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Synthesis and Biologic Activity of a C-Ring Analogue of Vitamin D_3: Biologic and Protein Binding Properties of 11α-Hydroxyvitamin D_3†

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ABSTRACT: The influence of C-ring substituents on the biologic activity and protein binding properties of vitamin D_3 has not been systematically investigated. To this end, we dehydrogenated cholesta-5,7-dien-3β-ol (**1**) to the 5,7,9(11)-triene (**3**). After protection of the 5,7-diene with a 4-phenyl-1,2,4-triazoline-3,5-dione Diels–Alder adduct, oxidation of the unprotected 9(11)-olefin gave epoxide **5**. Reverse Diels–Alder and reductive ring opening of epoxide **5** gave cholesta-5,7-diene-3β,11α-diol (**6**). Photolysis of **6** to the previtamin followed by thermal rearrangement resulted in 11α-hydroxyvitamin D_3 (**8**). We found that vitamin **8** increased calcium transport at a dose of 500 pmol/rat but failed to increase bone calcium mobilization at a dose as high as 50000 pmol/rat. Under the same conditions, corresponding doses of vitamin D_3 and 25-hydroxyvitamin D_3 increased bone calcium mobilization and intestinal calcium transport. The new vitamin analogue, **8**, was slightly less efficient (B-50 = 6.8×10^{-8} M) than 25-hydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3, and 25(*S*),26-dihydroxyvitamin D_3 (7.1×10^{-9} M, 7.7×10^{-9} M, and 7.9×10^{-9} M, respectively) in displacing radiolabeled 25-hydroxyvitamin D_3 from rat plasma vitamin D binding protein. On the other hand, vitamin analogue **8** showed significantly greater binding efficiency than 1α-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, and vitamin D_3 (B-50 = 2.5×10^{-6} M, 9.84×10^{-8} M, and 5.46×10^{-7} M, respectively), under these same conditions. Vitamin analogue **8** displayed approximately the same efficiency as vitamin D_3 in displacing radiolabeled 1,25-dihydroxyvitamin D_3 from a chick intestinal cytosol receptor but was less effective than 25-hydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3, 25(*S*),26-dihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3. We conclude that introduction of an 11α-hydroxyl group into the C-ring of vitamin D_3 results in a vitamin analogue with moderate vitamin D_3 agonist activity in the intestine but no activity with respect to bone calcium mobilization at the levels tested. 11α-Hydroxyvitamin D_3 does not have improved binding affinity to the intestinal cytosol receptor when compared to vitamin D_3. The new vitamin analogue shows significantly greater binding affinity to plasma vitamin D binding protein than vitamin D_3 (6.79×10^{-8} M vs. 5.46×10^{-7} M) or 1α-hydroxyvitamin D_3 (6.79×10^{-8} M vs. 2.5×10^{-6} M), suggesting that the presence of an extra hydroxyl group sufficiently removed from the 3β-hydroxyl is important in the binding of vitamin D analogues to vitamin D binding protein.

The vitamin D_3 endocrine system plays a central role in calcium and phosphorus homeostasis in many species (DeLuca & Schnoes, 1976, 1983). In order to act physiologically, vitamin D_3 first undergoes C-25-hydroxylation in the liver and subsequently C-1-hydroxylation in the kidney (DeLuca & Schnoes, 1976, 1983). 25-Hydroxyvitamin D_3 is considerably more active than vitamin D_3 in vivo, and 1,25-dihydroxyvitamin D_3 in turn is more active than 25-hydroxyvitamin D_3. Thus, the introduction of a hydroxyl group at C-25 and an α-hydroxyl group at C-1 plays an important role in determining the biological properties of the vitamin D_3 molecule (DeLuca & Schnoes, 1976, 1983). It is also known that shortening or lengthening of the side chain (Holick et al., 1975; Lam et al., 1975; Norman et al., 1979; Koizumi et al., 1979, Esvelt & DeLuca, 1981), alteration of the side chain by introduction of various functionalities (Onisko et al., 1979), expansion of the A-ring (Gerdes et al., 1981), alteration of the triene structure (Wecksler & Norman, 1980), and removal of or prevention of the introduction of hydroxyl groups at C-1, C-25, or C-3 decrease the biological activity of vitamin D_3 (Okamura et al., 1974; Lam et al., 1974; DeLuca & Schnoes, 1976, 1983). In contrast, certain C-24-, C-26-, and C-27-

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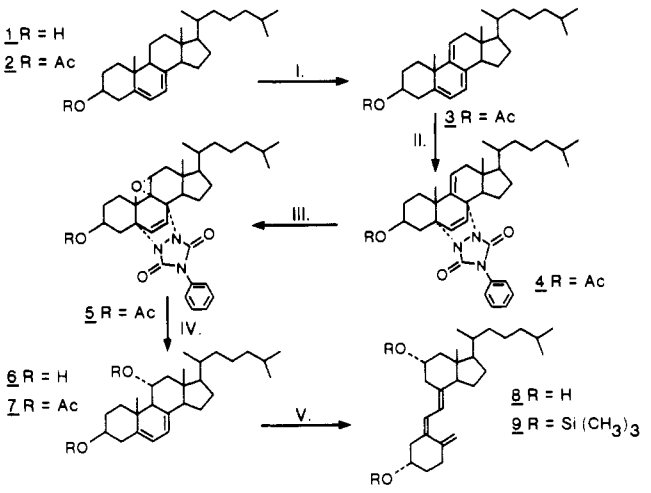

FIGURE 1: Synthesis of 11α-hydroxyvitamin D_3 (**8**): (I) oxidation with mercury(II) acetate; (II) Diels–Alder reaction with 4-phenyl-1,2,4-triazoline-3,5-dione; (III) oxidation with *m*-chloroperbenzoic acid; (IV) retro Diels–Alder reaction and reduction with LAH in THF; (V) photolysis and thermal rearrangement.

fluorinated analogues of 1,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 are more potent than their nonfluorinated parent compounds in some bioassay systems (Ikekawa, 1983).

Little is known of the biological effects of C-ring-substituted vitamin D_3 analogues. The synthesis of 11α-hydroxyvitamin D_3 from 3,11-dioxo-5β-cholestane has been reported; however, systematic biologic studies and protein binding studies were not carried out (Velluz & Amiard, 1961). We now report an efficient synthesis of 11α-hydroxyvitamin D_3[1] (**8**) from cholesta-5,7-dien-3β-ol (**1**) in five steps (Figure 1). In addition, we report its biologic activity in vivo and its binding properties in vitro.

Materials and Methods

General. Ultraviolet (UV) spectra were taken in ethanol with a Beckman Model 35 recording spectrophotometer (Beckman Instruments, Palo Alto, CA). Mass spectra were obtained on a Kratos MS50/DS-55 mass spectrometer–computer system (Kratos Instruments, U.K.). High-performance liquid chromatography (HPLC) was performed on a Waters liquid chromatograph equipped with two Model M-6000A pumps, a Model 450 UV detector, a Model 660 gradient programmer (all from Waters Associates, Milford, MA), and a Model 3380A Hewlett-Packard integrator (Hewlett-Packard, Avondale, PA). Nuclear magnetic resonance spectra (NMR) were obtained in deuterated chloroform with 0.1% tetramethylsilane on an IBM NR-80 Fourier-transform nuclear magnetic resonance spectrometer (IBM Instruments, Danbury, CT). $^{45}CaCl_2$ was obtained from New England Nuclear (New England Nuclear, Boston, MA). 25-Hydroxy[26,27-^{3}H]vitamin D_3 (23 Ci/mmol) and 1,25-dihydroxy[26,27-^{3}H]vitamin D_3 (158 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). 25-Hydroxyvitamin D_3, 25(*S*),26-dihydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and 1α,25-dihydroxyvitamin D_3 were gifts from Dr. Milan Uskokovic, Hoffman-La Roche (Nutley, NJ). Cholesta-5,7-dien-3β-ol and vitamin D_3 were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Biological Measurements. Serum calcium was measured in 0.1% lanthanum chloride as diluent with a Perkin-Elmer 2380 atomic absorption spectrometer (Perkin-Elmer Instruments, Norwalk, CT). Calcium transport in the duodenum was determined by using the everted gut sac method of Martin & DeLuca (1969). Radioligand binding assays using rat plasma vitamin D binding protein and chick intestinal cytosol receptor were performed as described previously (Kumar et al., 1979). The rats were administered the appropriate dose of the vitamin D_3 compound dissolved in 50 μL of ethanol 24 h before the experiment. Control rats received vehicle alone.

Animals. Male, weanling, albino rats (50–60 g) were obtained from the Holtzman Co. (Madison, WI). They were maintained in individual overhanging wire cages and fed a 0.02% calcium, 0.3% phosphorus, vitamin D deficient diet ad libitum for 4 weeks prior to use.

Syntheses (Figure 1). *(1) Acetylation of Cholesta-5,7-dien-3β-ol* (**1**). Cholesta-5,7-dien-3β-ol (25 g, 65 mmol) was acetylated with acetic anhydride and pyridine. After workup, 3β-acetoxycholesta-5,7-diene (**2**) (24 g, 56 mmol, 87%) was obtained as colorless platelets: mp 123–126 °C; UV λ_{max} 294 nm, 282, 272, 260; NMR δ 2.04 (s, 3 H, 3β-OOCCH$_3$), 4.70 (m, 1 H, 3α-H), 5.39, 5.57 (AB q, 2 H, J = 7.5 Hz, 6,7-H); mass spectrum m/z (assignment, relative intensity) 426 [M^+ ($C_{29}H_{46}O_2$: calcd 426.3486, found 426.3918), 1.5], 366 (M^+ – CH_3COOH, 100), 351 (M^+ – CH_3, 15), 253 (366 – side chain, 16).

(2) Dehydrogenation of 3β-Acetoxycholesta-5,7-diene (**2**) *(Windaus & Linsert, 1928).* 3β-Acetoxycholesta-5,7-diene (**2**) (23 g, 54 mmol) was dissolved in 900 mL of absolute ethanol with 0.5 mL of glacial acetic acid. Recrystallized mercury(II) acetate (55 g) was added, refluxed for 45 min, cooled to room temperature, and filtered to remove crystalline mercury(II) acetate. After concentration and addition of ethyl acetate (50 mL), the reaction mixture was filtered and concentrated under reduced pressure to a yellow oil, which was crystallized from methanol to result in 3β-acetoxycholesta-5,7,9(11)-triene (**3**) (9.4 g, 22 mmol, 40%) as colorless crystals: mp 84–86 °C; UV λ_{max} 340 nm, 325, 310; NMR δ 2.03 (s, 3 H, 3β-OOCCH$_3$), 4.70 (m, 1 H, 3α-H), 5.40 (m, 1 H, 11β-H), 5.40, 5.70 (AB q, 2 H, J = 6.4 Hz, 6,7-H); mass spectrum m/z (assignment, relative intensity) 424 [M^+ ($C_{29}H_{44}O_2$: calcd 424.2978, found 424.3389), 7], 364 (M^+ – CH_3COOH, 100), 349 (364 – CH_3, 30), 251 (364 – side chain, 14).

(3) Diels–Alder Reaction of 3β-Acetoxycholesta-5,7,9-(11)-triene (**3**) *with 4-Phenyl-1,2,4-triazoline-3,5-dione (Barton et al., 1971; Cookson et al., 1967).* 3β-Acetoxycholesta-5,7,9(11)-triene (**3**) (2.0 g, 4.7 mmol) was dissolved in a mixture of 30 mL of $CHCl_3$ and 30 mL of acetone (solvents anhydrous, freshly distilled). Under a positive pressure of argon, the solution was cooled to −78 °C in a dry ice–acetone bath. The addition of 4-phenyl-1,2,4-triazoline-3,5-dione (0.82 g, 4.7 mmol) dissolved in 10 mL of acetone was carried out dropwise over a period of 20 min. With the addition of the last drop, a faint pink color persisted. After being warmed to room temperature, the solvent was removed under reduced pressure to give a colorless gel, which was crystallized from aqueous acetone to result in 3β-acetoxy-5α,8α-(4-phenyl-1,2-urazolo)cholesta-6,9(11)-diene (**4**) (2.7 g, 4.6 mmol, 98%): mp 136–137 °C; NMR δ 2.02 (s, 3 H, 3β-OOCCH$_3$), 5.3 (m, 1 H, 3α-H), 5.60 (m, 1 H, 11-H), 6.25, 6.56 (AB q, 2 H, J = 11 Hz, 6,7-H), 7.39 (m, 5 H, aromatic); mass spectrum m/z (assignment, relative intensity) 599 [M^+, 0 (not seen)], 424 [M^+ – Diels–Alder adduct (calcd 424.2978, found 424.3353), 8], 364 (424 – CH_3COOH, 100), 349 (364 – CH_3, 18).

[1] Abbreviation: 11α-hydroxyvitamin D_3, 9,10-seco-5(*Z*),7(*E*),10(19)-cholestatriene-3β,11α-diol.

(*4*) *Oxidation of 3β-Acetoxy-5α,8α-(4-phenyl-1,2-urazolo)cholesta-6,9(11)-diene* (**4**) *with m-Chloroperbenzoic Acid.* A solution of **4** (2.7 g, 4.5 mmol) in 120 mL of anhydrous $CHCl_3$ with *m*-chloroperbenzoic acid (7.8 g, 45 mmol) was stirred at room temperature for 72 h. The reaction mixture was diluted with 200 mL of $CHCl_3$, extracted 4 times with 20% aqueous K_2CO_3, and extracted 3 times with water. The $CHCl_3$ solution was dried, filtered, and concentrated to a colorless foam, which was purified by chromatography (5 cm × 50 cm column packed with silica gel 60 with 5% ethyl acetate/95% $CHCl_3$ as an eluant). The resulting amorphous solid was crystallized from aqueous acetone to give 3β-acetoxy-5α,8α-(4-phenyl-1,2-urazolo)-9α,11α-epoxycholest-6-ene (**5**) (2.1 g, 3.4 mmol, 76%) as colorless needles: mp 147–148 °C; NMR δ 2.02 (s, 3 H, 3β-OOCCH$_3$), 3.30 (m, 1 H, 11-H), 5.40 (m, 1 H, C-3), 6.30, 6.53 (AB q, 2 H, J = 11 Hz, 6,7-H), 7.34 (m, 5 H, aromatic); mass spectrum m/z (assignment, relative intensity) 615 [M^+ ($C_{37}H_{49}N_3O_5$: calcd 615.3660, found 615.3565), 9], 423 [M^+ – (Diels–Alder adduct + OH), 8], 422 [M^+ – (Diels–Alder adduct + H_2O), 5], 363 (423 – CH_3COOH, 14), 362 (422 – CH_3COOH, 19), 177 (Diels–Alder adduct + 2H, 100).

(*5*) *Reduction of 3β-Acetoxy-5α,8α-(4-phenyl-1,2-urazolo)-9α,11α-epoxycholest-6-ene* (**5**) *with Lithium Aluminum Hydride.* A solution of **5** (300 mg, 0.49 mmol) with 500 mg of lithium aluminum hydride in 40 mL of anhydrous tetrahydrofuran was refluxed for 15 h. The reaction mixture was quenched with aqueous ethyl acetate, filtered, dried over Na_2SO_4, and concentrated under reduced pressure to a yellow oil, which was purified on a silica gel 60H column with an eluant of 15% ethyl acetate/85% $CHCl_3$. The resulting amorphous solid was crystallized from hexane to give cholesta-5,7-diene-3β,11α-diol (**6**) (90 mg, 22.5 mmol, 44%) as colorless needles: mp 146–148 °C; $[\alpha]^{20}_D$ –28° (ethanol); UV λ_{max} 294 nm, 282, 272, 263; NMR δ 3.60 (m, 1 H, 3α-H), 4.25 (m, 1 H, 11β-H), 5.38, 5.54 (AB q, 2 H, J = 8 Hz, 6,7-H); mass spectrum m/z (assignment, relative intensity) 400 [M^+ ($C_{27}H_{44}O_2$: calcd 400.3321, found 400.3222), 14], 383 (M^+ – OH, 16), 382 (M^+ – H_2O, 55), 367 (382 – CH_3, 18), 365 (382 – OH, 16), 364 (M^+ – $2H_2O$, 49), 349 (364 – CH_3, 45).

(*6*) *Acetylation of Cholesta-5,7-diene-3β,11α-diol* (**6**) (*Lardon & Reichstein, 1954*). A solution of **6** (10 mg, 0.025 mmol) was acetylated with pyridine and acetic anhydride. After workup, 3β,11α-diacetoxycholesta-5,7-diene (**7**) (7 mg, 0.015 mmol, 60% yield) was obtained as an amorphous solid: NMR δ 2.02 (s, 3 H, 11α-OOCCH$_3$), 2.04 (s, 3 H, 3β-CH_3COO), 4.70 (m, 1 H, 3α-H), 5.40 (m, 1 H, 11β-H), 5.40, 5.59 (AB q, 2 H, J = 8 Hz, 6,7-H); mass spectrum m/z (assignment, relative intensity) 484 [M^+ ($C_{31}H_{48}O_4$), O (not seen)], 424 (M^+ – CH_3COOH, 5), 364 (M^+ – $2CH_3COOH$, 100), 349 (364 – H_2O, 18).

(*7*) *Photolysis of Cholesta-5,7-diene-3β,11α-diol* (**6**) (*Dauben & Phillips, 1982*). A solution of **6** (25 mg, 0.0625 mmol) in 100 mL of anhydrous ether at 0–2 °C was irradiated at λ = 253.7 nm for 2000 s. Irradiation was repeated at λ = 350.0 nm for another 2000 s. The solvent was removed under reduced pressure to give a yellow oil, which was purified by preparative HPLC (Varian Micropak MCH-10 column with 10% 2-propanol/90% hexane, 8 mL/min) to give 11α-hydroxyvitamin D_3 (**8**) (3.2 mg,[2] 0.007 mmol, 11%): UV λ_{max} 262 nm, λ_{min} 228 nm ($\lambda_{max}/\lambda_{min}$ = 1.76); NMR δ 0.51 (s, 3 H, 18-CH_3), 3.90 (m, 2 H, 3α-H and 11β-H), 4.80 [m, 1 H, 19(*E*)-H], 5.03 [m, 1 H, 19(*Z*)-H], 6.13, 6.24 (AB q, J = 20 Hz, 6,7-H); mass spectrum m/z (assignment, relative intensity) 400 [M^+ ($C_{27}H_{44}O_2$: calcd 400.3330, found 400.3239), 0.3], 382 (M^+ – H_2O, 100), 364 (M^+ – $2H_2O$, 12), 349 (364 – CH_3, 4.2), 269 (382 – side chain, 28), 251 (269 – H_2O, 19), 136 (A-ring plus carbons 6 and 7, 67), 118 (136 – H_2O, 78).

Bis(tetramethylsilyl) Ether Derivative of 11α-Hydroxyvitamin D_3 (**9**). **9** gave the following mass spectral data: mass spectrum m/z (assignment, relative intensity) 544 (M^+, 36), 454 [M^+ – $(CH_3)_3SiOH$, 55], 439 (454 – CH_3, 10), 364 [M^+ – $2(CH_3)_3SiOH$, 7.5], 349 (364 – CH_3, 10), 251 (364 – side chain, 25), 208 (A-ring plus carbons 6 and 7, 12), 118 [208 – $(CH_3)_3SiOH$, 50].

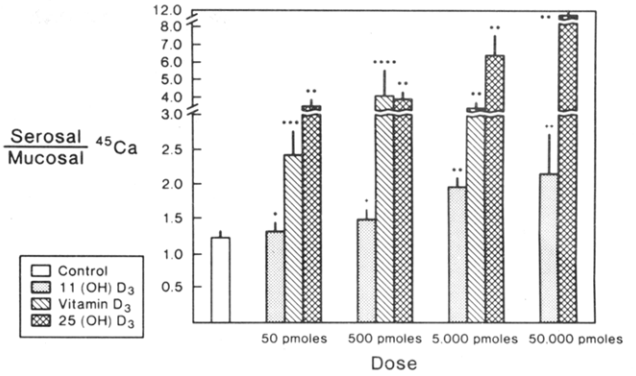

FIGURE 2: Intestinal calcium transport responses following the administration of varying doses of 11α-hydroxyvitamin D_3, vitamin D_3, or 25-hydroxyvitamin D_3 intrajugularly to rats raised on a vitamin D deficient, low-calcium diet: (*) P = NS; (**) P = ≤0.001; (***) P = 0.005; (****) P = 0.013; (+) P = 0.02; (++) P = 0.06.

Results

We report an efficient, five-step synthesis of 11α-hydroxyvitamin D_3 (**8**) from cholesta-5,7-dien-3β-ol (**1**) (Aldrich). Oxidation of 5,7-diene **2** with mercury(II) acetate gives 5,7,9(11)-triene **3** in 40% yield (Windaus & Linsert, 1928). 4-Phenyl-1,2,4-triazoline-3,5-dione reacts with triene **3** in Diels–Alder fashion to give cyclo adduct **4** in 98% yield (Barton et al., 1971; Cookson et al., 1967). The 5,7-diene protecting group serves to direct the epoxidation to the 9-(11)-olefin. Oxidation of protected triene **4** with *m*-chloroperbenzoic acid results in 9α,11α-epoxide **5** in 76% yield. The reaction of **5** with lithium aluminum hydride in refluxing tetrahydrofuran results in a retro Diels–Alder reaction and a reductive epoxide ring opening to give 11α-hydroxy-5,7-diene **6** in 44% yield. The photolysis of **6** followed by thermal rearrangement gives 11α-hydroxyvitamin D_3 (**8**) in 11% yield.

As shown in Figure 2, the ability of **8** to increase intestinal calcium transport is less than vitamin D_3 or 25-hydroxyvitamin D_3; however, **8** is able to elicit a response at a dose of 500 pmol/rat. Vitamin **8** has no biologic activity when tested in a bone calcium mobilization system at the doses tested (Table I). The B-50 value[3] of each vitamin tested (Figure 3) is listed in order of decreasing ability to displace radiolabeled 25-hydroxyvitamin D_3 from rat plasma vitamin D binding protein: 25-hydroxyvitamin D_3 (7.1×10^{-9} M), 24(*R*),25-dihydroxyvitamin D_3 (7.7×10^{-9} M), 25(*S*),26-dihydroxyvitamin D_3 (7.9×10^{-9} M), *11α-hydroxyvitamin D_3* (*6.79×10^{-8} M*), 1,25-dihydroxyvitamin D_3 (9.84×10^{-8} M), vitamin D_3 (5.46×10^{-7} M), and 1α-hydroxyvitamin D_3 (2.5×10^{-6} M). The B-50 value of each vitamin tested (Figure 4) is listed in order of decreasing ability to displace radiolabeled 1,25-dihydroxy-

[2] Weight of purified vitamin was determined from UV measurements by assuming the ε of **8** to be 18 200.

[3] B-50 value is defined as the concentration of material necessary to cause 50% displacement of the radiolabel.

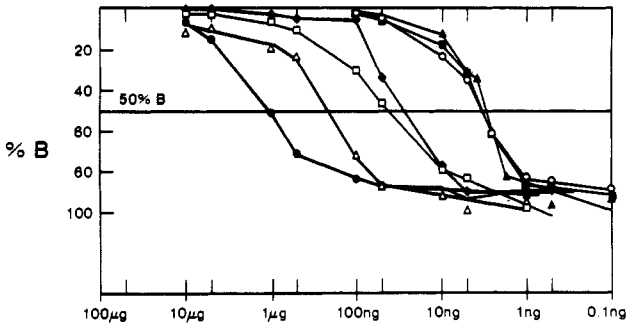

FIGURE 3: Competitive binding assay using rat plasma vitamin D binding protein and various vitamin D_3 compounds. [^{3}H]-25-Hydroxyvitamin D_3 was used as the radioligand; figures on abscissa refer to amount of analogue added per tube: (△) D_3; (♦) 11(OH)D_3; (○) 25,26(OH)$_2D_3$; (▲) 25(OH)D_3; (●) 1α(OH)D_3; (■) 24,25-(OH)$_2D_3$; (□) 1,25(OH)$_2D_3$.

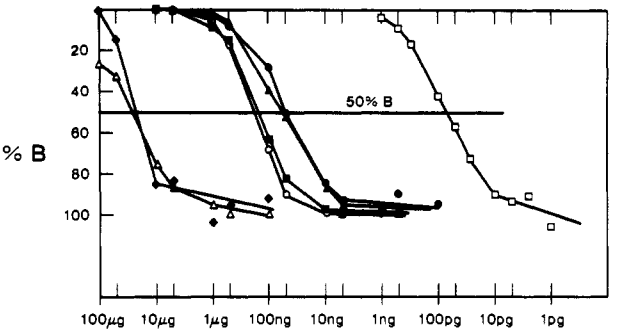

FIGURE 4: Competitive binding assay using chicken intestine cytosol receptor preparation and various vitamin D_3 compounds. [^{3}H]-1,25-Dihydroxyvitamin D_3 was used as the radioligand; figures on abscissa refer to amount of analogue added per tube: (△) D_3; (♦) 11(OH)D_3; (○) 25,26(OH)$_2D_3$; (▲) 25(OH)D_3; (●) 1α(OH)D_3; (■) 24,25(OH)$_2D_3$; (□) 1,25(OH)$_2D_3$.

Table I: Serum Calcium Values (mg/dL) 24 h following the Administration of the Various Vitamin D_3 Compounds[a]

dose/ rat (pmol)	11α(OH)D_3	vitamin D_3	25(OH)D_3
50	2.51 ± 0.06 (9)[b]	2.60 ± 0.14 (9)[b]	3.16 ± 0.13 (8)[b]
500	2.63 ± 0.08 (9)[b]	4.03 ± 0.17 (6)[c]	3.63 ± 0.11 (6)[c]
5000	2.58 ± 0.07 (8)[b]	3.87 ± 0.14 (7)[c]	3.53 ± 0.16 (6)[d]
50000	2.60 ± 0.10 (7)[b]	3.46 ± 0.12 (5)[d]	3.90 ± 0.10 (5)[c]

[a] The values are mean ± SE. The values of serum calcium for rats that had received vehicle alone were 2.84 ± 0.12 mg/dL. Figures in parentheses refer to the number of animals in each group. [b] P = NS. [c] $P < 0.001$. [d] $P \leq 0.004$.

vitamin D_3 from a chick intestinal cytosol receptor preparation: 1,25-dihydroxyvitamin D_3 (1.62×10^{-10} M), 1α-hydroxyvitamin D_3 (1.19×10^{-7} M), 25-hydroxyvitamin D_3 (1.38×10^{-7} M), 24(*R*),25-dihydroxyvitamin D_3 (3.48×10^{-7} M), 25(*S*),26-dihydroxyvitamin D_3 (4.16×10^{-7} M), vitamin D_3 (6.45×10^{-5} M), and *11α-hydroxyvitamin D_3* (**8**) (5.3×10^{-5} *M*).

Discussion

Vitamin analogue **8** is a vitamin D_3 agonist that is effective in increasing intestinal calcium transport at doses as low as 500 pmol/rat. At the corresponding and higher doses, **8** does not increase bone calcium mobilization. The inability of **8** to mobilize bone calcium may be due to either a unique property of this vitamin or the relative insensitivity of bone when compared to intestinal calcium transport. Higher doses may have elicited bone calcium mobilization.

Examination of the binding properties of the vitamin D_3 analogues to rat plasma vitamin D_3 binding protein (Figure 3) reveals several interesting facts. Vitamin D_3 itself exhibits poor binding properties. The addition of a second hydroxyl group on the A-ring results in an analogue (1α-hydroxyvitamin D_3) with decreased binding efficiency. The addition of a second hydroxyl group further away from the 3β-hydroxy group on the C-ring results in an analogue (11α-hydroxyvitamin D_3) with greatly increased binding efficiency. The addition of the second hydroxyl group in the side chain even further away from the 3β-hydroxy group results in an analogue (25-hydroxyvitamin D_3) with even better binding properties. The addition of a second hydroxyl group to the side chain of 25-hydroxyvitamin D_3 results in analogues [24(*R*),25-dihydroxyvitamin D_3 and 25(*S*),26-dihydroxyvitamin D_3] that show similar binding properties to 25-hydroxyvitamin D_3. The addition of a second hydroxyl group to the A-ring of 25-hydroxyvitamin D_3 results in an analogue (1α,25-dihydroxyvitamin D_3) that shows poorer binding efficiency than 25-hydroxyvitamin D_3. In contrast to the improved binding properties of 11α-hydroxyvitamin D_3 (**8**) to rat plasma vitamin D binding protein, **8** did not show improved binding characteristics to the intestinal cytosol receptor.

In view of the above observations, the following tentative generalization seems to be valid. The binding efficiency of vitamin D_3 analogues to rat plasma binding protein is dependent upon the presence of a second hydroxyl group positioned a critical distance away from the 3β-hydroxyl group of the parent vitamin D_3. Those analogues with a second hydroxyl group at C-25 show optimal binding properties. Those analogues with a second hydroxyl group positioned an intermediate distance away (e.g., C-11) show intermediate binding properties. Vitamin D_3 analogues with a second hydroxyl group positioned in close proximity to the 3β-hydroxyl group show poorest binding properties. The above observations could be interpreted to mean that there are only two active sites on each binding protein unit and that the intramolecular hydroxyl group distance on 11α-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3 closely approximates the distance between these active sites. With the synthesis and testing of new C- and D-ring analogues of vitamin D_3, one could formulate more accurately the relationship between the binding properties and intramolecular hydroxyl group distance of vitamin D_3 analogues. One could obtain in this manner additional indirect information concerning the structure and distance between active sites on the binding protein.

The reductive ring opening of 9α,11α-epoxide **5** is novel from several standpoints. We suggest that epoxide **5** opens to give the 11α-hydroxy product **6** rather than the predicted tertiary alcohol 9α-hydroxy product because the intermediate involved in the formation of **6** features a carbonium ion at C-9 conjugated to the 5,7-diene as a pentadienyl cation. In support of this argument, an analogous steroid reaction is given. The reductive ring opening of a 9α,11α-epoxy-substituted estriol under similar reducing conditions also gives the atypical 11α-hydroxy-substituted product (Tsuda et al., 1966). Note that a postulated intermediate would also feature a carbonium at C-9, in this case stabilized as a benzyl cation. In those analogous reactions in which resonance stabilization of the tertiary carbonium ion is not present, ring opening gives the tertiary hydroxy-substituted steroid (Djerassi, 1963). Although a careful isolation by HPLC and spectroscopic analysis of the minor products failed to reveal the presence of the predicted 9α-hydroxy tertiary alcohol, the triene cholesta-5,7,9(11)-trien-3β-ol was found to be present. It is reasonable to argue that the triene could have arisen from the dehydration of the

9α-hydroxy product. Also, the fact that the isolated yield of the 11α-hydroxy product **6** is only 44% leads one to speculate that the 9α-hydroxy product may not have survived the reaction conditions.

The stereochemistry of the 11-hydroxy substituent in **6** has been assigned as "α" on the basis of the following data. (I) The peracid oxidation of 9(11)-olefinic steroids usually results in 9α,11α-epoxides (CIBA, Ltd., 1956; von Heusser et al., 1951; Henbest & Wrigley, 1957; Djerassi et al., 1952). (II) The chemical shift of the 11-H (δ 4.2) is consistent with the chemical shift of 11-H of previously reported 11α-hydroxy steroids (Anastasia et al., 1983; Shoppee et al., 1968). (III) Diol **6** is acetylated at 20 °C to give the diacetate derivative **7**. Under similar conditions, 3β,11β-diol steroids usually react to give selective acetylation at the 3β-position (Lardon & Reichstein, 1954). (IV) The specific rotation of **6**, $[\alpha]^{20}_D$ = −28° (ethanol), reveals that the 11α-hydroxy substituent exerts a positive influence on the optical rotation of cholesta-5,7-dien-3β-ol (**1**), $[\alpha]^{20}_D$ = −69° (ethanol). 1α-Hydroxy-substituted **1** shows a similar positive influence on the optical rotation of unsubstituted **1** (Barton et al., 1973).

In conclusion, we have synthesized 11α-hydroxyvitamin D_3 (**8**) in five steps from cholesta-5,7-dien-3β-ol (**1**). The scheme effectively utilizes 4-phenyl-1,2,4-triazoline-3,5-dione as a Diels–Alder adduct and protecting group during epoxidation. Also, the synthesis features an unusual reductive ring opening of 9α,11α-epoxide **5** by lithium aluminum hydride to give 11α-hydroxy provitamin **6**. The biological properties of **8** were tested in vitamin D deficient rats raised on a low calcium diet. Vitamin analogue **8** displays vitamin D_3 agonist activity in the intestine but does not mobilize bone calcium in the doses we used. Whereas vitamin D_3 analogue **8** binds more effectively than vitamin D_3 to the rat plasma vitamin D binding protein, it shows no appreciable binding differences when compared to vitamin D_3 in a chick intestinal receptor system.

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