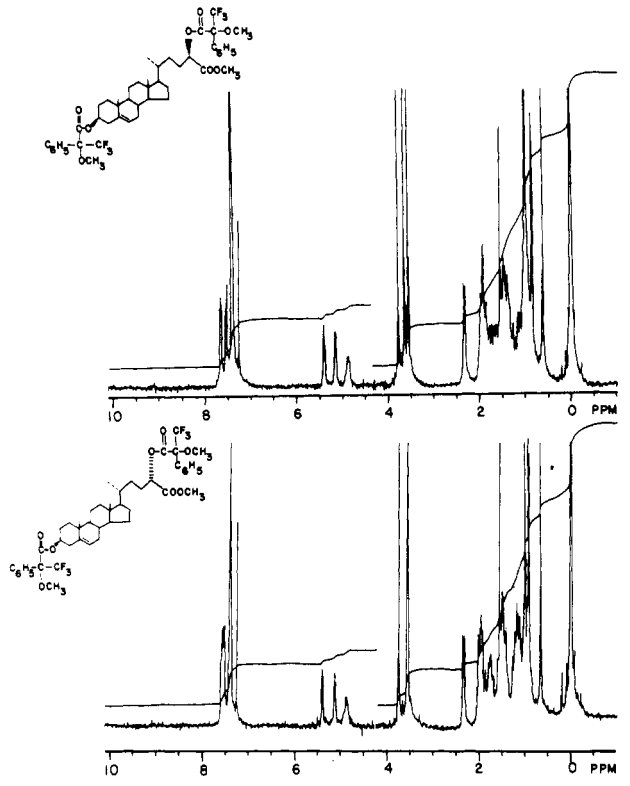

FIGURE 4: NMR spectrum of (+)-MTPA-(24*R*)-24- and (+)-MTPA-(24*S*)-24-hydroxyhomocholenic acid esters **4b** and **4c**. The only significant difference between the two spectra is that the methoxy peaks of the 24*R* compound appear as two singlets (2× 3H) while the methoxy signals of the 24*S* isomer appear as one singlet (6H).

column (Zorbax-SIL, Du Pont, 0.94 × 25 cm) into two peaks by development with 10% ethyl acetate in hexane. Peak I eluted at 39.2 mL [and later proved to be the 24*R* epimer (**4b**)] and peak II eluted at 43.2 mL (24*S*) (**4c**), to give a total of peak I (62 mg) (24*R*) (**4b**) and peak II (56 mg) (24*S*) (**4c**). (Peak I (**4b**) and peak II (**4c**)). Anal. Calcd. for $C_{46}H_{56}O_8F_6$: C, 64.93; H, 6.63. Found: C, 64.89; H, 6.68; *Peak I* (24*R*): NMR ($CDCl_3$) δ 0.68 (s, 18-CH_3), 0.93 (d, J = 6.0 Hz, 21-CH_3), 1.01 (s, 19-CH_3), 3.55 (s, OCH_3 of 3β-MTPA), 3.65 (s, OCH_3 of 24-MTPA), 3.78 (s, $COOCH_3$), 4.95 (m, 3α-H), 5.18 (m, 24-H), 5.40 (m, 6-H), 7.28-7.68 (m, phenyl). *Peak II* (24*S*): NMR ($CDCl_3$) δ 0.68 (s, 18-CH_3), 0.93 (d, J = 6.0 Hz, 21-CH_3), 1.01 (s, 19-CH_3), 3.58 (s, 2x $-OCH_3$ of 3β and 24-MTPA), 3.78 (s, $COOCH_3$), 4.96 (m, 3α-H), 5.18 (m, 24-H), 5.40 (m, 6-H), 7.28–7.68 (m, phenyl).

A comparison of the NMR spectra of the 24*R* and 24*S* epimers is shown in Figure 4. Note that the 24*R* epimer gives two singlets of 3H each at 3.55 and 3.65 ppm for the 3β-MPTA and 24-MPTA groups, while the 24*S* epimer gives a singlet of 6H at 3.58 ppm for both 3-MPTA and 24-MPTA groups.

(*4*) *(24R)-3β,24-Dihydroxy-25-homo-5,7-choladienic Acid Methyl Ester 3,24-Di-MTPA Ester* (**5b**). (24*R*)-3β,24-Di-MTPA ester **4b** (43.8 mg), $NaHCO_3$ (23 mg), and 1,3-dibromo-5,5-dimethylhydantoin (10 mg) in hexane (5 mL) were refluxed under nitrogen for 30 min. The reaction mixture was filtered while hot, the precipitate was washed with hexane, and the combined filtrates were evaporated under reduced pressure. The residue was dissolved in a solution of 2,4,6-trimethylpyridine in xylene (2 mL) and refluxed under nitrogen for 1.5 h. The reaction mixture was cooled, diluted with ether, washed successively with 1 N HCl, 10% $NaHCO_3$, and water, dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by flash chromatography through a short silica gel column (MN silica gel, 1 × 8 cm) and eluted with 20% ethyl acetate in hexane. The 2,4,6-triene 24-MTPA ester, a byproduct formed from the 4,6-diene, eluted with the front, and then 5,7-diene (24*R*)-3β,24-di-MTPA (**5b**) (peak I) (17 mg) was obtained. This was further purified by an HPLC microparticulate silica gel column (0.94 × 25 cm, Zorbax-SIL, Du Pont) eluting at 40 mL with a 12% ethyl acetate in hexane mixture: NMR ($CDCl_3$) δ 0.68 (s, 18-CH_3), 0.93 (d, J = 6.0 Hz, 21-CH_3), 1.01 (s, 19-CH_3) 3.55 (s, $-OCH_3$ of 3β-MTPA), 3.65 (s, $-OCH_3$ of 24-MTPA), 3.78 ($COOCH_3$), 4.95 (m, 3α-H), 5.15 (m, 24-H), 5.38 (m, 6-H), 5.58 (m, 7-H), 7.28–7.68 (m, phenyl); UV max (EtOH) 294, 282, 272, 264 (shoulder) nm.

(*5*) *(24S)-3β,24-Di-MTPA-oxy-25-homo-5,7-choladienic Acid Methyl Ester* (**5c**) *(Peak II)*. Diene **5c** was prepared from **4c** by the procedure described in the previous experiment. The product eluted under the same conditions as peak I at 44 mL: NMR ($CDCl_3$) δ 0.68 (s, 18-CH_3), 0.93 (d, J = 6.0 Hz 21-CH_3), 1.01 (s, 19-CH_3), 3.58 (s, 2× $-OCH_3$ of 3β and 24-OCH_3 of MTPA), 3.78 ($COOCH_3$), 4.95 (m, 3α-H), 5.15 (m, 24-H), 5.38 (m, 6-H), 5.58 (m, 7-H), 7.28–7.68 (m, phenyl); UV max (EtOH) 294, 282, 272, 264 (shoulder) nm.

(*6*) *(24S)-24,25-Dihydroxyvitamin D_3* (**9b**). (24*S*)-3β,24-Di-MTPA ester **5c** (peak II, 2.9 mg) was dissolved in anhydrous ether (2 mL) and a 2.85 M CH_3MgBr solution in ether (1.0 mL) added. The mixture was stirred under nitrogen for 2 h, carefully decomposed with ice-cold NH_4Cl solution, and extracted with ethyl acetate. The ethyl acetate was washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was chromatographed on an HPLC microparticulate silica gel column (Zorbax-SIL, Du Pont, 0.94 × 25 cm) eluted with 20% 2-propanol in hexane. Elution volume of the product was 32 mL for 8 min. The pooled fractions were evaporated to give 1.0 mg of (24*S*)-5,7-cholestadiene-3β,24,25-triol. A solution of this material (1.0 mg), in 120 mL of ether and 30 mL of benzene under argon, cooled to 0 °C was irradiated with a Hanovia lamp through a Vycor filter (220 nm) for 5 min. The solvents were evaporated under reduced pressure, and the residue was dissolved in ethanol (5 mL) and refluxed under argon for 2 h. The ethanol was evaporated and the residue purified by HPLC (Zorbax SIL, Du Pont, 0.94 × 25 cm) eluted with 10% 2-propanol in hexane to obtain product **9b** (unlabeled). The purified **9b** was then silylated as before, and the silyl derivative was identical with (24*S*)-24,25-$(OH)_2D_3$-tris(Me_3Si) (**8b**).

Results

The synthesis of (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 (**9a**) (Figure 1) proceeded from 3β-(tetrahydropyranyloxy)-5-cholenic acid methyl ester (**1b**) which was obtained from the readily available 3β-hydroxy-5-cholenic acid methyl ester (**1a**) with 2,3-dihydropyran in dichloromethane and *p*-toluenesulfonic acid as catalyst. Homologation with simultaneous α-hydroxylation of **1b** was achieved by the Pummerer rearrangement of the corresponding β-keto sulfoxide **2** (Pummerer, 1909; Corey & Chajkovsky, 1962; Irinchijima et al., 1974, 1975). Ester **1b** was converted to the β-keto sulfoxide **2** with the methylsulfinyl carbanion formed from phenyl methyl sulfoxide, *n*-butyllithium, and diisopropylamine in anhydrous tetrahydrofuran. β-Keto sulfoxide **2** underwent the Pummerer rearrangement by treatment with acetic anhydride in toluene followed by triethylamine treatment in benzene to give intermediate thio ester **3**. Subsequent hydrolysis of thio ester **3** with potassium hydroxide in methanol and tetrahydrofuran gave crude 24ξ-hydroxyhomocholenic acid which was converted to the methyl ester with diazomethane in ether, and the

tetrahydropyranyl protecting group (some of the THP protecting group is removed during the Pummerer rearrangement) was removed with a catalytic amount of *p*-toluenesulfonic acid in dichloromethane and methanol to give **4** ester as an epimeric mixture of the (24*R*)- and (24*S*)-hydroxy compound in 50–52% yield from **2** (the above procedure starting from **2** does not require any purification of the intermediates to give **4**). The synthesis of **4** has been previously reported by a different method (Kobayashi et al., 1980). Ester **4** was acetylated with acetic anhydride in pyridine to give **4a**. We were unable to separate this epimeric mixture on HPLC. However, when **4** was acylated with the chiral acid chloride (+)-MTPA, we were able to separate **4b**,**c** into corresponding (24*R*)- and (24*S*)-24-hydroxyhomocholenic acid methyl esters **4b** and **4c** as discussed below.

Diacetoxy ester **4a** was brominated at carbon 7 with dibromantin in a modified Hunziker–Müllner procedure (Hunziker & Müllner, 1958) and dehydrobrominated with 2,4,6-collidine to give a mixture of 4,6- and 5,7-diene esters. The crude reaction mixture was treated with *p*-toluenesulfonic acid in dioxane which caused elimination of the 3-ester from the allylic 4,6-diene (Partridge et al., 1981), but not from the homoallylic 5,7-diene, thus facilitating separation of the two products by silica gel column chromatography, giving the provitamin derivative **5a** which was further purified by HPLC. The UV spectrum of **5a** was typical of steroidal 5,7-dienes with maxima at 264, 272, 282, and 294 nm. The NMR spectrum was in good agreement with the above structure.

Brief irradiation of **5a** followed by thermal rearrangement gave the expected vitamin D_3 precursor **6a** together with some tachysterol derivative and other irradiation byproducts which were separated by HPLC (Zorbax SIL, Du Pont, 0.94 × 25 cm). The immediate unlabeled precursor **6a** of the radiohormone **9a** was characterized by UV, NMR, and high-resolution mass spectrometry. The UV absorbance maximum at 262 nm and a minimum at 228 nm are characteristic of the 5,6-*cis*-triene chromophore, and the NMR spectrum with doublets at δ 6.03 and 6.23 (protons at C-6 and C-7, respectively) and signals at δ 4.82 and 5.05 (C-19 hydrogens) confirmed the assignment. The NMR spectrum further exhibited the expected signals for the acetyl groups (singlet δ 2.01, singlet δ 2.12), the methyl ester group (singlet, δ 3.68), and the carbinyl proton at δ 4.95 (C-24 hydrogen). The high-resolution mass spectrum indicated a molecular ion of 500.3130 which corresponds to the formula $C_{30}H_{44}O_6$. Peaks 440.2884 (M^+ – AcOH), 425.2668 (M^+ – AcOH – CH_3), 313.2175 (M^+ – side chain), and 136.118 are characteristic of the compound.

The next synthetic step was the introduction of the tritium by the reaction of ester **6a** with C^3H_3MgBr (80 ± 10 Ci/mmol). This reaction gave the desired product **7** as an epimeric mixture which was separated from large amounts of radioactive byproduct by two chromatographic steps. A Sephadex LH-20 column was calibrated with enzymatically prepared (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 (Knutson & DeLuca, 1974; Ghazarian & DeLuca, 1974; Ghazarian et al., 1975), and then the crude radiolabeled mixture was chromatographed. Of the 500 mCi applied to the column about 100 mCi was collected, corresponding in elution volume to the authentic (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 used for column calibration. The collected product was further purified by HPLC microparticulate silica gel (Zorbax-SIL, Du Pont) which was calibrated with (24*R*)-24,25-$(OH)_2D_3$. A total of 38 mCi of 24ξ,25-$(OH)_2$[26,27-^{3}H]D_3 (38 mCi) was obtained.

In order to separate the 24-OH diastereomeric mixture, **7** was silylated with (trimethylsilyl)imidazole (TMSI) in hexane (Tanaka et al., 1975). After workup, tris(Me_3Si) derivatives **8a** and **8b** were separated by HPLC (Zorbax-SIL, Du Pont, 0.94 × 25 cm column) using a solvent mixture of 0.2% ethyl acetate in hexane. The retention time of (24*S*)-24,25-$(OH)_2$[26,27-^{3}H]D_3-tris(Me_3Si) (**8b**) was 23 min (peak I, less polar) and for (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3-tris(Me_3Si) (**8a**), 24 min (peak II, or more polar). **8a** comigrated with the silylated derivative (24*R*)-24,25-$(OH)_2D_3$-tris(Me_3Si) prepared from authentic (24*R*)-24,25-$(OH)_2D_3$.

Table I: Biological Activity of Synthetic (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3[a]

compound	serum calcium (mg/dL)
ethanol	5.1 ± 0.2
crystalline (24*R*)-24,25-$(OH)_2D_3$	6.0 ± 0.1[b]
(24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3	6.1 ± 0.2[c]

[a] Female weanling rats were fed a vitamin D deficient diet containing 0.47% calcium and 0.3% phosphorus for 3 weeks. They were dosed with the indicated compounds (125 ng) intrajugularly in 0.05 mL of ethanol. The rats were killed 24 h later and their serum calcium levels determined. Data are reported as mean ± SEM for four to five animals per group. [b] Significantly different from control, $P < 0.005$. [c] Significantly different from control, $P < 0.005$.

The separated tris(Me_3Si) diastereoisomers **8a** and **8b** were then desilylated with methanol containing a catalytic amount of HCl and purified again by HPLC as before to give 10 mCi of (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 (**9a**), with a specific activity of 178 Ci/mmol, and 9 mCi of (24*S*)-24,25-$(OH)_2$-[26,27-^{3}H]D_3 (**9b**). The method was worked out with CH_3-MgBr, and the end product, (24*R*)-24,25-$(OH)_2D_3$, was in all respects (NMR, mass spectrometry) identical with authentic (24*R*)-24,25-$(OH)_2D_3$.

The synthetic (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 is equal to authentic (24*R*)-24,25-$(OH)_2D_3$ in elevating serum calcium of vitamin D deficient rats (as shown in Table I). The rat vitamin D transport protein binds the (24*R*)-24,25-$(OH)_2$-[26,27-^{3}H]D_3 as well as (24*R*)-24,25-$(OH)_2D_3$, 25-OH-[26,27-^{3}H]D_3, and 25-OH-D_3 as shown in Figure 4. These results provide biological evidence that the radiolabeled (24*R*)-24,25-$(OH)_2D_3$ is identical with in vivo generated (24*R*)-24,25-$(OH)_2D_3$ despite (HPLC separation from unlabeled (24*R*)-24,25-$(OH)_2D_3$.

In an attempt to separate the 24-hydroxy epimer mixture of **4** prior to conversion to the secosteroid, it was acylated with (+)-MTPA chloride [(+)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride] (Dale et al., 1969) to give the 3,24-bis-MTPA esters **4b** and **4c**, which were easily separated on an HPLC microparticulate silica gel column (Zorbax-SIL, Du Pont, 0.94 × 25 cm) with the solvent mixture 10% ethyl acetate in hexane into peak I (**4b**) and peak II (**4c**). The retention time for **4b** was 11 min and for **4c**, 12 min. The separated epimers were then brominated and dehydrobrominated as before for the acetyl derivative. After dehydrobromination with collidine in xylene, the expected 5,7-diene **5b** (or **5c**) was obtained together with some 2,4,6-triene, a product of allylic elimination of the MPTA ester from the expected 4,6-diene.

The provitamin derivative **5b** was purified by HPLC and then irradiated, followed by thermal rearrangement. Unfortunately the irradiation step gave less than 10% yield of the expected **6b**, an observation made before with bulky 3β-protecting groups, making this approach for the labeled compound impractical. In another approach provitamin derivative **5c** (peak II) was first treated with methyl magnesium bromide to obtain the corresponding 24,25-dihydroxyprovitamin. After irradiation and thermal isomerization, (24*S*)-24,25-$(OH)_2D_3$ (**9b**, unlabeled form) was obtained, which after trimethyl-

silylation gave **8b** (unlabeled), identical with the previously prepared (24*S*)-24,25-$(OH)_2D_3$-tris(Me_3Si).

Discussion

Readily obtainable radiolabeled 24,25-$(OH)_2D_3$ of high specific activity is essential to continued progress in understanding the biochemical role of this major metabolite of vitamin D_3. For example, it is required for (1) investigation of tissue distribution of 24,25-$(OH)_2D_3$, (2) investigation of subcellular localization, (3) checking for possible receptors for 24,25-$(OH)_2D_3$ in tissues where it preferentially localizes, (4) investigation of further metabolism, and (5) analytical use in binding assay. However, the sole method of producing radiolabeled 24,25-$(OH)_2D_3$ has been enzymatic conversion of radiolabeled 25-hydroxyvitamin D_3 (25-OH-D_3) (Knutson & DeLuca, 1974; Ghazarian & DeLuca, 1974). The necessity of a biochemical conversion using homogenates from chicks given vitamin D_3 has limited the quantity and distribution of the labeled metabolite.

A chemical route to 24,25-$(OH)_2$[26,27-^{3}H]D_3 has several notable features: it does not rely on enzymatic conversion of 25-OH-[26,27-^{3}H]D_3 to 24,25-$(OH)_2$[26,27-^{3}H]D_3 but yields 24,25-$(OH)_2$[2l827-^{3}H]D_3 directly with a specific activity of 170 Ci/mmol. Though a separation of the 24-OH diastereomeric mixture is needed after the introduction of the radioactivity, the separation is relatively simple and does not require elaborate synthetic steps.

The same synthesis could also provide 24,25-$(OH)_2$-[26,27-^{14}C]D_3 from precursor **6a** with a specific activity of 120 mCi/mmol or 26,26,26,27,27,27-hexadeuterio-24,25-$(OH)_2D_3$. Several stereoselective syntheses of (24*R*)-24,25-$(OH)_2D_3$ have been published; however, none of them made radioactive labeling in the terminal C-26,27-dimethyl groups practical (Partridge et al., 1976; Takayama et al., 1980), since the C-26,27-dimethyl groups were introduced early in the synthesis and multistep handling of highly radioactive intermediates in elaborate synthetic steps would have been necessary. An elegant stereoselective synthesis of (24*R*)-24,25-$(OH)_2D_3$ (N. Koizumi et al., unpublished results) that allowed for tritium introduction in the final step unfortunately could not be considered, since only one tritium atom per mole could have been introduced and an additional chiral center at C-25 would have been created. The final choice for precursor for (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 was 24-hydroxyvitamin D_3 ester **6a**. The corresponding 24-hydroxyhomocholenic ester **4** has been prepared previously as an intermediate in the synthesis of 24-fluoro-25-hydroxyvitamin D_3 (Kobayashi et al., 1980), but it was longer and more tedious than the current synthesis. The application of the Pummerer rearrangement achieves the simultaneous α-hydroxylation and homologation of the commercially available 3β-hydroxy-5-cholenic acid in few steps and in high yield. No purification of the intermediates is necessary.

The separation of the epimeric mixture of 24-hydroxyhomocholenic ester **4** was achieved through their (+)-MTPA esters. The use of this chiral acid was a rational choice, since the C-24 position is far from the C-20 chiral center, and it behaves as if it were an achiral molecule. The diastereoisomeric separation was then achieved by the use of HPLC. However, the irradiation step converting provitamin derivatvie **5b** to vitamin derivative **6b** gave extremely poor yield, due to the bulkiness of the esterifying group at the 3β-position, making this approach impractical. Hydrolysis of the C-24 acyloxy group would have given epimeric mixtures. We decided to separate the epimers through the silylation of the final product, followed by desilylation of the separated silyl diastereoisomers. The method used was a slight modification of a previously published procedure (Ikekawa & Koizumi, 1976).

An alternate procedure would have been to treat **5b** with the radiolabeled Grignard reagent and to irradiate the radiolabeled provitamin followed by heat isomerization to the desired radiolabeled vitamin D_3 derivative. Although this reaction worked well on the unlabeled intermediates and did not epimerize the 24-OH group, we did decide against this method, since the irradiation of the highly radioactive provitamin followed by heat isomerization and a rather difficult HPLC separation of the irradiation products was less attractive than the method we ultimately used.

In competitive binding assays using the rat plasma transport protein system (Shepard et al., 1979), (24*R*)-24,25-$(OH)_2$-[26,27-^{3}H]D_3 exhibits a binding affinity identical with that of natural (24*R*)-24,25-$(OH)_2D_3$. As in the case of other highly tritium-labeled compounds (Napoli et al., 1980), there is, however, a slight chromatographic separation between radiolabeled and unlabeled metabolite on efficient columns, and that isotope effect must be borne in mind when cochromatography is used for establishing product identity. Nevertheless, the (24*R*)-24,25-$(OH)_2$[26,27-^{3}H]D_3 is identically active in increasing serum calcium levels in vitamin D deficient rats (Table I). This likely results from both intestinal calcium absorption and bone calcium mobilization. Thus, this preparation is suitable to perform the many experiments directed to determining the biological significance of (24*R*)-24,25-$(OH)_2D_3$.

Acknowledgments

We are indebted to Connie Smith for her invaluable technical assistance.

Registry No. **1a**, 20231-57-6; **1b**, 66414-42-4; **2** (*R*)-sulfoxide, 91743-52-1; **2** (*S*)-sulfoxide, 91743-53-2; (24*R*)-**4**, 91839-82-6; (24*S*)-**4**, 91839-83-7; (24*R*)-**4a**, 91797-77-2; (24*S*)-**4a**, 78857-22-4; **4b**, 91743-54-3; **4c**, 91743-55-4; (24*R*)-**5a**, 91797-78-3; (24*S*)-**5a**, 78857-23-5; **5b**, 91743-56-5; **5c**, 91743-57-6; (24*R*)-**6a**, 91743-58-7; (24*S*)-**6a**, 91743-59-8; (24*R*)-**7** unlabeled, 55721-11-4; (24*S*)-**7** unlabeled, 55700-58-8; **8a** unlabeled, 91743-60-1; **8b** unlabeled, 91743-61-2; **9a** ^{3}H labeled, 91743-62-3; **9b** ^{3}H labeled, 91743-63-4; C^3H_3Br, 53969-20-3; phenyl methyl sulfoxide, 1193-82-4; 2,3-dihydropyran, 110-87-2; (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride, 20445-33-4; (24*S*)-5,7-cholestadiene-3β,24,25-triol, 55700-57-7.

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8-Chloroguanosine: Solid-State and Solution Conformations and Their Biological Implications†

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ABSTRACT: The three-dimensional structure of 8-chloroguanosine dihydrate was determined by X-ray crystallography. The crystals belong to the orthorhombic space group $P2_12_12_1$, and the cell dimensions are a = 4.871 (1) Å, b = 12.040 (1) Å, and c = 24.506 (1) Å. The structure was determined by direct methods, and least-squares refinement, which included all hydrogen atoms, converged at R = 0.031 for 1599 observed reflections. The conformation about the glycosidic bond is syn with $\chi_{CN} = -131.1°$. The ribose ring has a C(2′)-endo/C(1′)-exo ($^2T_1$) pucker, and the gauche$^+$ conformation of the $-CH_2OH$ side chain is stabilized by an intramolecular O(5′)–H···N(3) hydrogen bond. Conformational analysis by means of ^{1}H NMR spectroscopy showed that, in dimethyl sulfoxide, the sugar ring exhibits a marked preference for the C(2′)-endo conformation (~70%) and a conformation about the glycosidic bond predominantly syn (~90%), hence similar to that in the solid state. However, the conformation of the exocyclic 5′-CH_2OH group exhibits only a moderate preference for the gauche$^+$ rotamer (~40%), presumably due to the inability to form the intramolecular hydrogen bond to N(3) in a polar medium. The conformational features are examined in relation to the behavior of 8-substituted purine nucleosides in several enzymatic systems, with due account taken of the steric bulk and electronegativities of the 8-substituents.

Following the demonstration, some years ago, that 8-bromoadenosine and 8-bromoguanosine are in the syn conformation about the glycosidic bond in the solid state (Tavale & Sobell, 1970) and in solution (Sarma et al., 1974), it was widely assumed that a bulky substituent at C(8) of a purine nucleoside or nucleotide restricts the conformation to syn in solution. For 8-bromoadenosine, this inference derived support from theoretical and hard-sphere calculations [see Birnbaum & Shugar (1978)], suggesting that the entire anti range was excluded by close contacts between the bromine and ribose atoms. Its validity was subsequently placed in doubt by the demonstration that 8-bromoadenosine diphosphate ribose, cocrystallized with alcohol dehydrogenase, shows the adenine moiety in the same anti conformation as in adenosine diphosphate ribose (ADP-ribose) and in NAD$^+$ (Abdallah et al., 1975). This led to the synthesis of purine nucleosides with C(8) substituents sufficiently bulky as to unequivocally exclude existence of the anti conformation (e.g., Birnbaum & Shugar, 1978). Concurrently it has been established that, while 8-

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