BIOORGANIC CHEMISTRY 9, 187–198 (1980)

# Inhibitors of the 25-Hydroxylation of Vitamin $D_3$ in the Rat

BRUCE L. ONISKO, HEINRICH K. SCHNOES, AND HECTOR F. DELUCA

*Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin, 53706*

*Received December 4, 1978*

Two new sidechain-modified analogs of vitamin $D_3$, 25-azavitamin $D_3$ and 25-fluorovitamin $D_3$, were prepared; both compounds were found to inhibit the *in vivo* 25-hydroxylation of vitamin $D_3$ in the rat. 25-Azavitamin $D_3$ was chemically synthesized from a degradation product of stigmasterol by a six-step process. The desired carbon skeleton was efficiently assembled by alkylation of a suitably protected C-20 bromomethylpregnane with the enolate of *N,N*-dimethylacetamide (70%). The completion of the synthesis utilized the known photochemistry of steroidal 5,7-dienes to prepare the vitamin D triene system. In contrast, 25-fluorovitamin $D_3$ was prepared by direct vitamin modification. 25-Hydroxyvitamin $D_3$ 3-acetate was fluorinated with diethylaminosulfur trifluoride to give 25-fluorovitamin $D_3$ 3-acetate (59%); saponification provided the desired analog. When vitamin D-deficient rats on a low calcium diet were dosed with [3-$^3$H]vitamin $D_3$ (0.05 $\mu$g), 10% of the dose was found in serum as 25-hydroxyvitamin $D_3$ 4 hr after administration. If 25-azavitamin $D_3$ (50 or 200 $\mu$g) was given 2 hr before the radiolabeled vitamin $D_3$, however, serum 25-hydroxyvitamin $D_3$ concentration was markedly reduced. 25-Fluorovitamin $D_3$ caused similar reduction when administered at much lower doses.

## INTRODUCTION

The biological responses elicited by vitamin $D_3$ in vertebrates are now known to be the result of the direct cellular action of one of its metabolites, 1$\alpha$,25-dihydroxyvitamin $D_3$ (1$\alpha$,25-$(OH)_2D_3$)[1] (*1*). The two-step metabolic activation of vitamin D is essential, and consists of (1) the hydroxylation of vitamin $D_3$, primarily in the liver, to produce 25-hydroxyvitamin $D_3$ (25-OH-$D_3$), and (2) subsequent hydroxylation at C-1 to 1$\alpha$,25-$(OH)_2D_3$ in the kidney. Inhibition of vitamin $D_3$ metabolism is thus expected to lead to the abolition of the physiological response. Inhibition of the 25-hydroxylation of vitamin $D_3$ would be most effective since the alternative, inhibition of the kidney 1$\alpha$-hydroxylase, might not prevent accumulation of 25-OH-$D_3$, a metabolite which can act directly on target tissues at high concentration (*2, 3*). Inhibitors of 25-hydroxylation, therefore, can provide useful tools for the study of vitamin D metabolism, vitamin D toxicity, and the mode of action of vitamin D metabolites in various tissues. There is also considerable clinical interest in such compounds as potentially effective agents for the reduction of the hypercalcemia caused by a variety of disorders (*4, 5*).

[1] Abbreviations used: 1$\alpha$,25-$(OH)_2D_3$, 1$\alpha$,25-dihydroxyvitamin $D_3$; 25-OH-$D_3$, 25-hydroxyvitamin $D_3$.



0045-2068/80/020187-12$02.00/0

Glucocorticoids (*6*) and anticonvulsants (*7*) are known to antagonize the vitamin $D_3$-mediated intestinal calcium transport response. The primary effects of these substances, however, complicate their application as research tools for the study of vitamin D action, and severely limit their use as therapeutic agents for the maintenance of calcium homeostasis. Since analogs structurally related to vitamin D should avoid this problem, we have undertaken the preparation of two compounds—25-azavitamin $D_3$ and 25-fluorovitamin $D_3$—conceived specifically as inhibitors of the 25-hydroxylation of vitamin D. This report describes the preparation of these substances and demonstrates the ability of each compound (**7, 11**), to inhibit the 25-hydroxylation of vitamin D *in vivo*.

## MATERIALS AND METHODS

*Instruments.* Mass spectra were obtained on an AEI Model MS-902 mass spectrometer at 70 eV using a direct probe for introduction of samples (source temperature 110–130°C above ambient); high-resolution mass spectra were measured on the same instrument coupled to an AEI Model DS-50 data system and using perfluoro-kerosene as internal mass standard. uv absorption spectra were recorded on a Beckman Model 25 instrument; nmr spectra were taken on either a Bruker 270 MHz or Bruker 90 MHz spectrometer using $CDCl_3$ as solvent and tetramethylsilane as internal standard; ir absorption spectra were obtained with a Perkin-Elmer Model 567 instrument; optical rotations were measured on a Perkin-Elmer Model 141 polarimeter with a Brinkman Instruments Model K-2/R temperature controller. Gas–liquid chromatography was carried out on a Packard Model 417 chromatograph equipped with a glass column (2 mm × 2 m) packed with 3% OV-101 on Chromsorb 30 (100/120 mesh), operated isothermally at indicated temperatures with a $N_2$ flow rate of 30 ml/min. Melting points were measured on a Thomas Hoover appartus and are uncorrected. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Illinois.

*Solvents.* Commercial Skellysolve B was distilled and the fraction boiling at 67–69°C was used. Pyridine and benzene were distilled before use and dried over molecular sieves. Tetrahydrofuran was dried by distillation from lithium aluminum hydride. Other solvents were reagent grade and used as such.

*Chromatography.* Column chromatography was done on either silica gel (Davison Chemical, grade 923, 100-200 mesh), Sephadex LH-20 (Pharmacia, swelled in column solvent 24 hr prior to packing), or neutral Alumina (BioRad, AG-7, minus 200 mesh). For analytical tlc, air-dried silica gel G or Aluminum oxide G plates (0.25 mm thick) were prepared. The former were used unless otherwise stated. For preparative tlc, air-dried plates (20 × 20 cm, 0.75 mm thick) were prepared from a degassed slurry of 1/1 silica gel H and silica gel PF-254 in either water or 0.5 *M* aqueous KOH (*8*).

*Synthesis of 25-Azavitamin $D_3$* (**7**)

*(20S)-6β-Methoxy-20-(p-toluenesulfonoxymethyl)-3α,5-cyclo-5α-pregane* (**1**).

This tosylate was obtained in overall yield of 61% from stigmasterol according to the method of Partridge *et al.* (*9*). Crystallization from acetonitrile gave white crystals of **1** exhibiting: mp 144–145°C; $[\alpha]_D^{20} = +34°$ (*c* 0.98, $CHCl_3$); ir ($CCl_4$) 1190 and 1180 $cm^{-1}$ (sulfonate), 1100 $cm^{-1}$ (CO); nmr (90 MHz) δ 7.79 and 7.34 (AB, $J$ = 8 Hz, 4H, tosylate), 3.98 (d of d, $J$ = 9.0 and 3.4 Hz, 1H, C-22), 3.78 (d of d, $J$ = 9.0 and 5.9 Hz, 1H, C-22), 3.31 (s, 3H, OMe), 2.76 (d of d, $J$ = 2.7 and 2.7 Hz, 1H, C-6), 2.45 (s, 3H, tosylate), 1.01 (s, 3H, C-19), 0.98 (d, $J$ = 6 Hz, 3H, C-21), 0.68 (s, 3H, C-18), 0.42 (d of d, $J$ = 8 and 5 Hz, 1H, C-4); *m/e* (rel. intensity) 500 ($M^+$, 19), 485 (12), 468 (68), 445 (21), 296 (45), 281 (28), 91 (100); homogeneous on tlc ($R_f$ = 0.50, 20% ethyl acetate in Skellysolve B); *Anal.* Calcd for $C_{30}H_{44}O_4S$: C, 71.96; H, 8.86. Found: C, 71.36; H, 8.93. Lit. mp 144–145°C; $[\alpha]_D^{25} = +30.8°$ (*c* 1.0, $CHCl_3$), nmr ($CDCl_3$) δ 7.50, 3.85, 3.25, 2.70, 2.38, 0.95, 0.93, 0.62 (*9*).

*(20S)-20-Bromomethyl-6β-methoxy-3α,5-cyclo-5α-pregnane* (**2**). A mixture of 2.30 g tosylate **1**, 314 ml of acetonitrile, and 3.51 g of dry powdered lithium bromide was heated at reflux for 6.5 hr. The solvent was evaporated and the solids were dissolved in 150 ml of $H_2O$ and extracted with dichloromethane (3×, 50 ml each). The combined extracts were washed twice with 50 ml of $H_2O$, once with 50 ml of saturated salt, dried over sodium sulfate, and flash evaporated. Crystallization from aqueous ethanol gave 1.88 g (85%) of **2**: mp 63.5–65°C; $[\alpha]_D^{20} = +55°$ (*c* 1.60, $CHCl_3$); ir ($CCl_4$) 1100 $cm^{-1}$ (CO); nmr (90 MHz) δ 3.52 (d of d, $J$ = 9.8 and 2.8 Hz, 1H, C-22), 3.34 (d of d, $J$ = 9.8 and 5.4 Hz, 1H, C-22), 3.32 (s, 3H, OMe), 2.77 (d of d, $J_1 = J_2$ = 2.9 Hz, 1H, C-6), 1.09 (d, $J$ = 6.3 Hz, 3H, C-21), 1.02 (s, 3H, C-19), 0.75 (s, 3H, C-18), 0.66 (d of d, $J$ = 7.9 and 4.0 Hz, 1H, C-4), 0.43 (d of d, $J$ = 7.9 and 5.0 Hz, 1H, C-4); mass spectrum, *m/e* (rel. intensity) 410 (52), 408 (48), 395 (40), 393 (42), 378 (98), 376 (100), 355 (78), 353 (85); homogeneous on tlc ($R_f$ = 0.70, 10% ethyl acetate in Skellysolve B); 98% pure by gc ($t_R$ = 4.1 min, oven 250°C isothermal); *Anal.* Calcd for $C_{23}H_{37}OBr$: C, 67.47; H, 9.11. Found: C, 67.49; H, 9.23.

*6β-Methoxy-3α,5-cyclo-5α-cholanic acid dimethylamide* (**3**). To a dry flask charged with 14.5 ml of tetrahydrofuran, 3.6 ml of hexane, and 1.55 ml of diisopropylamine was added 4.9 ml of 1.79 *M* *n*-butyl lithium which had been titrated according to the procedure of Gilman and Cartledge (*10*). The mixture was maintained under $N_2$ at 0°C for 0.5 hr, then cooled to −78°C; 1.63 ml of dimethylacetamide was added, and the mixture was stirred at −78°C for 1.25 hr. The bromide (**2**, 1.2 g in 15 ml of tetrahydrofuran) was added, and the mixture stirred at 0°C for 21 hr. A few chips of ice and 50 ml of 5% HCl were added, followed by extraction with dichloromethane (3×, 50 ml each). The combined organic extracts were washed with saturated salt, dried ($Na_2SO_4$), and flash evaporated. The residue was chromtographed on a column of silica gel (250 g). Elution with ethyl acetate gave 0.85 g (70%) of **3** as an oil (tubes 34 to 67, 15 ml fractions). The oil, which resisted crystallization, showed: ir ($CCl_4$) 1655 $cm^{-1}$ (amide), 1100 $cm^{-1}$ (CO); nmr (90 MHz) δ 3.32 (s, 3H, OMe), 3.00 (s, 3H, NMe), 2.93 (s, 3H, NMe), 2.77 (d of d, $J_1 = J_2$ = 2.9 Hz, 1H, C-6), 1.02 (s, 3H, C-19), 0.94 (d, $J$ = 5.9 Hz, 3H, C-21), 0.72 (s, 3H, C-18), 0.43 (d of d, $J$ = 8.3 and 4.8 Hz, 1H, C-4); *m/e* (rel. intensity) 415 ($M^+$, 6), 400 (7), 383 (26), 368 (8), 360 (13), 100 (32), 87 (100); high-resolution mass spectrum calcd for $C_{27}H_{45}NO_2$: 415.345.

Found: 415.343; homogeneous on tlc ($R_f$ = 0.55, ethyl acetate); 91% pure by gc ($t_R$ = 10.1 min, oven 250°C isothermal).

*3β-Acetoxy-5-cholenic acid dimethylamide* (**4**). A solution of 1.86 g of cyclosteroid **3** in 75 ml of glacial acetic acid was heated to 70°C for 18 hr. The mixture, after cooling, was neutralized with 10% aqueous sodium hydroxide and extracted three times with ethyl acetate (100 ml each). The combined organic extracts were washed three times with 10% aqueous sodium hydroxide (50 ml each), once with 50 ml of water and once with 50 ml of saturated salt solution. Flash evaporation of solvent gave an amorphous solid that was crystallized from hexane to provide 1.84 g (93%) of **4**: mp 192–193.5°C; $[\alpha]_D^{20} = -41°$ (*c* 1.1, $CHCl_3$); ir ($CCl_4$) 1733 and 1245 $cm^{-1}$ (acetate), 1651 $cm^{-1}$ (amide); nmr (90 MHz) δ 5.37 (m, 1H, C-5), 4.62 (m, 1H, C-3), 3.00 (s, 3H, NMe), 2.93 (s, 3H, NMe), 2.03 (s, 3H, acetate), 1.02 (s, 3H, C-19), 0.95 (d, $J$ = 5.9 Hz, 3H, C-21), 0.68 (s, 3H, C-18); *m/e* (rel. intensity) 443 ($M^+$, 0.8), 428 (0.6), 383 (65), 368 (6), 100 (70), 87 (100); homogeneous on tlc ($R_f$ = 0.55, ethyl acetate); 96% pure by gc ($t_R$ = 28.6 min, oven 250°C isothermal); *Anal.* Calcd for $C_{28}H_{45}NO_3$: C, 75.80; H, 10.22; N, 3.16. Found: C, 75.50; H, 10.30; N, 3.09. Lit. mp 184–186.5°C; $[\alpha]_D^{26} = -41.°C$ (*11*).

*4-Phenyl-1,2,4-triazolin-3,5-dione adduct of 3β-acetoxy-cholane-5,7-dienoic acid dimethylamide* (**5**). A mixture of 500 mg amide **4**, 45 ml of carbon tetrachloride, 665 mg sodium bicarbonate, and 194 mg 1,3-dibromo-5,5-dimethylhydantoin was refluxed under nitrogen for 0.3 hr. The solution was cooled to 0°C and the solid hydantoin was removed by filtration. The filtrate was evaporated, redissolved in 5.0 ml of xylene, and added dropwise to a mixture of 300 mg collidine in 6.5 ml of xylene at 140°C. The reaction mixture was maintained at this temperature under $N_2$ for 1.5 hr, cooled, diluted with 100 ml of benzene, and washed with 50 ml each of 5% HCl, 4% $NaHCO_3$, and finally saturated salt solution. After drying over $Na_2SO_4$ and evaporating the solvent at reduced pressure, the resulting oil was crystallized with 5.0 ml of acetone. The crystals (containing both 4,6 and 5,7 dienes) were dissolved in 50 ml of ethyl acetate, cooled to 0°C, and titrated with 10.5 ml of a 2 mg/ml solution of 4-phenyl-1,2,4-triazolin-3,5-dione in ethyl acetate. After removing the solvent, the residue was purified by preparative tlc (silica gel) developed with 3% MeOH in $CHCl_3$. Crystallization from hexane provided 87 mg (13%) of **5**: mp 122–125°C; $[\alpha]_D^{20} = 77°$ (*c* 0.49, $CHCl_3$); uv (EtOH) $\lambda_{max}$ 255 nm (ε 3,900); ir ($CCl_4$) 1736 and 1242 $cm^{-1}$ (acetate), 1757 and 1705 $cm^{-1}$ (triazoline), 1652 $cm^{-1}$ (amide); nmr (90 MHz) δ 7.39 (m, 5H, phenyl), 6.42 and 6.22 (AB, $J$ = 8 Hz, 2H, C-6 and C-7), 5.42 (t of t, $J$ = 10 and 5 Hz, 1H, C-3), 3.28 (d of d, $J$ = 13 and 5 Hz, 1H, C-4), 3.00 (s, 3H, NMe), 2.93 (s, 3H, NMe), 2.01 (s, 3H, acetate), 0.98 (s, 3H, C-19), 0.95 (d, $J$ = 5 Hz, 3H, C-21), 0.81 (s, 3H, C-18); *m/e* (rel. intensity) 616 ($M^+$, absent), 439 (2), 381 (35), 366 (35), 311 (11), 177 (22), 100 (33), 87 (100); homogeneous on tlc ($R_f$ = 0.53, 3% MeOH in $CHCl_3$).

*25-Aza-7-dehydrocholesterol* (**6**). To a mixture of 61.1 mg adduct **5** in 13 ml of dry distilled tetrahydrofuran was added 76 mg of lithium aluminum hydride. The mixture was refluxed under $N_2$ for 7.0 hr, then the reaction was terminated by the addition of 5.0 ml of water. The white precipitate was removed by filtration and water (20 ml) was added to the filtrate to give a two-phase system. The organic

layer was removed and the aqueous phase extracted three times with dichloromethane (20 ml each). The combined organic extracts were dried over $Na_2SO_4$, solvents were removed at reduced pressure, and the residue was chromatographed on 75 g of aluminum oxide. Elution with 3% MeOH in $CHCl_3$ gave (in tubes 23 to 34, 3.5 ml fractions) an amorphous solid which after crystallization from hexane provided 32 mg (84%) of **6**: mp 141.5–143°C; $[\alpha]_D^{20} = -118°$ (*c* 0.27, $CHCl_3$); uv (EtOH) $\lambda_{max}$ 262 ($\epsilon$ 11,300), 281 (11,400), 291 (6,400); ir ($CHCl_3$) 3630 $cm^{-1}$ (OH), 2820 and 2780 and 1460 $cm^{-1}$ ($-N(CH_3)_2$), 1600 and 1655 $cm^{-1}$ (diene), 1040 and 1020 $cm^{-1}$ (CO); nmr (270 MHz) $\delta$ 5.57 (d of d, $J$ = 5.9 and 2.4 Hz, 1H, C-6), 5.39 (d of t, $J_1$ = 5.8, $J_2$ = 2.1 Hz, 1H, C-7), 3.64 (t of t, $J$ = 11.1 and 4.1 Hz, 1H, C-3), 2.24 (s, 6H, $-N(Me)_2$), 0.95 (d, $J$ = 6 Hz, 3H, C-21), 0.94 (s, 3H, C-19), 0.62 (s, 3H, C-18); *m/e* (rel. intensity) 385 ($M^+$, 26), 370 (5), 352 (2), 84 (5), 71 (4), 58 (100); homogeneous on tlc ($R_f$ = 0.64, 3% MeOH in $CHCl_3$, aluminum oxide G); 98% pure by gc ($t_R$ = 12.4 min, oven 260°C); *Anal.* Calcd for $C_{26}H_{43}NO$: C, 80.98; H, 11.24; N, 3.63. Found: C, 80.81; H, 11.45; N, 3.58; high-resolution mass spectrum calcd for $C_{26}H_{43}NO$: 385.3345, found: 385.3350.

*25-Azavitamin $D_3$* (**7**). Provitamin **6** (47 mg in 100 ml of ethyl ether) was irradiated under $N_2$ for 10.5 min using an ice bath, vigorous stirring, Vycor filter, water-cooled quartz irradiation apparatus, and a mercury arc lamp (Hanau TQ 150 Zz). The solvent was removed by rotary evaporation and the resulting oil was purified on four preparative tlc plates that had been impregnated with KOH. After developing the plates with 5% methanol in chloroform, the major band was eluted with 5% methanol in chloroform to give the previtamin fraction ($\lambda_{max}$ 262 nm, 23 mg). The oil was dissolved in 10 ml of ethanol and heated to 70°C under $N_2$ for 3.0 hr. The solvent was removed by rotary evaporation, and the residue was purified on two KOH-impregnated preparative tlc plates using 5% methanol in chloroform to develop the plates and elute the major band. Evaporation of solvent provided 8.7 mg (19%) of 25-azavitamin $D_3$ (**7**) as an oil exhibiting: uv (EtOH) $\lambda_{max}$ 265 nm ($\epsilon$ 17,200), $\lambda_{min}$ 230 nm; nmr (270 MHz) $\delta$ 6.24 and 6.03 (AB, $J$ = 11 Hz, 2H, C-6,7), 5.05 (d of t, $J$ = 2 and 1 Hz, 1H, C-19), 4.82 (d, $J$ = 2 Hz, 1H, C-19), 3.95 (t of t, $J$ = 7.4 and 3.7 Hz, 1H, C-3), 2.58 (d of d, $J$ = 12 and 3 Hz, 1H, C-4), 2.31 (s, 6H, $-N(Me)_2$), 0.93 (d, $J$ = 6 Hz, 3H, C-21), 0.54 (s, 3H, C-18); *m/e* (rel. intensity) 385 ($M^+$, 15), 370 (3), 352 (1), 249 (2), 136 (2), 118 (2), 84 (10), 71 (4), 58 (100); homogeneous on tlc ($R_f$ = 0.69, 3% methanol in chloroform, aluminum oxide G); 99% pure by gc ($t_R$ = 9.6 and 10.5 min for pyro- and isopyro-derivatives, oven 260°C); high-resolution mass spectrum calcd for $C_{26}H_{43}NO$: 385.3345, found: 385.3340.

## *Synthesis of 25-Fluorovitamin $D_3$* (**11**)

*25-Hydroxyvitamin $D_3$, 3-acetate* (**9**). To 105 mg 25-OH-$D_3$ (**8**) dissolved in 1.3 ml of dry pyridine was added 110 mg acetic anhydride. After heating at 50°C under nitrogen for 5.0 hr, volatiles were removed by rotary evaporation. The resulting oil was dissolved in 15 ml of ethyl acetate then washed with an equal volume of 5% HCl, followed by 5% $NaHCO_3$ and finally saturated brine. After drying ($Na_2SO_4$), the solution was applied to a preparative tlc plate made of silica gel.

Development with 20% ethyl acetate in Skellysolve B and elution of the major band with ethyl acetate gave 92.5 mg (79%) of monoacetate (**9**) as a colorless oil: uv (EtOH) $\lambda_{max}$ 265 nm ($\epsilon$ 15,000); ir ($CCl_4$) 3620 (OH), 3080 (exocyclic methylene), 1735 and 1242 $cm^{-1}$ (acetate); nmr (270 MHz) $\delta$ 6.22 and 6.03 (AB, $J$ = 11 Hz, AB, C-6,7), 5.06 (d of t, $J_1$ = 2.2, $J_2$ = 1.1 Hz, 1H, C-19), 4.94 (t of t, $J$ = 8.1 and 4 Hz, 1H, C-3), 4.84 (d, $J$ = 2.2 Hz, 1H, C-19), 2.04 (s, 3H, acetate), 1.21 (s, 6H, C-26,27), 0.93 (d, $J$ = 5.4 Hz, 3H, C-21), 0.54 (s, 3H, C-18); *m/e* (rel. intensity) 442 ($M^+$, 57), 382 (82), 368 (30), 253 (39), 158 (77), 118 (100), 59 (75); homogeneous on tlc ($R_f$ = 0.43, 20% ethyl acetate in Skellysolve B); high-resolution mass spectrum calcd for $C_{29}H_{46}O_3$: 442.3447, found: 442.3421.

*25-Fluorovitamin $D_3$ 3-acetate* (**10**). A solution of 25-hydroxyvitamin $D_3$ 3-acetate (**9**, 15 mg) in 0.5 ml of dichloromethane was added dropwise to a cooled (dry ice/2-propanol) mixture of diethylaminosulfur trifluoride (30 mg) in 0.5 ml of dichloromethane. The mixture was stirred for 5 min; the cooling bath was removed, and the contents were warmed to ambient temperature (15 min). Five milliliters of 4% aqueous $NaHCO_3$ and 10 ml dichloromethane were then added. The organic phase was separated, washed with water, dried ($Na_2SO_4$), and solvents were removed by flash evaporation. This produced a yellow oil that was applied to a prepartive tlc plate made of silica gel. After developing the plate with 10% ethyl acetate in Skellysolve B, the major product was eluted with ethyl acetate. Solvent removal gave tertiary fluoride **10** (9.0 mg, 59%), as a colorless oil: uv (EtOH) $\lambda_{max}$ 265 nm ($\epsilon$ 16,000); ir ($CCl_4$) 3080 (exocyclic methylene), 1740 and 1240 $cm^{-1}$ (acetate); nmr (270 MHz) $\delta$ 6.21 and 6.03 (AB, $J$ = 11 Hz, 2H, C-6,7), 5.06 (m, 1H, C-19), 4.94 (t of t, $J$ = 8 and 4 Hz, 1H, C-3), 4.83 (d, $J$ = 2.2 Hz, 1H, C-19), 2.04 (s, 3H, acetate), 1.34 (d, $J_{H,F}$ = 21.7 Hz, 6H, C-26,27), 0.93 (d, $J$ = 6 Hz, 3H, C-21), 0.54 (s, 3H, C-18); *m/e* (rel. intensity) 444 ($M^+$, 12), 384 (58), 369 (10), 364 (6), 253 (22), 158 (44) 118 (100), 61 (22), 59 (1); homogeneous on tlc ($R_f$ = 0.56, 10% ethyl acetate in Skellysolve B); high-resolution mass spectrum calcd for $C_{29}H_{45}O_2F$: 444.3404, found: 444.3385.

*25-Fluorovitamin $D_3$* (**11**). Acetate **10** (7.5 mg) was saponified (0.1 *M* methanolic KOH, 25°C, 1 hr under $N_2$) and the hydrolysis product was isolated by addition of $H_2O$ and extraction with $CHCl_3$. The $CHCl_3$ phase was applied to a preparative silica gel tlc plate. Development with 20% ethyl acetate in Skellysolve B and elution of the predominant product with ethyl acetate provided 4.9 mg (72%) of the desired fluorovitamin (**11**) as a colorless oil: uv (EtOH) $\lambda_{max}$ 265 nm; ir ($CCl_4$) 3620 (OH), 3080 $cm^{-1}$ (exocyclic methylene); nmr (270 MHz) $\delta$ 6.24 and 6.03 (AB, $J$ = 11 Hz, 2H, C-6,7), 5.05 (m, 1H, C-19), 4.82 (d, $J$ = 2.6 Hz, 1H, C-19), 3.92 (t of t, $J$ = 7.1 and 3.6 Hz, 1H, C-3), 1.34 (d, $J_{H,F}$ = 21.3 Hz, 6H, C-26,27), 0.93 (d, $J$ = 6 Hz, 3H, C-21), 0.54 (s, 3H, C-18); *m/e* (rel. intensity) 402 ($M^+$, 13), 369 (4), 271 (4), 253 (5), 136 (100), 118 (88), 61 (12), 59 (1); homogeneous on tlc ($R_f$ = 0.38, 20% ethyl acetate in Skellysolve B) and gc ($t_R$ = 6.3 and 7.0 min for the pyro- and isopyro-derivatives, oven 260°C); high-resolution mass spectrum calcd for $C_{27}H_{43}FO$: 402.3298; found: 402.3284.

*Biological Assay: Inhibition of 25-Hydroxylation of vitamin $D_3$*

*Animals*. Male albino weanling rats obtained from the Holtzmann Co. (Madi-

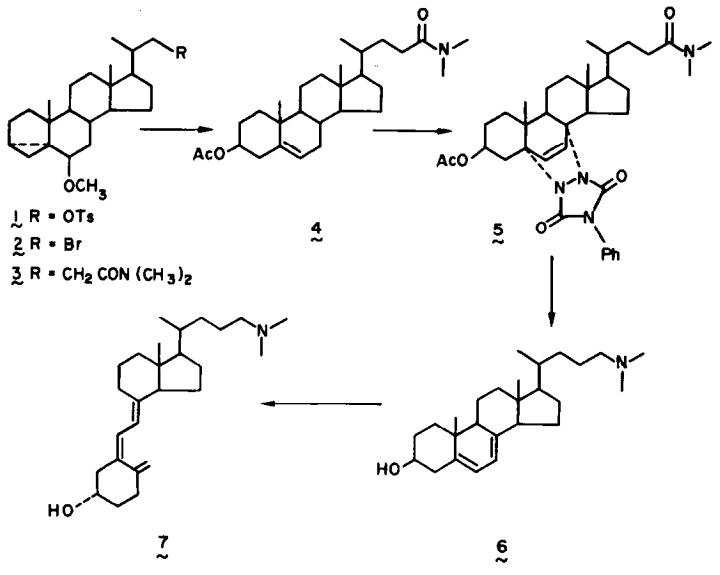
son, Wis.) were housed in overhanging wire cages. They were allowed *ad libitum* both distilled water and a low calcium, vitamin D-deficient diet (*12*) for 2.5 weeks. At this time they were weighed (83 ± 6 g, mean ± SD, $n = 12$), then dosed intrajugularly under light ether anesthesia with either ethanol (0.05 ml, normal controls) or 0.05 ml of ethanol containing graded amounts of synthetic **7** or **11**. Two hours after the first dose, each animal received 0.05 $\mu$g of [3$\alpha$-$^3$H]vitamin $D_3$ (7.9 Ci/mmol) in 0.05 ml of ethanol by the same route. The animals were sacrificed 4 hr after the second dose under ether anesthesia by jugular exsanguination. The collected blood was allowed to clot, then centrifuged to give serum.

*Serum 25-OH-$D_3$ content.* The serum was extracted as described by Bligh and Dyer (*13*), then the organic phase was chromatographed on Sephadex LH-20 (25 g, 2 × 23-cm column). The columns were eluted with chloroform/Skellysolve B (1 : 1); 50 fractions (3.2 ml each) were collected. The columns were then washed with 200 ml of chloroform/Skellysolve B (3 : 1) and reequilibrated with 100 ml of chloroform/Skellysolve B (1 : 1). After removing solvent from the column fractions by evaporation, the residues were dissolved in scintillation counting solution [2 g of 2,5-diphenyl-oxazole and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)benzene per liter of toluene]. Tritium content was determined with a Packard Tri-Carb Model 3375 liquid scintillation counter. On the average, 86% of the applied radioactivity was recovered from the Sephadex LH-20 columns, 6% of which was present in the column wash. The 25-OH-$D_3$ eluted in fractions 20 to 36. (Authentic samples of vitamin $D_3$ and 25-OH-$D_3$ were found to peak in fractions 12 and 28, respectively.) Since the total serum volume of a rat in milliliters is approximately 3% of its body weight in grams (*14*), the serum 25-OH-$D_3$ content, given as a percentage of the total administered dose, is equal to 3(dpm in 25-OH-$D_3$ peak) (body weight in g)/(ml serum extracted) (dpm in total dose).

## RESULTS

### *Analog Synthesis and Characterization*

The preparation of 25-azavitamin $D_3$ (**7**) is outlined in Scheme 1. The starting material, tosylate *1* was obtained from stigmasterol by the method of Partridge *et al.* (*9*), then converted to bromide **2** with LiBr in refluxing acetonitrile. The enolate of *N,N*-dimethylacetamide, generated with lithium diisopropylamide, reacted with **2** to give the amide **3** in 70% yield. Heating **3** in acetic acid gave cholenic acid dimethylamide **4** in nearly quantitative yield. The $\Delta^5$-steroid **4** was brominated (1,3-dibromo-5,5-dimethylhydantoin), then dehydrobrominated (collidine) to a mixture of dienes from which the desired $\Delta^{5,7}$-diene was isolated as adduct **5** after Diels-Adler reaction with 4-phenyl-1,2,4-triazoline-3,5-dione (*15*). Reduction of **5** with lithium aluminum hydride effected removal of the triazoline adduct (*16*) and simultaneously converted acetate to alcohol and amide to amine giving diene **6** in 84% yield after chromatography on neutral alumina. Irradiation of an ether solution of **6** yielded the previtamin derivative, which, after

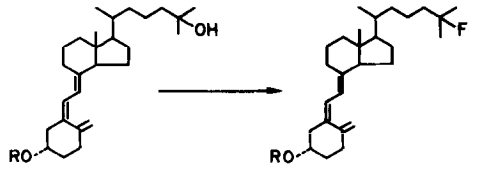



SCHEME 1. Synthesis of 25-azavitamin $D_3$ (7).

purification, was isomerized by gentle heating to the desired 25-azavitamin $D_3$ (7).

25-OH-$D_3$ (8) served as starting material for the synthesis of 25-fluorovitamin $D_3$ (11), as illustrated in Scheme 2. Selective acetylation of 25-OH-$D_3$ (8) gave monoacetate 9. Reaction of 9 with diethylaminosulfur trifluoride (*17*) provided tertiary fluoride 10 in good yield (59%); saponification then afforded 25-fluorovitamin $D_3$ (11).

Synthetic 7 and 11 gave the expected physical data. Spectral parameters (see Figs. 1 and 2 and experimental part for assignments) are in complete agreement with structural assignments. Gas chromtography of both 7 and 11 gave the expected twin peaks indicative of isomerization to pyro- and isopyro-derivatives analogous to the thermal isomerization of vitamin $D_3$ itself under these conditions (*18*). The glc pattern, the ultraviolet absorption spectra, and nmr data (Fig. 2) established the purity of the preparations. Noteworthy is the strong influence of the amino nitrogen on the mass spectral fragmentation pattern of 7 (Fig. 1). The




SCHEME 2. Synthesis of 25-fluorovitamin $D_3$ (11).

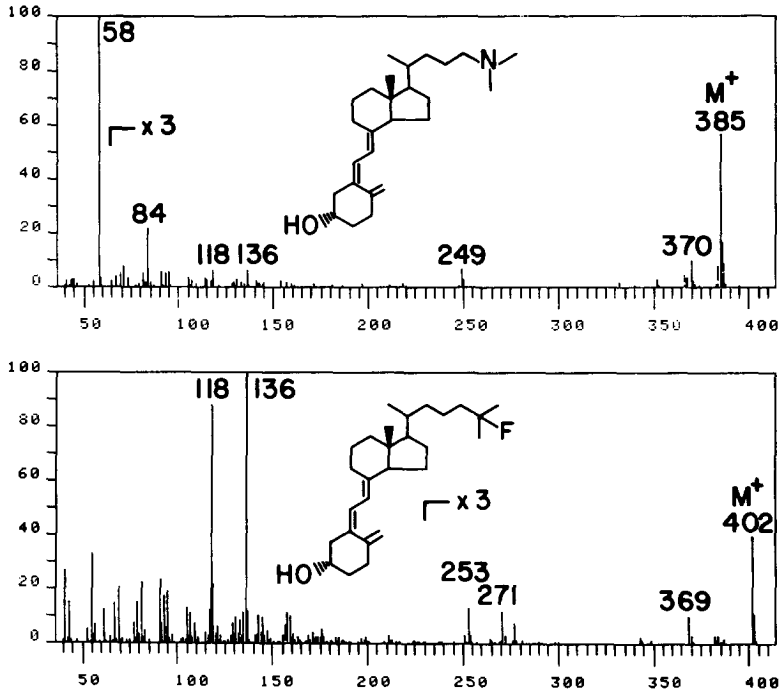



FIG. 1. Mass spectra of 25-azavitamin $D_3$ (top), and 25-fluorovitamin $D_3$ (bottom).

ions of *m/e* 136 and 118 which dominate and characterize the mass spectra of 5,7,10(19)-trienes containing monohydroxylated A-rings occur in only minor abundance in the spectrum of aza-compound **7**, where instead the immonium ion of *m/e* 58 ($CH_2 = N^+(CH_3)_2$) is the most prominent fragment.

### *Inhibition of the 25-Hydroxylation of Vitamin $D_3$*

The ability of compounds **7** and **11** to inhibit the 25-hydroxylation of vitamin D was examined. Vitamin D-deficient rats, maintained on a low calcium diet, were dosed intrajugularly with [3$\alpha$-$^3$H]vitamin $D_3$ (0.05 $\mu$g). Four hours later, the serum 25-OH-$D_3$ content was measured by extraction of the serum and chromatography of the extract on a column of Sephadex LH-20. Previously it was demonstrated that the product measured is in fact 25-OH-$D_3$ (*19*). As shown in Table 1, approximately 10% of the initial dose is present in serum as 25-OH-$D_3$. 25-Azavitamin $D_3$, injected 2 hr before the radiolabeled vitamin $D_3$ dose, did not appreciably effect serum 25-OH-$D_3$ content when administered at either 0.05, 0.5, or 5.0 $\mu$g amounts, but 50 $\mu$g of **7** decreased serum 25-OH-$D_3$ level by more than a factor of two and 200 $\mu$g of **7** produced greater than 10-fold reduction. A dose of 25-fluorovitamin $D_3$ (**11**) as low as 0.5 $\mu$g decreased the amount of 25-OH-$D_3$ in serum, and the highest dose tested (50 $\mu$g) reduced serum 25-OH-$D_3$ by more than a factor of 20, compared to normal controls that received only solvent.

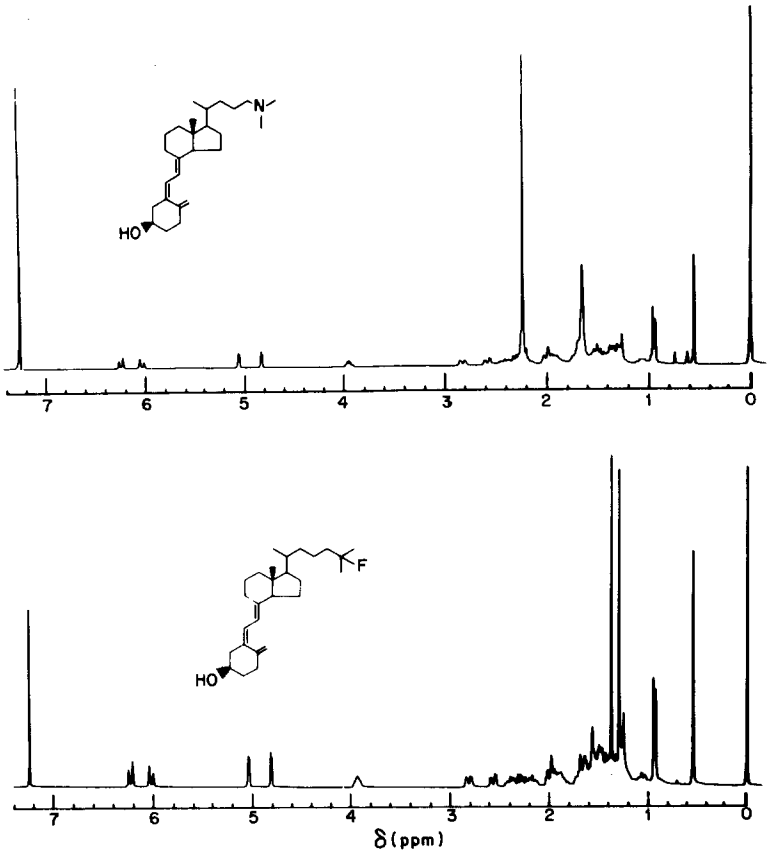



FIG. 2. Proton magnetic resonance spectra of 25-azavitamin $D_3$ (top), and 25-fluorovitamin $D_3$ (bottom).

TABLE 1

ABILITY OF POTENTIAL ANTAGONISTS TO INHIBIT THE *in Vivo* 25-HYDROXYLATION OF VITAMIN $D_3$

| | Serum 25-OH-$D_3$ levels[a] of rats given | |
|---|---|---|
| Antagonist dose ($\mu$g) | 25-Azavitamin $D_3$ | 25-Fluorovitamin $D_3$ |
| 0 | 10.4 ± 0.6[b] | — |
| 0.05 | 11.9 | 12.9 |
| 0.5 | 9.5 | 5.8 |
| 5.0 | 10.4 | 1.9 |
| 50.0 | 4.5 | 0.44 |
| 200.0 | 0.80 | — |

[a] Expressed as percentage of total dose of administered [$^3$H]vitamin $D_3$ present in serum as 25-OH[$^3$H]$D_3$.

[b] Mean ± SE, $n$ = 3; each of the other values in this table represent the findings from a single animal.

## DISCUSSION

Central to the synthesis of 25-azavitamin $D_3$ (**7**) from stigmasterol was the construction of the desired amino side chain. We found the direct introduction of a dimethylacetamide residue via alkylation of the corresponding amide enolate (*20, 21*) with a $C_{22}$-bromide (reaction **2** → **3**, Scheme 1) to be a very satisfactory and efficient approach for accomplishing this task. Remaining steps of the synthesis essentially followed established practice. Intermediate **4** has previously been prepared by the reaction of 3$\beta$-acetoxy-5-cholenic acid and dimethylamine (*22*). Louw *et al.* (*23*) attempted the conversion of **4** to **7** but were unable to purify the 7-dehydro derivative of **4**.

25-Fluorovitamin $D_3$ (**11**), was prepared (Scheme 2) by a different approach—the modification of a precursor possessing the intact vitamin D triene system. Most syntheses of vitamin D metabolites (or analogs) are based on the photochemical conversion of an appropriately substituted steroidal 5,7-diene to the previtamin derivative, and the thermal isomerization of the latter to the desired vitamin (*24*). These final steps often give poor yields and occasionally fail completely (*25*). Analog **11** and the preparation of 1$\alpha$-hydroxy-25-fluorovitamin derivatives (*26*) illustrate the synthetic utility of direct vitamin modification which in combination with recently developed methodology for direct C-1 functionalization of vitamin D derivatives (*27*) offers a useful simplification in the synthesis of a variety of vitamin D metabolites and analogs.

The two C-25 blocked analogs **7** and **11** were designed as potential inhibitors of the 25-hydroxylase that converts vitamin $D_3$ to 25-OH-$D_3$. The data of Table 1 show that both compounds markedly diminish the level of 25-OH[$^3$H]$D_3$ in the blood of vitamin D-deficient rats given [$^3$H]vitamin $D_3$, strongly suggesting inhibition of the enzymatic conversion of $D_3$ to 25-OH-$D_3$. 25-Fluorovitamin $D_3$ (**11**) is approximately 100 times more potent than 25-azavitamin $D_3$ (**7**) in our assay, since doses of 0.5 $\mu$g of **11** and 50 $\mu$g of **7** achieved roughly equivalent depressions of serum 25-OH[$^3$H]$D_3$ content. The lower potency of 25-azavitamin $D_3$ may be a consequence of unfavorable coulombic interaction with the hydroxylase enzyme due to the presence of a charged amino function, but poor liver uptake, or rapid clearance of the compound are alternative explanations.

As effective inhibitors of vitamin $D_3$ 25-hydroxylation, analogs **7** and **11**, would be expected to antagonize the biological responses (calcium transport, bone calcium mobilization) elicited by vitamin $D_3$, since metabolism of the vitamin is a prerequisite for function. Detailed biochemical studies have shown that the aza compound (**7**) is indeed an effective antagonist of vitamin $D_3$, but the fluoro derivative, the more potent inhibitor of 25-hydroxylation, does not prevent vitamin D action *in vivo*. These results and their biochemical basis will be the subject of a forthcoming report.

## ACKNOWLEDGMENTS

We are indebted to Drs. Joseph Napoli and John Chu for the preparation of the diethylaminosulfur

trifluoride used for the synthesis of the fluoroanalog. We thank Mr. Sean Hehir for the nmr data and the Upjohn Co. (Kalamazoo, Mich.) for a generous gift of 25-OH-$D_3$. This work was supported by grant No. AM-14881 and training grant No. GM-00236 BCH from the National Institute of Health and the Harry Steenbock Research Fund.

## REFERENCES

1. H. F. DeLuca and H. K. Schnoes, *Ann. Rev. Biochem.* **45,** 631 (1976).
2. E. B. Olson and H. F. DeLuca, *Science* **165,** 405 (1969).
3. L. G. Raisz, C. L. Trummel, M. F. Holick, and H. F. DeLuca, *Science* **175,** 768 (1972).
4. B. E. C. Nordin, "Metabolic Bone and Stone Disease," pp. 96–132. Williams & Wilkins Co., Baltimore, 1973.
5. C. R. Paterson, "Metabolic Disorders of Bone," pp. 62–128. Blackwell Scientific Publications, Oxford, 1974.
6. D. V. Kimberg, R. D. Baerg, E. Gershon, and R. T. Grandusius, *J. Clin. Invest.* **50,** 1309 (1971).
7. H. C. Harrison and H. E. Harrison, *Proc. Soc. Exp. Biol. Med.* **153,** 220 (1976).
8. E. Stahl, "Thin-Layer Chromatography," p. 37. Springer-Verlag, Berlin, 1965.
9. J. J. Partridge, S. Faber, and M. R. Uskokovic, *Helv. Chim. Acta* **57,** 764 (1974).
10. H. Gilman, and F. K. Cartledge, *J. Organometal. Chem.* **2,** 447 (1964).
11. R. E. Counsell, P. D. Klimstra, L. N. Nysted, and R. E. Ranney, *J. Med. Chem.* **8,** 45 (1964).
12. T. Suda, H. F. DeLuca, and Y. Tanaka, *J. Nutr.* **100,** 1049 (1970).
13. E. G. Bligh and W. J. Dyer, *Canad. J. Biochem. Physiol.* **37,** 911 (1959).
14. G. Ponchon and H. F. DeLuca, *J. Clin. Invest.* **48,** 1273 (1969).
15. R. C. Cookson, S. S. H. Gilani, and D. R. Stevens, *J. Chem. Soc.* (*C*), 1905 (1967).
16. D. H. R. Barton, T. Shioiri, and D. A. Widdowson, *J. Chem. Soc.* (*C*), 1968, (1971).
17. W. J. Middleton, *J. Org. Chem.* **40,** 574 (1975).
18. H. Ziffer, W. J. A. van den Heuvel, E. O. A. Haahti, and E. C. Horning, *J. Amer. Chem. Soc.* **82,** 6411 (1960).
19. M. F. Holick and H. F. DeLuca, *J. Lipid Res.* **12,** 460 (1971).
20. B. M. Trost and R. A. Kunz, *J. Org. Chem.* **39,** 2475 (1974).
21. T. Cuvigny, P. Hullot, M. Larcheveque, and H. Normant, *C. R. Acad. Sci.* **278,** 1105 (1974).
22. D. F. Louw, J. Strating, and H. J. Backer, *Rec. Trav. Chim. Pays-Bas* **73,** 667 (1954).
23. D. F. Louw, J. Strating, and H. J. Backer, *Rec. Trav. Chim. Pays-Bas* **73,** 1540 (1955).
24. P. E. Georghiou, *Chem. Rev.* **6,** 83 (1977).
25. M. Sheves and Y. Mazur, *Chem. Commun.* 21 (1977).
26. J. L. Napoli, M. Fivizzani, H. K. Schnoes, and H. F. DeLuca, *Steroids* **32,** 453 (1978).
27. H. E. Paaren, D. E. Hamer, H. K. Schnoes, and H. F. DeLuca, *Proc. Nat. Acad. Sci. U.S.A.* **75,** 2080 (1978).