Synthesis and Biological Activity of Novel Vitamin D Analogues: 24,24-Difluoro-25-hydroxy-26,27-dimethylvitamin D_3 and 24,24-Difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3†

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We synthesized 24,24-difluoro-25-hydroxy-26,27-dimethylvitamin D_3 (16), and 24,24-difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3 (21), from 3β-hydroxy-22,23-dinorcholenic acid 3-acetate. Compound 16 was found to be a highly potent vitamin D analogue with bioactivity similar to that of 25-hydroxyvitamin D_3 in vivo. Compound 16 was bound by vitamin D binding protein with an affinity slightly less than that of 25-hydroxyvitamin D_3. It was bound to the intestinal cytosol receptor for 1,25-dihydroxyvitamin D_3 with approximately the same affinity as that of 25-hydroxyvitamin D_3. In the organ-culture duodenum, 16 induced the synthesis of calcium binding protein with a potency approximately $^1/_{20}$ that of 1,25-dihydroxyvitamin D_3. Compound 21 was also noted to be a highly potent vitamin D analogue with bioactivity in vivo similar to that of 1,25-dihydroxyvitamin D_3. It was bound to vitamin D binding protein with an affinity considerably less than that of 1,25-dihydroxyvitamin D_3. It was bound to the intestinal cytosol receptor for 1,25-dihydroxyvitamin D_3 with an affinity slightly less than that of the native hormone. In the organ-culture duodenum, 21 was noted to be about 3 times more active than 1,25-dihydroxyvitamin D_3 in the induction of calcium binding protein. The introduction of fluorines at C-24 and extension of the sterol side chain at C-26 and C-27 by methylene groups results in vitamin D analogues that have biological activity in vivo similar to those of the respective nonfluorinated natural sterols.

The physiological and biochemical transformations required in order to activate vitamin D_3 include sequential hydroxylations at C-25 and C-1. The resulting sterol, 1α,25-dihydroxyvitamin D_3, is the most potent naturally occurring sterol.[1-3] In the past, several studies have delineated the structural requirements for biological activity of the sterol. It has been shown, for example, that removing methylene groups in the side chain, lengthening the side chain by addition of methylene groups, or the introduction of carboxylic acids on side-chain-shortened analogues results in a decrease in the biological activity of the vitamin.[4-8] Compounds such as 25-azavitamin D_3 are less active than their C-25 counterparts.[9] It has also been established that alterations in the structure of the triene, expansion of the A ring, and removal of various hydroxyl groups at C-1, C-3, or C-25, result in analogues of vitamin D that are less active than the parent sterol.[10-14] Fluorinated sterols with fluorine atoms at C-24 or C-26 and C-27 are either equipotent with or more active than the corresponding natural compounds.[15-20] Conversely, fluorine substitutions at other sites decrease or do not alter the bioactivity of the vitamin.[14,21-29] A fluorine substitution at C-6 results in an antivitamin.[30,31] 11α-Hydroxyvitamin D_3, a C ring analogue of vitamin D_3, is more active than vitamin D_3 itself.[32] Considerable work has also been done on the effects of alterations of the structure of vitamin D sterols on the binding to the receptor for 1,25-dihydroxyvitamin D_3 or vitamin D binding protein.[14,32-39]

We and others recently synthesized 25-hydroxy-26,27-dimethylvitamin D_3 and 1,25-dihydroxy-26,27-dimethylvitamin D_3 and showed that these compounds were highly potent analogues of vitamin D_3.[40,41] In addition, we showed that 1,25-dihydroxy-26,27-dimethylvitamin D_3 had a slightly increased duration of action when compared with 1,25-dihydroxyvitamin D_3. As fluorination at C-24 reduces the biological degradation of vitamin D_3 analogues by interfering with 24-hydroxylation, we reasoned that C-24 fluorination of the above mentioned analogues would result

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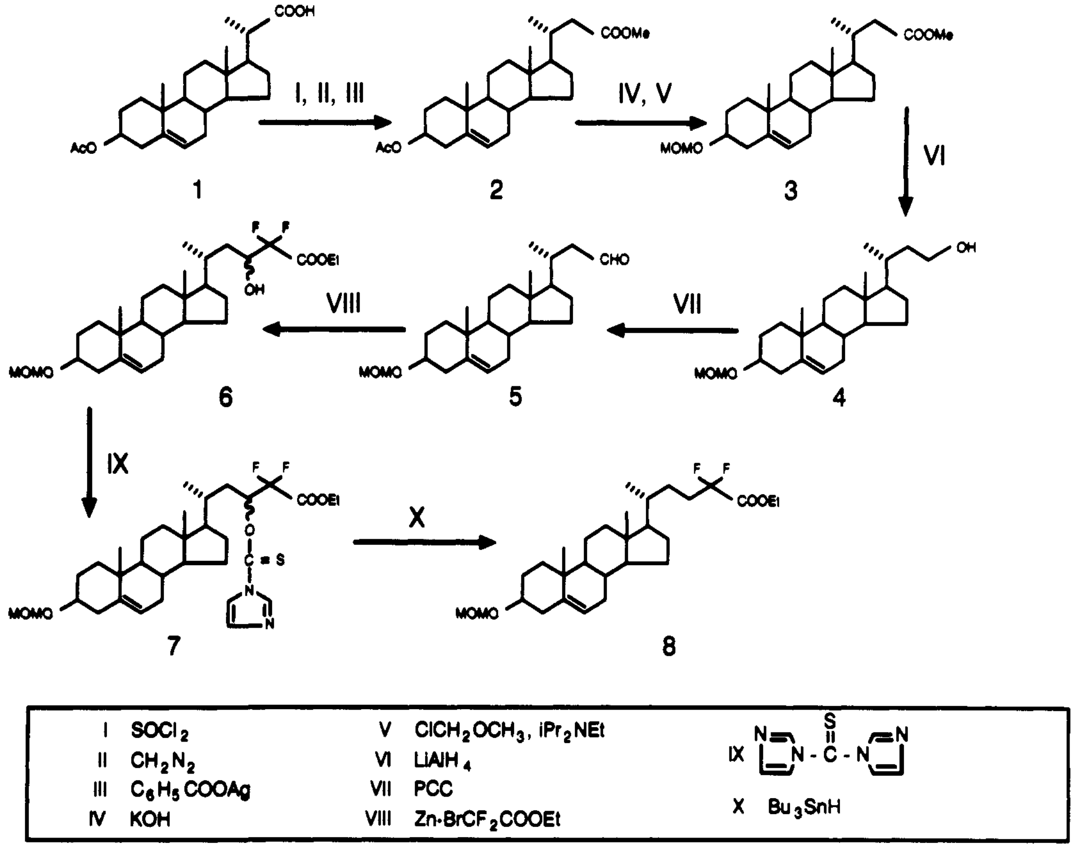
Scheme I. Synthesis of Intermediate 8



in even more potent analogues of vitamin D_3. To test this hypothesis, we synthesized 24,24-difluoro-25-hydroxy-26,27-dimethylvitamin D_3 and 24,24-difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3 (Schemes I–III) and tested their bioactivity in vivo and in vitro.

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Experimental Section

General Procedures. Ultraviolet spectra were obtained on a Beckman Model DU-70 recording spectrophotometer (Beckman Instruments, Palo Alto, CA). Infrared spectra were obtained with a Nicolet 5M-X Fourier transform infrared spectrophotometer (Nicolet Analytical Instruments, Schaumburg, IL). NMR spectra (^{1}H, ^{19}F) were recorded on an IBM NR-80 Fourier transform nuclear magnetic resonance spectrometer (IBM Instruments, Danbury, CT), using tetramethylsilane and fluorotrichloromethane as internal standards. Mass spectra were recorded using either a Kratos MS-50/DS-55 mass spectrometer–computer system or an AEI/Kratos MS-30 (Kratos Instruments, UK). Melting points were recorded on a Haake melting point apparatus (Haake-Buchler Instruments, Inc., Saddlebrook, NJ). Flash chromatography was performed with silica gel (Merck, grade 60, 230–400 mesh).[42] Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN). High-performance liquid chromatography (HPLC) was performed on a Waters liquid chromatograph equipped with two Model M-6000A pumps, a Model 660 gradient programmer from Waters Associates (Milford, MA), and a Kratos Model 783 ultraviolet detector (Kratos Instruments, Ramsey, NJ). [^{45}Ca]Calcium chloride was obtained from New England Nuclear (Boston, MA). [26,27-^{3}H]25-hydroxyvitamin D_3 (23 Ci/mmol) and [26,27-^{3}H]1,25-dihydroxyvitamin D_3 (158 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). 25-Hydroxyvitamin D_3, (25*S*)-25,26-dihydroxyvitamin D_3, (24*R*)-24,25-dihydroxyvitamin D_3, 1α-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3 were gifts from Dr. Milan Uskokovic, Hoffmann-La Roche (Nutley, NJ).

Animals. Male, weanling, albino rats (50–60 g) were obtained from the Holtzman Company (Madison, WI). They were maintained in individual overhanging wire cages in an ultraviolet light free environment. They were fed a 0.02% calcium, 0.3% phosphorus diet *ad libitum* for a period of 3 weeks when they were used for the experiments noted below.[43,44]

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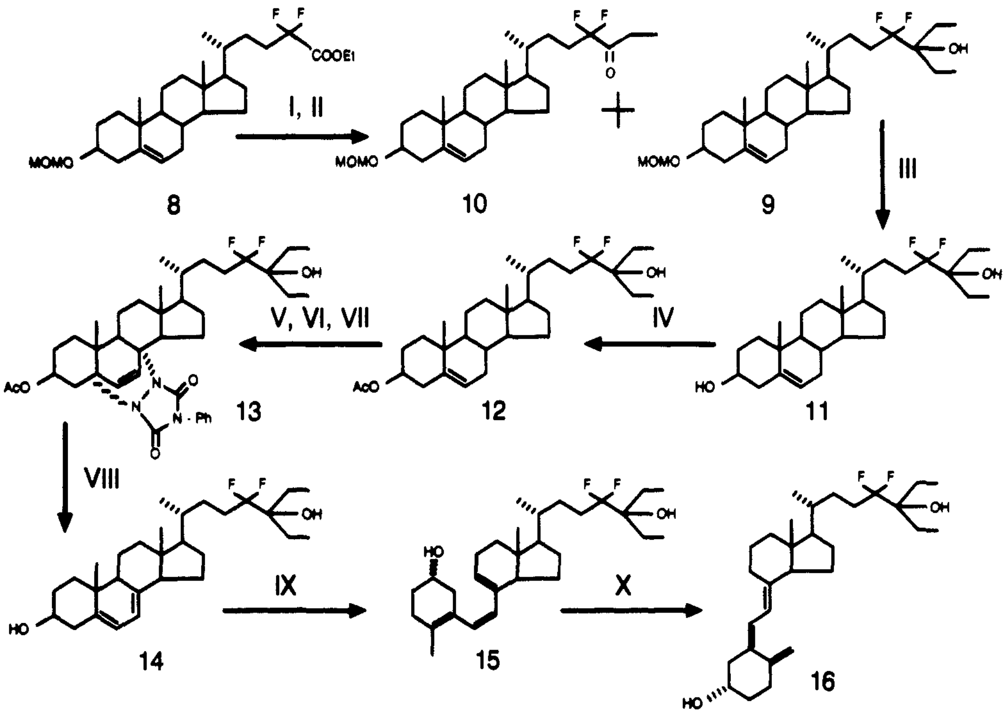
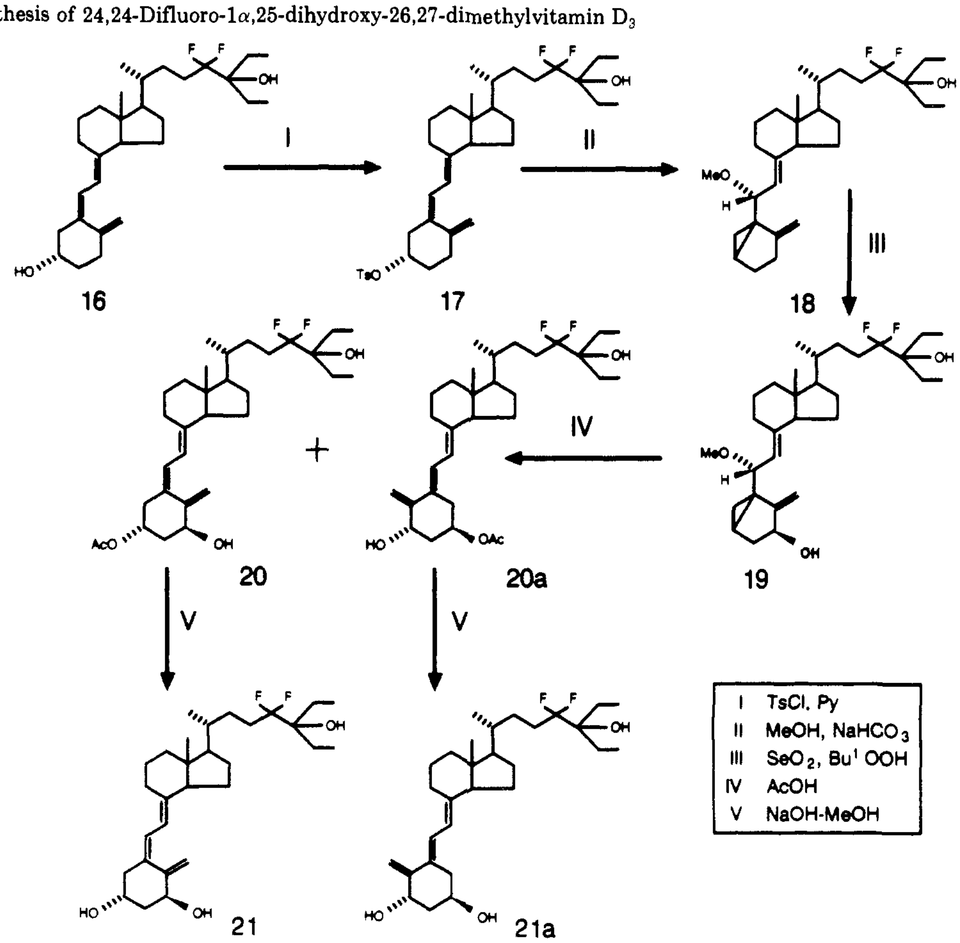
Scheme II. Synthesis of 24,24-Difluoro-25-hydroxy-26,27-dimethylvitamin D_3



I	EtMgBr	V	NBS	IX	hν
II	PCC, MS-3A	VI	Collidine	X	Ethanol, reflux
III	TsOH	VII	4-Phenyl-1,2,4-triazoline-3,5-dione		
IV	Ac_2O-Py	VIII	$LiAlH_4$		

Scheme III. Synthesis of 24,24-Difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3



I	TsCl, Py
II	MeOH, $NaHCO_3$
III	SeO_2, Bu^tOOH
IV	AcOH
V	NaOH-MeOH

Serum Calcium. Serum calcium was measured using atomic absorption spectrometry with a Perkin-Elmer 2380 atomic absorption spectrometer (Perkin-Elmer Instruments, Norwalk, CT).

Intestinal-Calcium Transport. This was measured by using the everted gut sac method of Martin and DeLuca.[45]

Animal Dosing Procedures. Under light ether anesthesia, rats received varying amounts of either 16, 21, 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, or vehicle dissolved in 50 μL of absolute ethanol. At the appropriate times, the animals were sacrificed and blood was collected for the measurement of serum calcium. The duodena from the same animals were used for the determination of active calcium transport.

Binding Assays. The ability of 16, 21, 25-hydroxyvitamin D_3 (25-(OH)-D_3), 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3), vitamin D_3 (D_3), 24(*R*),25-dihydroxyvitamin D_3 (24*R*,25-$(OH)_2$-D_3), 25(*S*),26-dihydroxyvitamin D_3 (25*S*,26-$(OH)_2$-D_3), and 1α-hydroxyvitamin D_3 (1α-(OH)-D_3) to bind to rat vitamin D binding protein and chick intestinal cytosol receptor for 1,25-dihydroxyvitamin D_3 were assessed as described earlier.[14,32,46]

Induction of Calcium Binding Protein in Cultured Duodena. This was performed as described by Corradino.[47,48]

Statistical Analysis. This was performed as described by us in our earlier publication.[40] Briefly, the effects of dose, concentration, and time of administration on serum calcium and intestinal calcium transport were assessed with a two-factor response surface design.

Methyl 3β-Acetoxy-24-norchol-5-en-23-oate (2). 3β-Hydroxy-22,23-dinorcholenic acid 3-acetate, (1, 6.0 g, 15.4 mmol) was cooled to −18 °C and thionyl chloride (14.0 mL) was added dropwise over a period of 36 min. The reaction was stored at room temperature for 3 h. Thionyl chloride was removed under reduced pressure. The residue was dissolved in benzene and the solvent was distilled at low pressure in order to remove the last traces of thionyl chloride. The acid chloride was dissolved in benzene (40 mL) and added dropwise to an ethereal solution of diazomethane at 0 °C in 28 min. Diazomethane was prepared from Diazald (17.14 g, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide), potassium hydroxide (4.7 g), water (15.90 mL), diethyleneglycol monoethyl ether (28.11 mL), and ether (112 mL). The reaction was stirred at room temperature for 15 h. Ether and benzene were removed under reduced pressure, and the yellow solid obtained displayed absorption at 2106 cm^{-1} in the infrared spectrum. The crude diazoketone was dissolved in methylene chloride (80 mL) and methanol (120 mL). A solution of silver benzoate (0.8257 g) in triethylamine (8.0 mL) was added over a period of 79 min. After the addition of silver benzoate, no more evolution of nitrogen was observed. The reaction was stirred overnight at room temperature. The reaction was poured into water and the mixture was filtered under suction. The filtrate was extracted with methylene chloride and the organic extract was filtered through a short Celite column. The organic extract was washed with 5% HCl and water and then dried (Na_2SO_4) and evaporated. The solid obtained was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:9) to give **2** (4.5182 g, 70%): mp 133.3–133.8 °C (from methanol), (lit[49] mp 126.0–127.5 °C); IR ($CHCl_3$) 1726 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.72 (3 H, s, 18-CH_3), 2.03 (3 H, s, $OCOCH_3$), 3.65 (3 H, s, COOMe), 4.58 (1 H, m, 3α-H), 5.35 (1 H, m, 6-H); MS m/z (assignment, relative intensity) 356 (M^+ − AcOH, 100), 341 (356 − CH_3, 19), 255 (356 − side chain, 11); high-resolution MS calcd for $C_{24}H_{36}O_2$ (M^+ − AcOH) 356.2706, found 356.2661.

Methyl 3β-(Methoxymethoxy)-24-norchol-5-en-23-oate (3). Compound **2** (3.0 g, 7.2 mmol) was dissolved in 0.1 M potassium hydroxide in methanol (230 mL) and THF (125 mL). The mixture was stirred at room temperature for 4 h. The reaction was poured into cold water and extracted with ethyl acetate. The organic extract was dried (Na_2SO_4) and evaporated. The residue (2.7 g) was dissolved in methylene chloride (25 mL) and diisopropylethylamine (1.877 g, 14.5 mmol) was added. The mixture was cooled to 0 °C and chloromethyl methyl ether (1.166 g, 14.5 mmol) was added dropwise in 15 min. The reaction was stirred at room temperature for 16 h, poured into water, and extracted with ethyl acetate. The organic layer was washed with cold 2.5% HCl and water and was then dried (Na_2SO_4) and evaporated. The crude product was purified by flash chromatography on silica gel (ethyl acetate/hexane 3:7) to give **3** (2.7517 g, 91%): mp 92.3–93.4 °C; IR ($CHCl_3$) 1728 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.73 (3 H, s, 18-CH_3), 3.37 (3 H, s, OCH_3), 3.39 (1 H, m, 3α-H), 3.67 (3 H, s, COOMe), 4.69 (2 H, s, OCH_2OCH_3), 5.36 (1 H, m, 6-H); MS m/z (assignment, relative intensity) 356 (M^+ − MOMOH, 100), 341 (356 − CH_3, 10), 255 (356 − side chain, 7); high-resolution MS calcd for $C_{24}H_{36}O_2$ (M^+ − MOMOH) 356.2706, found 356.2570.

3β-(Methoxymethoxy)-24-norchol-5-en-23-ol (4). A suspension of lithium aluminum hydride (0.814 g, 21.5 mmol) in THF (60 mL) was cooled to −78 °C and a solution of **3** (2.568 g, 6.1 mmol) in THF (20 mL) was added dropwise in 11 min. The reaction was stirred at room temperature for 3 h and quenched by the addition of wet ether, ethyl acetate, and water. The mixture was extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and evaporated, and the product was purified by flash chromatography on silica gel (ethyl acetate/hexane 3:7) to provide **4** (2.3013 g, 96%): mp 104.2–104.9 °C; IR (KBr) 3324 (OH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.70 (3 H, s, 18-CH_3), 3.36 (3 H, s, OCH_3), 3.40 (1 H, m, 3α-H), 3.67 (2 H, br t, J = 6.7 Hz, CH_2OH), 4.69 (2 H, s, OCH_2OCH_3), 5.37 (1 H, m, 6-H); MS m/z (assignment, relative intensity) 328 (M^+ − MOMOH, 100), 313 (328 − CH_3, 5), 255 (328 − side chain, 4); high-resolution MS calcd for $C_{23}H_{36}O$ (M^+ − MOMOH) 328.2757, found 328.2758.

3β-(Methoxymethoxy)-24-norchol-5-en-23-al (5). Sodium acetate (0.209 g, 2.6 mmol) was added to a solution of **4** (1.0 g, 2.6 mmol) in methylene chloride (30 mL). The mixture was cooled to 0 °C and pyridinium chlorochromate (PCC, 1.655 g, 7.7 mmol) was added over a period of 7 min. The reaction was stirred at room temperature for 4 h. Ether was added to the reaction and the mixture was filtered through a small column of florasil. The filtrate was evaporated and the residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:4) to yield **5** (0.8906 g, 90%): mp 106.1–106.7 °C; IR ($CHCl_3$) 1721 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.72 (3 H, s, 18-CH_3), 3.37 (3 H, s, OMe), 3.40 (1 H, m, 3α-H), 4.68 (2 H, s, OCH_2OCH_3), 5.35 (1 H, m, 6-H), 9.75 (1 H, t, J = 1.3 Hz, CHO); MS m/z (assignment, relative intensity) 326 (M^+ − MOMOH, 100), 311 (326 − CH_3, 5), 255 (326 − side chain, 2); high-resolution MS calcd for $C_{23}H_{34}O$ (M^+ − MOMOH) 326.2601, found 326.2639.

Ethyl 24,24-Difluoro-23-hydroxy-3β-(methoxymethoxy)-homochol-5-en-25-oate (6). Zinc dust (1.046 g, 0.02 g-atom) freshly activated by washing successively with 20% HCl, water, ether and drying was added to THF (20 mL) and the suspension was heated to reflux. A solution of **5** (1.8832 g, 4.8 mmol) and ethyl bromodifluoroacetate (2.95 g, 14.5 mmol) in THF (15 mL) was added dropwise to the above refluxing zinc dust in THF over a period of 36 min. The reaction was refluxed for 30 min, cooled to room temperature, and poured into potassium hydrogen sulfate (1 M, 100 mL). The mixture was extracted with ethyl acetate. The organic extract was washed with water and then was dried (Na_2SO_4) and evaporated. The residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:4) to provide **6** (1.5883 g, 64%) as a mixture of diastereoisomers as indicated by TLC and ^1H NMR: mp 86.2–98.5 °C; IR (KBr) 3405 (OH), 1771 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.70 (s, 18-CH_3), 0.71 (s, 18-CH_3), 1.35 (t, J = 6.7 Hz, $COOCH_2CH_3$), 3.37 (s, OCH_3), 3.42 (m, 3α-H), 3.89–4.56 (m, 23-CHOH), 4.32 (q, J = 6.7 Hz, $COOCH_2CH_3$), 4.68 (s, CH_3OCH_2O), 5.34 (m, 6-H); MS m/z (assignment, relative intensity) 450 (M^+ − MOMOH, 100), 435 (450 − CH_3, 7), 255 (450 − side chain, 5); high-resolution MS calcd for $C_{27}H_{40}F_2O_3$ (M^+ − MOMOH) 450.2935, found 450.2985.

Ethyl 24,24-Difluoro-23-[[imidazol-1-yl(thiocarbonyl)]-oxy]-3β-(methoxymethoxy)homochol-5-en-25-oate (7). Compound **6** (1.5745 g, 3.1 mmol) was dissolved in 1,2-dichloroethane (35 mL) and 1,1′-thiocarbonyldiimidazole (90%, 1.217 g, 6.1 mmol) was added in small portions over a period of 8 min. The mixture

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was heated at 72 °C for 17 h. The solvent was evaporated under a reduced pressure and the resulting yellow oily residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 4:6) to yield **7** as a thick oil (1.8763 g, 98%, mixture of diastereoisomers as indicated by TLC and ^{1}H NMR): IR ($CHCl_3$) 1769 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.64 (s, 18-CH_3), 0.70 (s, 18-CH_3), 1.27 (t, J = 6.7 Hz, $COOCH_2CH_3$), 3.35 (s, OCH_3), 3.38 (m, 3α-H), 4.29 (q, J = 6.7 Hz, $COOCH_2CH_3$), 4.68 (s, CH_3OCH_2O), 5.34 (m, 6-H), 6.25 (m, 23-H), 7.04 (br s, imidazole-H), 7.60 (br s, imidazole-H), 8.29 (br s, imidazole-H); MS m/z (assignment, relative intensity) 622 (M$^+$, 59), 560 (M$^+$ – MOMOH, 87), 432 (M$^+$ – MOMOH – $C_4H_4N_2OS$, 100), 255 (M$^+$ – MOMOH – side chain, 16); high-resolution MS calcd for $C_{33}H_{48}F_2N_2O_5S$ (M$^+$) 622.3240, found 622.3219.

Ethyl 24,24-Difluoro-3β-(methoxymethoxy)homochol-5-en-25-oate (8). Tributyltin hydride (1.691 g, 5.811 mmol) in toluene (80 mL) was heated to reflux and a solution of **7** (1.8096 g, 2.9055 mmol) in toluene (40 mL) was added dropwise in 22 min. The reaction was refluxed for 55 min and the solvent was removed under reduced pressure. The white, oily solid residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:5) to give **8** (1.3577 g, 94%): mp 65.5–66.1 °C (from ethanol); IR ($CHCl_3$) 1765 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.60 (3 H, s, 18-CH_3), 1.28 (3 H, t, J = 6.7 Hz, $COOCH_2CH_3$), 3.28 (3 H, s, OCH_3), 3.35 (1 H, m, 3α-H), 4.25 (2 H, q, J = 6.7 Hz, $COCH_2CH_3$), 4.61 (2 H, s, H_3COCH_2O, 5.27 (1 H, m, 6-H); ^{19}F NMR ($CDCl_3$) δ –106.35 (t, J = 16.7 Hz, CF_2); MS m/z (assignment, relative intensity) 434 (M$^+$ – MOMOH, 100), 419 (M$^+$ – CH_3, 9), 255 (434 – side chain, 10), 228 (4), 213 (9). Anal. Calcd for $C_{29}H_{46}F_2O_4$: C, 70.13; H, 9.33; F, 7.65. Found: C, 70.01; H, 9.42; F, 7.27.

24,24-Difluoro-3β-(methoxymethoxy)-26,27-dimethylcholest-5-en-25-ol (9) and 24,24-Difluoro-3β-(methoxymethoxy)-27-nor-26-methylcholest-5-en-25-one (10). A solution of **8** (2.5998 g, 5.23 mmol) in THF (50 mL) was cooled to –78 °C and ethylmagnesium bromide in THF (16.4 mL, 32.7 mmol, 2 M in THF) was added dropwise in 16 min. The reaction was maintained at –78 °C for 30 min and then the cooling bath was removed. The mixture was stirred at room temperature for 60 min and quenched by pouring it into cold 5% HCl. The reaction mixture was extracted with ethyl acetate. The organic extract was washed with water, dried (Na_2SO_4), and evaporated to give a white solid (2.5921 g). The crude product (2.5921 g) was dissolved in methylene chloride (60 mL) and powdered 3A molecular sieves (5.35 g) were added. Pyridinium chlorochromate (PCC, 4.63 g, 21.5 mmol) was added to the mixture in small amounts over a period of 10 min. The reaction was stirred at room temperature for 6$^1/_2$ h. The mixture was diluted with ether and filtered through a small column of Celite. The Celite was washed with ethyl acetate. The filtrate was evaporated under reduced pressure and the product was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:9, followed by 1:4) to give **9** (0.6554 g, 25%) and **10** (1.7366 g, 69%).

Characterization of 9: mp 85.6–86.7 °C; IR (Nujol mull) 3428 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.67 (3 H, s, 18-CH_3), 3.34 (3 H, s, OMe), 3.41 (1 H, m, 3α-H), 4.67 (2 H, m, H_3COCH_2O), 5.34 (1 H, m, 6-H); ^{19}F NMR ($CDCl_3$) δ –111.80 (t, J = 18.1 Hz, CF_2); MS (EI) m/z (assignment, relative intensity) 448 (M$^+$ – MOMOH, 100), 428 (448 – HF, 34), 408 (448 – 2HF, 11), 255 (448 – side chain, 26), 87 ($C_5H_{11}O$, 48); MS (CI, CH_4) m/z 511 (M$^+$ + 1); high-resolution MS calcd for $C_{29}H_{46}F_2O$ (M$^+$ – MOMOH) 448.3505, found 448.3504.

Characterization of 10: mp 108.1–108.6 °C; IR ($CHCl_3$) 1742 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.69 (3 H, s, 18-CH_3), 1.11 (3 H, t, J = 7.3 Hz, $CF_2COCH_2CH_3$) 2.69 (2 H, q, J = 7.3 Hz, $COCH_2$), 3.38 (3 H, s, OMe), 3.39 (1 H, m, 3α-H), 4.67 (2 H, s, H_3COCH_2O), 5.35 (1 H, m, 6-H); ^{19}F NMR ($CDCl_3$) δ –107.73 (t, J = 15.8 Hz, CF_2); MS m/z (assignment, relative intensity) 418 (M$^+$ – MOMOH, 100), 403 (418 – CH_3, 13), 255 (418 – side chain, 10); high-resolution MS calcd for $C_{27}H_{40}F_2O$ (M$^+$ – MOMOH) 418.3037, found 418.3032.

24,24-Difluoro-26,27-dimethylcholest-5-ene-3β,25-diol (11). Compound **9** (1.0946 g, 2.14 mmol) was dissolved in a mixture of methanol and THF (50 mL, methanol/THF 7:3) and the solution was cooled to 0 °C. p-Toluenesulfonic acid (1.63 g, 8.53 mmol) was added to the solution in 2 min. The reaction was stirred at room temperature for 60 h. The mixture was poured into water and extracted with ethyl acetate. The organic extract was washed with 5% sodium bicarbonate and water and then was dried (Na_2SO_4) and evaporated to give **11** (1.0 g, quantitative): mp 150.6–151.0 °C (from chloroform and hexane); IR (Nujol mull) 3393 cm^{-1} (OH); ^{1}H NMR ($CDCl_3$) δ 0.67 (3 H, s, 18-CH_3), 3.49 (1 H, m, 3α-H), 5.34 (1 H, m, 6-H); MS m/z (assignment, relative intensity) 466 (M$^+$, 21), 448 (M$^+$ – H_2O, 24), 433 (448 – CH_3, 19), 428 (448 – HF, 5), 419 (11), 381 (21), 355 (38), 273 (466 – side chain, 13), 255 (273 – H_2O, 28), 87 ($C_5H_{11}O$, 100); high-resolution MS calcd for $C_{29}H_{48}F_2O_2$ 466.3610, found 466.3620.

24,24-Difluoro-26,27-dimethylcholest-5-ene-3β,25-diol 3-Acetate (12). Compound **11** (1.0 g, 2.1 mmol) in pyridine was cooled to 0 °C and acetic anhydride (2.705 g, 26.5 mmol) was added in 5 min. The reaction was stirred at room temperature for 16 h and poured into water. The mixture was extracted with ethyl acetate. The organic extract was washed with 5% HCl and water. The crude product was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:4) to give **12** (0.998 g, 92%): mp 131.8–131.9 °C (from hexane); IR (Nujol mull) 3463 (OH), 1709 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.71 (3 H, s, 18-CH_3), 2.04 (3 H, s, $OCOCH_3$), 4.63 (1 H, m, 3α-H), 5.36 (1 H, m, 6-H); MS m/z (assignment, relative intensity) 448 (M$^+$ – AcOH, 71), 433 (448 – CH_3, 21), 419 (15), 255 (448 – side chain, 31), 147 (100), 87 (77). Anal. Calcd for $C_{31}H_{50}F_2O_3$: C, 73.2; H, 9.9; F, 7.5. Found: C, 73.7; H, 10.1; F, 7.6.

5α,8α-(3,5-Dioxo-4-phenyl-1,2,4-triazolino)-24,24-difluoro-26,27-dimethylcholest-6-ene-3β,25-diol 3-Acetate (13). N-Bromosuccinimide (NBS, 0.2 g, 1.1 mmol) and benzoyl peroxide (0.0159 g, 0.07 mmol) were added in one portion to a refluxing solution of **12** (0.50 g, 0.98 mmol) in carbon tetrachloride (65 mL). The mixture was refluxed for 20 min, cooled to 0 °C, and filtered under nitrogen. The filtrate was evaporated under vacuum and the residue was dried for 45 min. The crude bromide was dissolved in xylene (50 mL) and collidine (10.66 mL) and the solution was refluxed for 45 min. The reaction was poured into water and extracted with ethyl acetate. The organic extract was washed with 5% HCl and water and dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue (mixture of 4,6- and 5,7-dienes) was dissolved in methylene chloride (25 mL). A solution of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, 0.194 M) in methylene chloride was added dropwise to the mixture of 4,6- and 5,7-dienes until a faint red color of PTAD persisted. After addition of PTAD, the reaction was stirred at room temperature for 1 h. The solvent was evaporated and the reaction mixture was separated by flash chromatography on silica gel (ethyl acetate/hexane 4:6) to yield **13** (0.4745 g, 71%): mp 188.8–189.2 °C from ether/hexane); UV (ethanol) λ_{max} 256, 216 nm; IR (Nujol mull) 3468, 1753, 1734, 1701 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.81 (3 H, s, 18-CH_3), 2.01 (3 H, s, $OCOCH_3$), 5.42 (1 H, m, 3α-H), 6.19, 6.42 (2 H, AB q, J = 8.5 Hz, 6-H, 7-H), 7.38 (5 H, m, aromatic-H); MS m/z (assignment, relative intensity) 446 (M$^+$ – PTAD – AcOH, 100), 431 (446 – CH_3, 11), 417 (446 – C_2H_5, 11), 253 (446 – side chain, 17), 177 (18), 87 ($C_5H_{11}O$, 26); high-resolution MS calcd for $C_{29}H_{44}F_2O$ (M$^+$ – PTAD – AcOH) 446.3349, found 446.3320.

24,24-Difluoro-26,27-dimethylcholesta-5,7-diene-3,25-diol (14). A solution of **13** (0.4249 g, 0.62 mmol) in THF (50 mL) was cooled to 0 °C and lithium aluminum hydride (0.8794 g, 23.17 mmol) was added in 11 min. The reaction was refluxed for 3 h, cooled to 0 °C, and quenched by addition of wet ether followed by ethyl acetate and water. The lithium salts were filtered off and the filtrate was extracted with ethyl acetate. The organic extract was dried (Na_2SO_4) and evaporated, and the residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:1) to give **14** (0.2343 g, 81%): mp 132.9–133.6 °C; UV (ethanol) λ_{max} 294, 282, 272, 263 nm; IR (Nujol mull) 3389, 3287 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.63 (3 H, s, 18-CH_3), 3.62 (1 H, m, 3α-H), 5.38 (1 H, m, 6-H or 7-H), 5.58 (1 H, m, 7-H or 6-H); ^{19}F NMR ($CDCl_3$) δ –112.04 (t, J = 18.8 Hz, CF_2); MS m/z (assignment, relative intensity) 464 (M$^+$, 100), 446 (M$^+$ – H_2O, 51), 431 (M$^+$ – H_2O – CH_3, 98), 405 (54), 271 (464 – side chain, 22), 253 (271 – H_2O, 84), 87 ($C_5H_{11}O$, 44); high-resolution MS calcd for $C_{29}H_{46}F_2O_2$ (M$^+$) 464.3454, found 464.3444.

24,24-Difluoro-25-hydroxy-26,27-dimethylvitamin D_3 (16). A solution of **14** (0.100 g, 0.22 mmol) in ether (225 mL) was cooled to 0 °C and was deoxygenated by bubbling nitrogen through the solution. The cold solution was irradiated at 254 nm for 2000 s and at 350 nm for another 2000 s. The solvent was evaporated under reduced pressure. The residue containing previtamin **15** was dissolved in ethanol (100 mL) and the solution was refluxed for 2 h. Ethanol was removed under reduced pressure and the reaction mixture was purified by flash chromatography on silica gel (2-propanol/hexane 1:10). HPLC (Varian Micropak Si-10, 50 cm × 8 mm, 2-propanol/hexane 2:100, 6 mL/min) examination of the isolated product (35.74 mg) showed the presence of a small amount of impurity together with the required component. A portion (18.39 mg) of the isolated product was further purified by HPLC (same conditions as above) to give 16 (14.14 mg, 27% yield based on UV measurement assuming ϵ to be 18200): UV (ethanol) λ_{max} 264 nm, λ_{min} 228 nm; ^{1}H NMR ($CDCl_3$) δ 0.57 (3 H, s, 18-CH_3), 3.94 (1 H, m, 3α-H), 4.82 (1 H, m, 19-H), 5.04 (1 H, m, 19-H), 6.00 and 6.25 (2 H, ABq, J = 11.2 Hz, 6-H and 7-H); MS m/z (assignment, relative intensity) 464 (M^+, 46), 446 (M^+ − H_2O, 12), 431 (446 − CH_3, 47), 271 (M^+ − side chain, 17), 253 (271 − H_2O, 18), 136 (ring A plus C-6 and C-7, 100), 118 (136 − H_2O, 79), 87 ($C_5H_{11}O$, 47); high-resolution MS calcd for $C_{29}H_{46}F_2O_2$ 464.3454, found 464.3468.

24,24-Difluoro-25-hydroxy-26,27-dimethylvitamin D_3 3-Tosylate (17). A solution of 16 (0.07 g, 0.15 mmol) in pyridine (2.0 mL) was cooled to 0 °C and freshly recrystallized *p*-toluenesulfonyl chloride (0.176 g, 0.92 mmol) was added in 3 min. The reaction was stirred at 0–4 °C for 40 h and poured into cold, saturated sodium bicarbonate solution. The mixture was extracted with ethyl acetate and the organic extract was washed with 2.5% HCl, saturated $NaHCO_3$, and water and was then dried (Na_2SO_4). The solvent was evaporated and the residue showed one spot on TLC (ethyl acetate/hexane 3:7). Tosylate **17** was employed in the next reaction without any further purification or characterization.

24,24-Difluoro-25-hydroxy-26,27-dimethyl-3,5-cyclovitamin D_3 (18). Tosylate 17 (prepared via the preceding reaction) was treated with dry methanol (40 mL) and sodium bicarbonate (0.6073 g) at 55 °C for 8 h. Solvent was removed under reduced pressure and water was added to the residue. The product mixture was extracted with ether and benzene. The organic extract was washed with water, dried (Na_2SO_4), and evaporated. The residue was purified by preparative TLC on silica gel (ethyl acetate/hexane 15:85) to give 18 (0.0475 g, 66%): ^{1}H NMR ($CDCl_3$) δ 0.56 (3 H, s, 18-CH_3), 0.70 (1 H, m, 3-H), 3.25 (3 H, s, OMe), 4.15 (1 H, d, J = 9.0 Hz, 6-H), 4.86 (1 H, m, 19-H), 4.99 (1 H, d, J = 9.0 Hz, 7-H), 5.03 (1 H, m, 19-H); MS m/z (assignment, relative intensity) 478 (M^+ − side chain, 14), 253 (285 − MeOH, 94), 87 ($C_5H_{11}O$, 100); high-resolution MS calcd for $C_{30}H_{48}F_2O_2$ 478.3610, found 478.3620.

24,24-Difluoro-1α,25-dihydroxy-26,27-dimethyl-3,5-cyclovitamin D_3 (19). A solution of *tert*-butyl hydroperoxide (40 μL, 4.82 M) in methylene chloride was added dropwise to a suspension of selenium dioxide (6.31 mg, 0.057 mmol) in methylene chloride (6.0 mL) in 2 min. The mixture was stirred at room temperature for 30 min, diluted with methylene chloride (20 mL), and cooled to 0 °C. Compound 18 (0.037 g, 0.077 mmol) in methylene chloride (4 mL) was added dropwise over a period of 10 min. After 5 min, the cooling bath was removed and the reaction was stirred at room temperature for 30 min. The reaction was poured into 10% NaOH (30 mL) and the mixture was shaken vigorously. The organic layer was separated and the aqueous layer was extracted with ether. The combined organic extracts were washed with 10% NaOH, followed by water, and then dried (Na_2SO_4) and evaporated. The residue was purified by preparative TLC on silica gel (ethyl acetate/hexane 35:65) to yield **19** (14.10 mg, 37%): ^{1}H NMR ($CDCl_3$) δ 0.53 (3 H, s, 18-CH_3), 0.69 (1 H, m, 3-H), 3.23 (3 H, s, OMe), 3.91–4.39 (1 H, m, 1-H), 4.16 (1 H, d, J = 9.0 Hz, 6-H), 4.95 (1 H, d, J = 9.0 Hz, 7-H), 5.15 (1 H, m, 19-H), 5.20 (1 H, m, 19-H); MS m/z (assignment, relative intensity) 494 (M^+, 12), 476 (M^+ − H_2O, 4), 462 (M^+ − MeOH, 32), 444 (462 − H_2O, 10), 429 (444 − CH_3, 5), 269 (M^+ − MeOH − side chain, 18), 251 (269 − H_2O, 16), 87 ($C_5H_{11}O$, 55); high-resolution MS calcd for $C_{30}H_{48}F_2O_3$ 494.3559, found 494.3588.

24,24-Difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3 3-Acetate (20) and 5,6-Trans Isomer (20a). Compound **19** (12.50 mg, 0.025 mmol) in glacial acetic acid (1.0 mL) was heated at 55 °C for 15 min. The reaction was poured into cold saturated sodium bicarbonate and extracted with ether and benzene. The organic extract was washed with water, dried (Na_2SO_4), and evaporated. The product mixture was first purified by preparative TLC on silica gel (ethyl acetate/hexane 3:7) and then further purified by HPLC (Varian Micropak Si-10, 50 cm × 8 mm, 2-propanol/hexane 3.5:100, 6 mL/min) to give **20** (2.4601 mg, 19%): UV (ethanol) λ_{max} 264 nm, λ_{min} 227 nm; ^{1}H NMR ($CDCl_3$) δ 0.55 (3 H, s, 18-CH_3), 2.03 (3 H, s, $OCOCH_3$), 4.38 (1 H, m, 1-H), 5.01 (1 H, m, 19-H), 5.18 (1 H, m, 3α-H), 5.34 (1 H, m, 19-H), 6.00 and 6.35 (2 H, AB q, J = 11.2 Hz, 7-H and 6-H); MS m/z (assignment, relative intensity) 522 (M^+, 8), 504 (M^+ − H_2O, 40), 489 (504 − CH_3, 9), 462 (M^+ − AcOH, 78), 444 (M^+ − AcOH − H_2O, 100), 429 (444 − CH_3, 34), 269 (M^+ − AcOH − side chain, 28), 251 (269 − H_2O, 57), 134 (ring A plus C-6 and C-7-AcOH, 74), 117 (43), 87 ($C_5H_{11}O$, 55); high-resolution MS calcd for $C_{31}H_{48}F_2O_4$ 522.3521, found 522.3479.

The yield of **20a** was 1.6723 mg, 13%: UV (ethanol) λ_{max} 269 nm, λ_{min} 228 nm; ^{1}H NMR ($CDCl_3$) δ 0.57 (3 H, s, 18-CH_3), 2.01 (3 H, s, $OCOCH_3$), 4.47 (1 H, m, 1-H), 4.99 (1 H, m, 19-H), 5.13 (1 H, m, 19-H), 5.24 (1 H, m, 3-H), 5.81 (1 H, d, J = 11.8 Hz, 7-H), 6.57 (1 H, d, J = 11.8 Hz, 6-H); MS m/z (assignment, relative intensity) 462 (M^+ − AcOH, 40), 273 (6), 251 (M^+ − AcOH − H_2O − side chain, 17), 134 (ring A plus C-6 and C-7-AcOH, 100), 116 (134 − H_2O, 12), 87 ($C_5H_{11}O$, 41); high-resolution MS calcd for $C_{29}H_{44}F_2O_2$ (M^+ − AcOH) 462.3298, found 462.3335.

24,24-Difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3 (21). Compound **20** (2.1494 mg) was cooled to 0 °C and 10% methanolic sodium hydroxide (10 mL) was added dropwise in 10 min. The reaction was stirred at room temperature for 90 min and methanol was removed under reduced pressure. Water was added to the residue and the mixture was extracted with ether, benzene, and methylene chloride. The organic extract was washed with water and evaporated. The residue was purified by HPLC (Varian Micropak Si-10, 50 cm × 8 mm, 2-propanol/hexane 18:100, 6 mL/min) to give **21** (1.74 mg, 88%): UV (ethanol) λ_{max} 265 nm, λ_{min} 228 nm; ^{1}H NMR ($CDCl_3$) δ 0.56 (3 H, s, 18-CH_3), 4.18 (1 H, m, 3-H), 4.38 (1 H, m, 1-H), 4.99 (1 H, m, 19-H), 5.32 (1 H, m, 19-H), 6.00 and 6.38 (2 H, AB q, J = 12.0 Hz, 7-H and 6-H); MS m/z (assignment, relative intensity) 480 (M^+, 24), 462 (M^+ − H_2O, 28), 444 (M^+ − $2H_2O$, 29), 287 (M^+ − side chain, 10), 269 (287 − H_2O, 16), 251 (269 − H_2O, 27), 152 (ring A plus C-6 and C-7, 35), 134 (152 − H_2O, 100), 116 (134 − H_2O, 5), 87 ($C_5H_{11}O$, 60); high-resolution MS calcd for $C_{29}H_{48}F_2O_3$ 480.3415, found 480.3383.

5,6-*trans*-24,24-Difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3 (21a). Compound **20a** (1.57 mg) was treated with 10% methanolic sodium hydroxide and the product was purified by HPLC (as described above for **20** to **21**) to yield **21a** (1.3 mg, 87%): UV (ethanol) λ_{max} 273 nm, λ_{min} 230 nm; ^{1}H NMR ($CDCl_3$) δ 0.58 (3 H, s, 18-CH_3), 4.22 (1 H, m, 3-H), 4.50 (1 H, m, 1-H), 4.98 (1 H, m, 19-H), 5.11 (1 H, m, 19-H), 5.86 (1 H, d, J = 11.2 Hz, 7-H), 6.58 (1 H, d, J = 11.2 Hz, 6-H); MS m/z (assignment, relative intensity) 480 (M^+, 13), 462 (M^+ − H_2O, 13), 444 (M^+ − $2H_2O$, 6), 287 (M^+ − side chain, 9), 269 (287 − H_2O, 13), 251 (269 − H_2O, 15), 152 (ring A plus C-6 and C-7, 26), 134 (152 − H_2O, 100), 87 ($C_5H_{11}O$, 26); high-resolution MS calcd for $C_{29}H_{46}F_2O_3$ (M^+) 480.3415, found 480.3432.

Results

3β-Hydroxy-22,23-dinorcholenic acid 3-acetate (**1**) was subjected to Arndt–Eistert homologation[50,51] to provide **2** in 70% yield (Scheme I). The 3β-acetate was hydrolyzed and the alcohol function was protected by the base-stable methoxymethyl (MOM) group to produce **3** in 91% yield. Compound **3** was converted into **4** by reduction with lithium aluminum hydride in 96% yield. It was oxidized with pyridinium chlorochromate (PCC) to provide aldehyde **5** in 90% yield.[52] **5** was subjected to Reformatsky reaction[53] using activated zinc and ethyl bromodifluoro-

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Table I. Bone-Calcium Mobilization (serum calcium (mg/dL)) following the Administration of 25-(OH)-D_3 (A) or 24,24-$(F)_2$-25-(OH)-26,27-$(CH_3)_2$-D_3 (B)

		5 pmol	50 pmol	500 pmol	5000 pmol
2 h	A	4.32 ± 0.28 (5)[a]	4.48 ± 0.07 (5)	4.66 ± 0.13 (5)	4.64 ± 0.36 (5)
	B	4.40 ± 0.09 (5)	4.28 ± 0.09 (5)	4.22 ± 0.08 (5)	4.78 ± 0.17 (5)
4 h	A	4.14 ± 0.15 (5)	4.10 ± 0.19 (5)	4.22 ± 0.11 (5)	4.12 ± 0.31 (5)
	B	4.38 ± 0.19 (5)	4.18 ± 0.06 (4)	4.40 ± 0.11 (4)	4.56 ± 0.28 (5)
13 h	A	4.40 ± 0.07 (5)	4.86 ± 0.13 (5)	6.22 ± 0.17 (5)	6.82 ± 0.23 (5)
	B	4.20 ± 0.13 (5)	4.14 ± 0.10 (5)	5.14 ± 0.20 (5)	5.92 ± 0.16 (5)
24 h	A	4.34 ± 0.12 (5)	5.06 ± 0.24 (5)	5.50 ± 0.17 (5)	5.82 ± 0.14 (5)
	B	4.90 ± 0.18 (5)	4.72 ± 0.16 (5)	5.10 ± 0.12 (5)	5.90 ± 0.18 (5)
47 h	A	4.66 ± 0.12 (5)	4.68 ± 0.10 (5)	5.12 ± 0.14 (5)	6.24 ± 0.08 (5)
	B	4.34 ± 0.10 (5)	4.63 ± 0.29 (4)	4.50 ± 0.19 (5)	6.16 ± 0.25 (5)
72 h	A	4.32 ± 0.06 (5)	4.52 ± 0.09 (5)	5.04 ± 0.23 (5)	6.50 ± 0.23 (5)
	B	4.20 ± 0.14 (5)	4.64 ± 0.10 (5)	4.38 ± 0.14 (5)	5.84 ± 0.07 (5)

[a] Number of animals per group.

acetate, and **6** was isolated by flash chromatography in 64% yield as a mixture of diastereoisomers in a 1.4:1 ratio. Both diastereoisomers were detected by TLC and ^1H NMR spectroscopy. No attempt was made to separate the stereoisomers. The hydroxyl function at C-23 in **6** was deoxygenated by converting **6** into **7** (98% yield) and refluxing **7** with tributyltin hydride[54] to produce the key intermediate **8** in 94% yield. The structure of **8** was confirmed by ^{19}F NMR, ^1H NMR, IR, MS, and elemental analysis.

Treatment of **8** with ethylmagnesium bromide unexpectedly yielded tertiary alcohol **9** as minor product and the secondary alcohol (reduced form of **10**) as the major product. The best way of separating **9** from the reduced form of **10** was found to involve oxidation of the crude reaction mixture with pyridinium chlorochromate in the presence of 3A molecular sieves.[55] As a result, **9** and **10** were obtained in 25% and 69% yields, respectively. Ketone **10** on treatment with ethylmagnesium bromide again yielded **9** as the minor product and the secondary alcohol as the major product. The large production of the secondary alcohol compared to tertiary alcohol **9** on treatment of **8** with EtMgBr or **10** with EtMgBr is not well understood at present.

Compound **9** was converted to **11** quantitatively by treating with *p*-toluenesulfonic acid (Scheme II). The secondary alcohol in **11** was protected as an acetate to provide **12** in 92% yield. Allylic bromination of **12** with *N*-bromosuccinimide in the presence of benzoyl peroxide and dehydrobromination of the bromide with collidine gave a mixture of 4,6- and 5,7-dienes. The 5,7-diene was separated from the 4,6-diene by titrating the mixture with 4-phenyl-1,2,4-triazoline-3,5-dione. Product **13** was obtained in 71% yield. Protecting groups on **13** were removed with lithium aluminum hydride and 5,7-diene **14** was regenerated in 81% yield. Compound **14** was irradiated first[56,57] at 254 nm and then at 350 nm to provide previtamin **15**, which was not isolated. The photolysis mixture was refluxed in ethanol and the crude product was purified by flash chromatography and HPLC to give **16** in 27% yield. Vitamin D_3 analogue **16** displayed λ_{max} 264 nm and λ_{min} 228 nm in UV spectrum. Proton nuclear magnetic resonance spectrum (^1H NMR) of **16** showed the presence of a triene system. Mass spectrum displayed the molecular ion at 464 ($C_{29}H_{46}F_2O_2$) and a fragmentation pattern consistent with the structure.

Introduction of 1α-hydroxy function (Scheme III) in **16** was achieved by using the method of Sheves et al. and Paaren et al.[58,59] Tosylation of **16** with *p*-toluenesulfonyl chloride and pyridine at 0–4 °C gave **17**. Solvolysis of **17** with methanol in the presence of sodium bicarbonate at 55 °C for 8 h resulted in the formation of cyclovitamin **18** in 66% yield. Allylic hydroxylation of **18** with selenium dioxide and *tert*-butyl hydroperoxide provided **19** in 37% yield. Cycloreversion of **19** with glacial acetic acid gave **20** and trans isomer **20a** in 17% and 13% yields, respectively. Removal of protecting groups in **20** and **20a** with methanolic sodium hydroxide and purification by HPLC afforded **21** and **21a** in 88% and 87% yield. Vitamin D analogue **21** exhibited λ_{max} 265 nm and λ_{min} 228 nm, and trans isomer **21a** showed a λ_{max} of 273 nm and a λ_{min} of 230 nm in the UV spectrum. Proton nuclear magnetic spectra and mass spectra of **21** and **21a** were consistent with their respective structures.

Compound **16** was noted to increase serum-calcium concentrations within 13 h of its administration (Table I). At this time, lower doses of the analogue were inactive (5, 50 pmol) whereas higher doses were active (500, 5000 pmol). Consistent responses were observed at doses of 50–500 pmol at 24, 48, and 72 h. The response from a biological activity viewpoint was similar to that observed for 25-(OH)-D_3. Statistical analysis of the relationships between time, dose, and response for **16** and 25-(OH)-D_3, however, showed that the differences between **16** and 25-(OH)-D_3 with respect to serum calcium response ($p < 0.05$) were primarily reflected in differences between the quadratic dose concentration effects. At early times (4 h) there was a stronger quadratic response for **16**, while at 36 h and 72 h, the dose concentration response was essentially linear for 25-(OH)-D_3 but still quadratic for **16** (Figure 1). These differences were small and not of biological significance.

When intestinal calcium transport responses were determined for **16** and 25-(OH)-D_3, the results were as noted in Table II. Both compounds were biologically active at a dose of 500 pmol at 13 h. Statistical analysis of the relationships between time, dose, and response for the two analogues (Figure 2) for the intestinal calcium transport response showed that the effects of **16** and 25-(OH)-D_3 were similar early (4 h) but tended to diverge with time. This "interaction" of time and concentration was statis-

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Table II. Intestinal-Calcium Transport (Serosal/Mucosal ^{45}Ca) following the Administration of 25-(OH)-D_3 (A) or 24,24-$(F)_2$-25-(OH)-26,27-$(CH_3)_2$-D_3 (B)

		5 pmol	50 pmol	500 pmol	5000 pmol
2 h	A	2.03 ± 0.23 (5)[a]	1.75 ± 0.10 (5)	2.22 ± 0.17 (5)	2.93 ± 0.42 (5)
	B	1.89 ± 0.07 (5)	1.80 ± 0.15 (4)	2.58 ± 0.47 (5)	3.77 ± 0.32 (5)
4 h	A	2.42 ± 0.44 (5)	2.17 ± 0.27 (5)	2.83 ± 0.38 (5)	3.31 ± 0.81 (5)
	B	1.61 ± 0.19 (5)	1.97 ± 0.34 (4)	2.27 ± 0.18 (4)	3.18 ± 0.53 (5)
13 h	A	2.22 ± 0.10 (5)	3.75 ± 0.53 (5)	3.60 ± 0.66 (5)	5.43 ± 0.59 (5)
	B	2.30 ± 0.34 (5)	2.18 ± 0.40 (5)	5.85 ± 0.50 (5)	4.84 ± 0.83 (5)
24 h	A	2.84 ± 0.16 (5)	4.80 ± 0.69 (5)	7.19 ± 0.78 (5)	7.19 ± 0.99 (5)
	B	2.34 ± 0.34 (5)	6.16 ± 0.50 (5)	7.11 ± 0.36 (5)	8.65 ± 2.13 (5)
47 h	A	3.41 ± 0.48 (5)	11.54 ± 1.08 (5)	9.99 ± 0.76 (5)	11.01 ± 2.70 (5)
	B	2.59 ± 0.37 (5)	3.29 ± 0.51 (4)	8.04 ± 2.00 (5)	11.06 ± 1.81 (5)
72 h	A	3.59 ± 0.47 (5)	8.87 ± 0.95 (5)	9.89 ± 0.86 (5)	11.48 ± 0.88 (5)
	B	2.20 ± 0.34 (5)	2.84 ± 0.44 (5)	7.29 ± 1.28 (5)	9.39 ± 1.14 (5)

[a] Number of animals per group.

Table III. Bone-Calcium Mobilization (serum calcium (mg/dL)) following the Administration of 1α,25-$(OH)_2$-D_3 (A) or 24,24-$(F)_2$-1α,25-$(OH)_2$-26,27-$(CH_3)_2$-D_3 (B)

		5 pmol	50 pmol	500 pmol	5000 pmol
2 h	A	4.40 ± 0.04 (4)[a]	4.35 ± 0.12 (4)	4.76 ± 0.11 (5)	4.84 ± 0.07 (5)
	B	4.50 ± 0.11 (4)	4.42 ± 0.10 (5)	4.80 ± 0.11 (5)	5.10 ± 0.10 (5)
4 h	A	4.32 ± 0.16 (5)	4.58 ± 0.06 (4)	5.32 ± 0.24 (5)	5.60 ± 0.07 (5)
	B	4.28 ± 0.07 (5)	5.00 ± 0.29 (5)	5.10 ± 0.10 (5)	5.48 ± 0.12 (5)
12 h	A	4.38 ± 0.07 (5)	5.58 ± 0.19 (5)	6.10 ± 0.18 (5)	6.14 ± 0.07 (5)
	B	4.34 ± 0.17 (5)	5.50 ± 0.19 (4)	6.12 ± 0.04 (5)	6.40 ± 0.18 (5)
24 h	A	4.58 ± 0.12 (5)	5.00 ± 0.24 (5)	4.98 ± 0.21 (5)	5.50 ± 0.15 (5)
	B	4.38 ± 0.14 (5)	4.74 ± 0.05 (5)	5.58 ± 0.09 (5)	6.36 ± 0.07 (5)
48 h	A	4.08 ± 0.02 (5)	4.54 ± 0.20 (5)	4.35 ± 0.09 (4)	4.78 ± 0.19 (5)
	B	3.98 ± 0.08 (4)	4.08 ± 0.04 (5)	4.94 ± 0.14 (5)	5.86 ± 0.02 (5)
69 h	A	4.12 ± 0.13 (5)	4.55 ± 0.06 (4)	4.48 ± 0.22 (5)	4.78 ± 0.33 (5)
	B	4.13 ± 0.06 (4)	4.02 ± 0.18 (5)	4.87 ± 0.15 (3)	4.82 ± 0.08 (5)

[a] Number of animals per group.

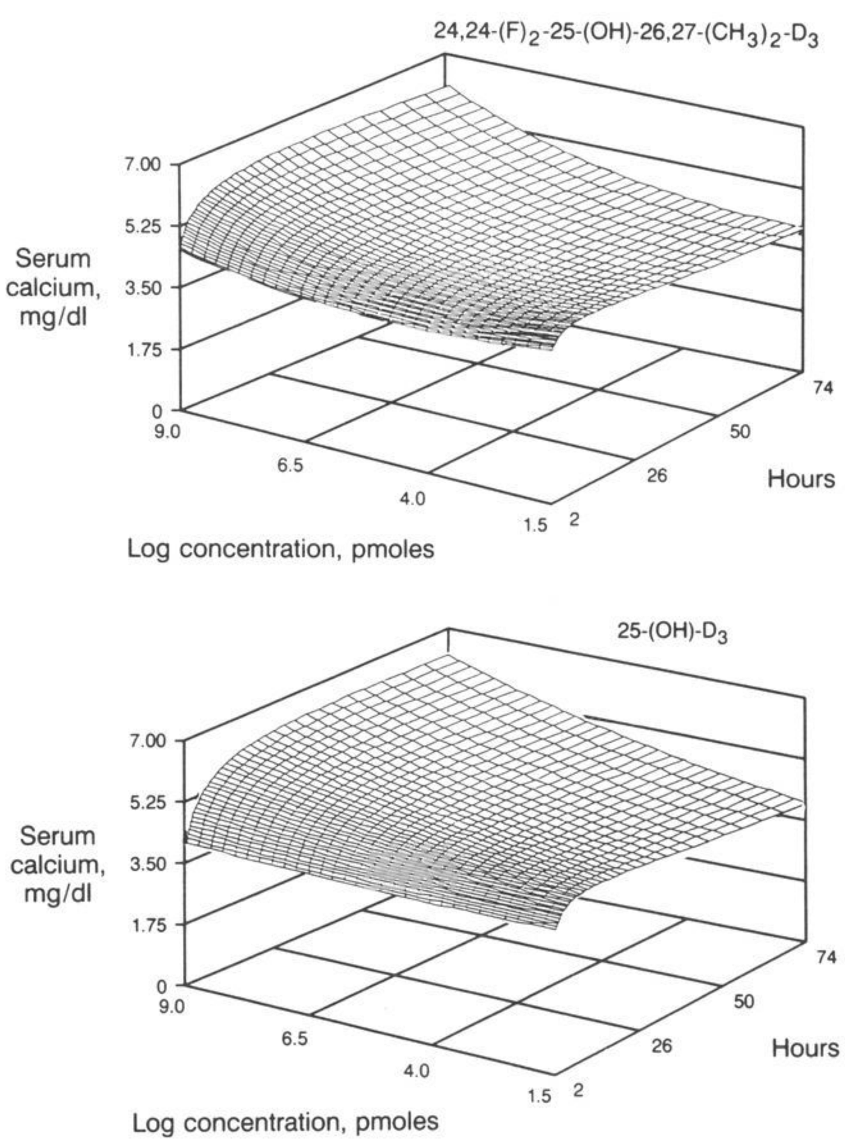

Figure 1. Serum calcium concentrations at various time points after the administration of varying doses of 25-hydroxyvitamin D_3 or analogue **16**.

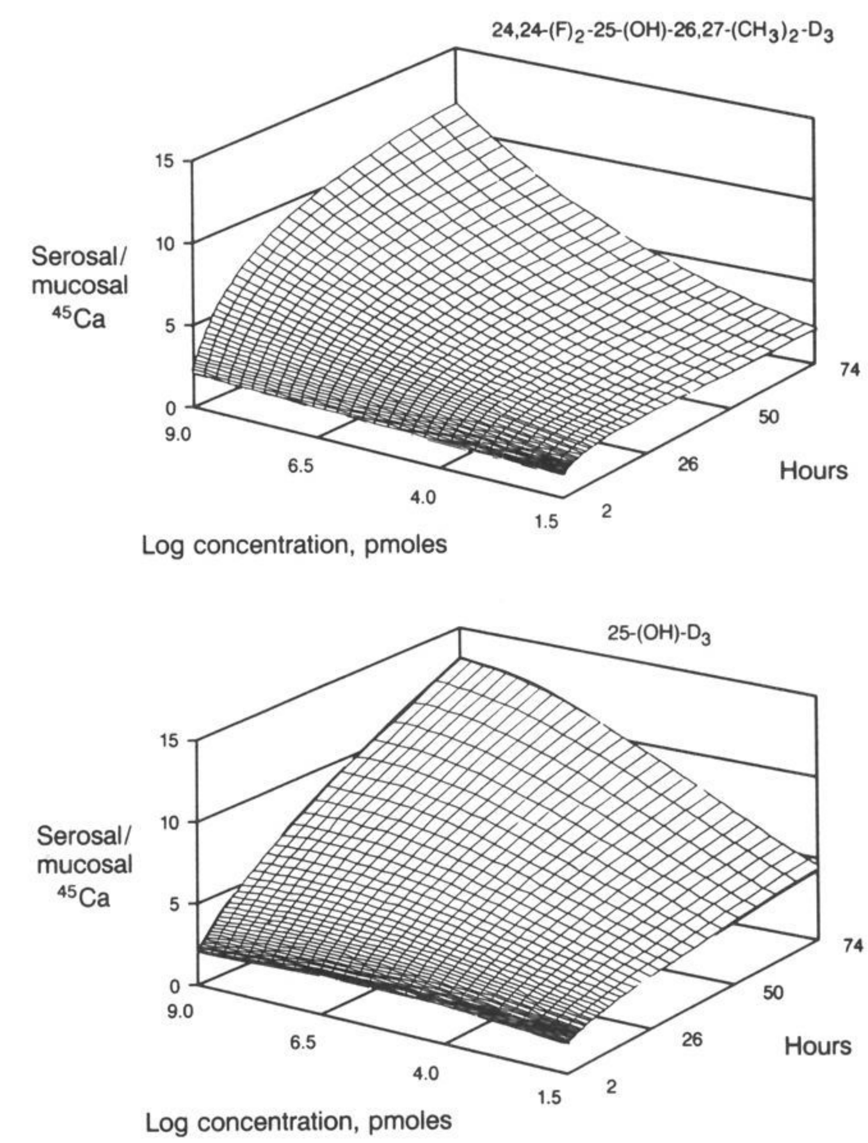

Figure 2. Intestinal calcium transport measured by the everted gut sac method at various time points after the administration of varying doses of 25-hydroxyvitamin D_3 or analogue **16**.

tically significant ($p < 0.01$).

The results of serum-calcium concentrations noted following the administration of **21** or 1α,25-$(OH)_2$-D_3 are shown in Table III. Both compounds were active at a dose of 50 pmol/rat within 4 h of administration. At a dose of 5000 pmol both compounds were active within 2 h. The

Table IV. Intestinal-Calcium Transport (Serosal/Mucosal ^{45}Ca) following the Administration of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ (A) or $24,24\text{-}(F)_2\text{-}1\alpha,25\text{-}(OH)_2\text{-}26,27\text{-}(CH_3)_2\text{-}D_3$ (B)

		5 pmol	50 pmol	500 pmol	5000 pmol
2 h	A	2.21 ± 0.17 (4)[a]	2.50 ± 0.19 (4)	3.10 ± 0.24 (5)	3.12 ± 0.58 (5)
	B	2.08 ± 0.11 (4)	2.27 ± 0.15 (5)	4.55 ± 0.81 (5)	3.70 ± 0.54 (5)
4 h	A	2.53 ± 0.17 (5)	5.09 ± 1.17 (4)	6.60 ± 0.61 (5)	7.66 ± 0.82 (5)
	B	3.36 ± 0.60 (5)	4.22 ± 0.45 (5)	6.44 ± 0.82 (5)	4.84 ± 1.05 (5)
12 h	A	2.62 ± 0.60 (5)	3.23 ± 0.40 (5)	4.06 ± 0.40 (5)	7.00 ± 0.32 (5)
	B	2.85 ± 0.56 (5)	4.41 ± 0.50 (4)	8.92 ± 0.88 (5)	8.39 ± 1.27 (5)
24 h	A	2.95 ± 0.42 (5)	4.34 ± 0.55 (5)	3.50 ± 0.36 (5)	4.71 ± 0.48 (5)
	B	2.14 ± 0.30 (5)	3.93 ± 0.18 (5)	7.30 ± 0.61 (5)	5.32 ± 1.22 (4)
48 h	A	3.19 ± 0.44 (5)	4.71 ± 0.39 (5)	5.98 ± 0.74 (4)	7.22 ± 1.05 (5)
	B	2.09 ± 0.24 (4)	4.25 ± 0.69 (5)	5.39 ± 0.49 (5)	8.00 ± 0.60 (5)
69 h	A	2.75 ± 0.19 (5)	5.69 ± 0.38 (4)	5.67 ± 0.56 (5)	4.99 ± 0.42 (5)
	B	2.34 ± 0.27 (4)	3.04 ± 0.56 (5)	5.46 ± 1.23 (3)	8.84 ± 0.46 (5)

[a] Number of animals per group.

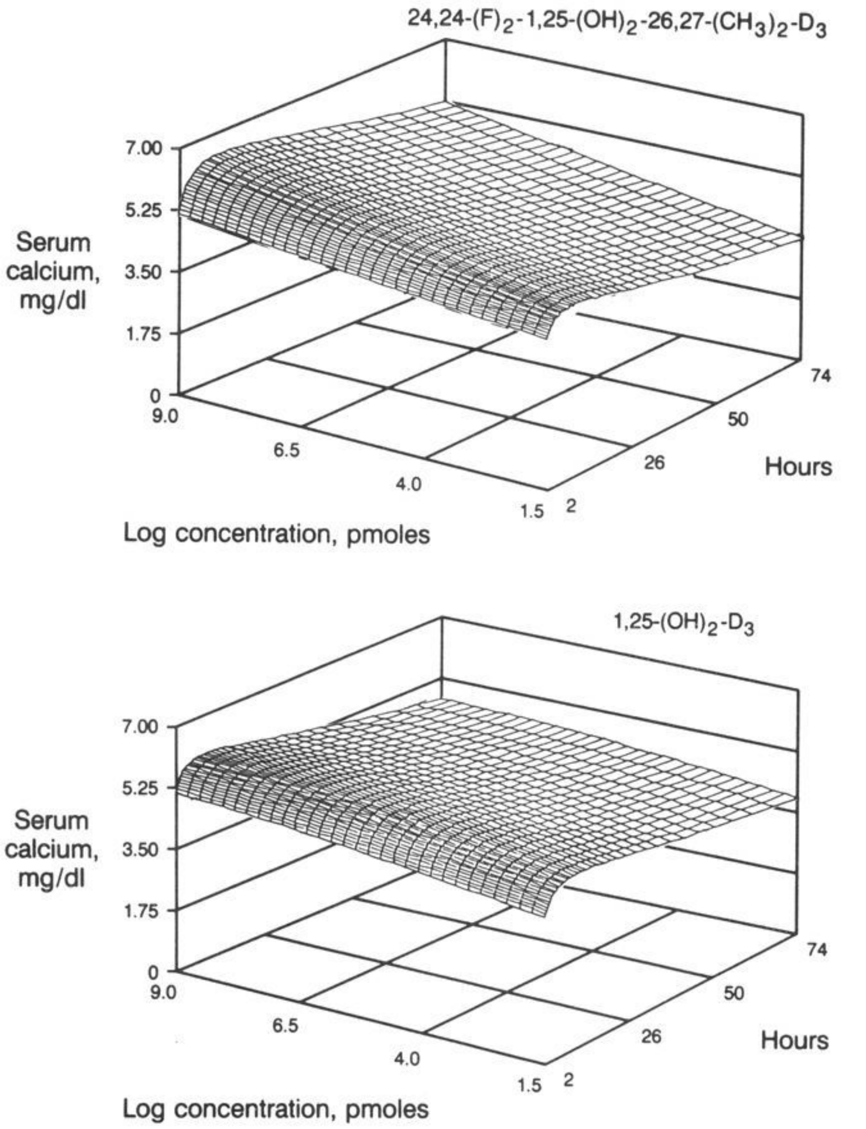

Figure 3. Bone-calcium mobilization at various times following the administration of varying doses of 1,25-dihydroxyvitamin D_3 or analogue **21**.

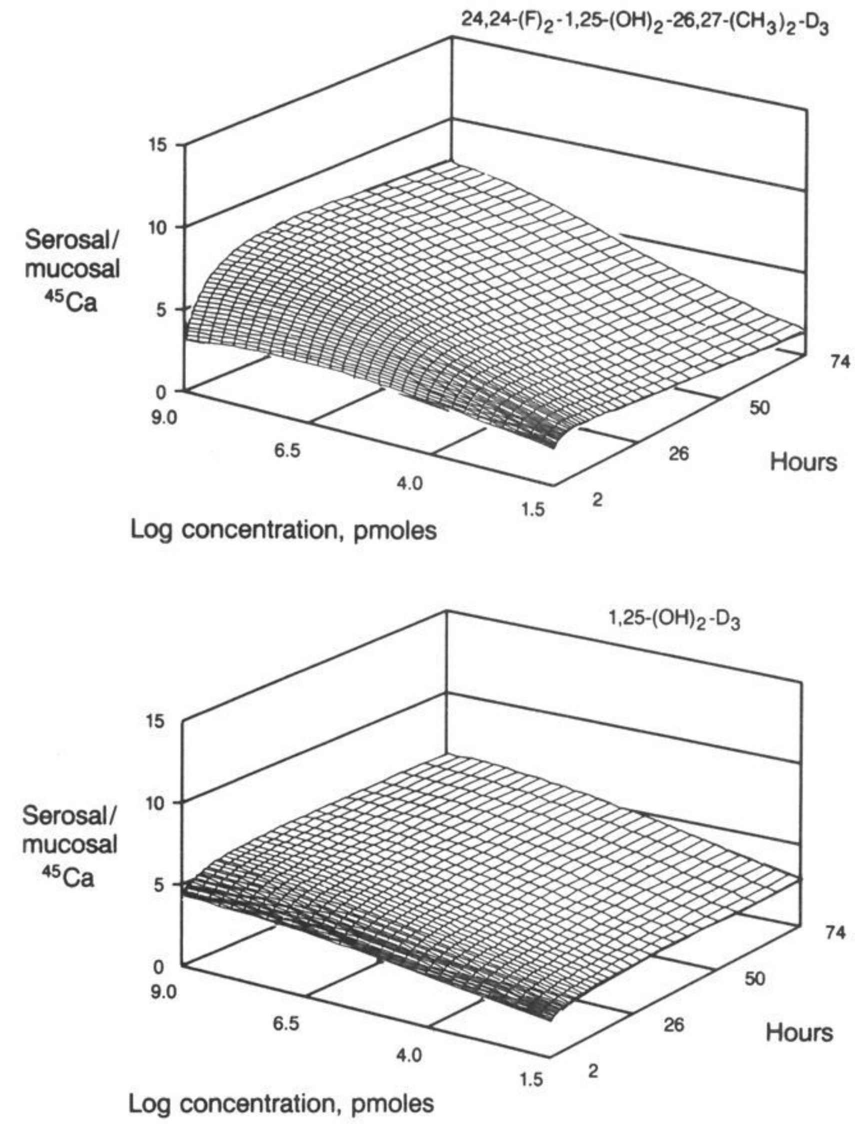

Figure 4. Intestinal-calcium transport at various times following the administration of varying doses of 1,25-dihydroxyvitamin D_3 or analogue **21**.

analysis of time, dose, and response relationships following the administration of **21** or $1\alpha,25\text{-}(OH)_2\text{-}D_3$ are shown in Figure 3. The dose concentration effects (overall) for **21** and $1\alpha,25\text{-}(OH)_2\text{-}D_3$ on serum calcium were similar over the dose range during 0–24 h but tended to diverge at low doses and somewhat more at higher doses, during 24–72 h. This "interaction" of dose concentration and time was statistically significant ($p < 0.001$).

Intestinal calcium transport responses for **21** and $1\alpha,25\text{-}(OH)_2\text{-}D_3$ are shown in Table IV. Both analogues were active within 2 h of the administration of a dose of 500 pmol. The intestinal calcium transport response (Figure 4) was essentially linear in dose concentration for **21** over 0–72 h. $1\alpha,25\text{-}(OH)_2\text{-}D_3$ yielded a "plateau" dose concentration response for the whole time range. These differences in the response surfaces for intestinal-calcium transport between the two compound were statistically significant ($p < 0.005$).

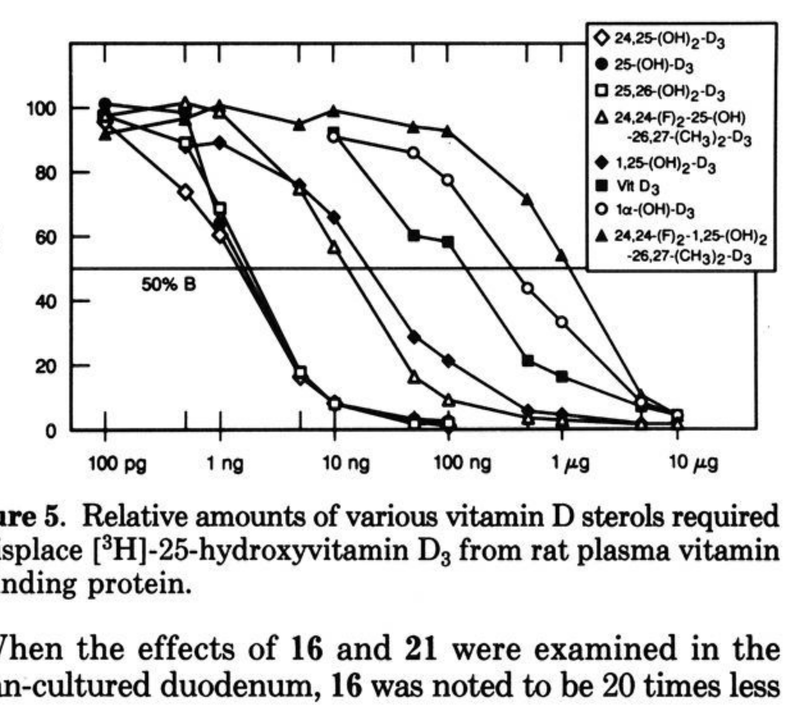

Figure 5. Relative amounts of various vitamin D sterols required to displace $[^3H]$-25-hydroxyvitamin D_3 from rat plasma vitamin D binding protein.

When the effects of **16** and **21** were examined in the organ-cultured duodenum, **16** was noted to be 20 times less

Table V. Calcium Binding Protein Induction in Duodenal Organ Culture: Relative Biopotency of 24,24-$(F)_2$-25-(OH)-26,27-$(CH_3)_2$-D_3 and 24,24-$(F)_2$-1,25-$(OH)_2$-26,27-$(CH_3)_2$-D_3

vitamin D_3 analogue	concn, nM	calcium binding protein[a,b] μg/100 mg of duodenum	relative potency compared to 1,25-$(OH)_2$-D_3[c]
1,25-$(OH)_2$-D_3	0.01	6.9 ± 0.9	
	0.1	13.8 ± 0.6	100
	1	32.8 ± 3.0	
	10	80.3 ± 5.4	
24,24-$(F)_2$-25-(OH)-26,27-$(CH_3)_2$-D_3	1	10.1 ± 0.4	
	10	22.4 ± 2.1	4.5
	100	66.7 ± 3.5	
24,24-$(F)_2$-1,25-$(OH)_2$-26,27-$(CH_3)_2$-D_3	0.01	11.9 ± 1.9	325
	0.1	21.7 ± 1.6	
	1	49.5 ± 3.9	
	10	94.0 ± 3.4	

[a] Values: mean ± SE; 6 duodena/treatment group. [b] Least-squares fit, log–log plot: $\log y = 0.358 \log x + 4.75$ ($r = 0.99$). [c] Values determined by inserting values of y (CaBP concentration) into the equation and solving for x (1,25-$(OH)_2$-D_3 concentration). The ratio of the actual concentration of the analogue used to the concentration of 1,25-$(OH)_2$-D_3 required to give the same response was then calculated. The potency of 1,25-$(OH)_2$-D_3 was arbitrarily set to 100 and the relative potencies of the two analogues were determined by dividing the calculated ratios into 100.

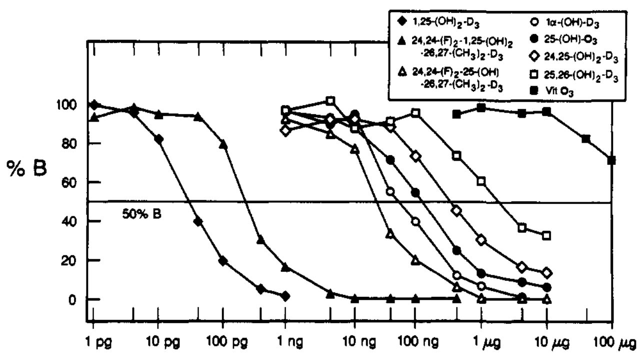

Figure 6. Relative amounts of various vitamin D sterols required to displace [^{3}H]-1,25-dihydroxyvitamin D_3 from chick intestinal cytosol receptor.

active than 1,25-$(OH)_2$-D_3. Compound **21**, however, was 3 times more active than 1α,25-$(OH)_2$-D_3 (Table V).

The ability of **16**, **21**, and related vitamin D_3 analogues to displace [^{3}H]25-(OH)·D_3 from rat plasma vitamin D-binding protein are shown in Figure 5. The B_{50} values (the amount of analogue at which 50% of tracer is displaced) for the respective analogues are as follows: 24*R*,25-$(OH)_2$-D_3, 3.22×10^{-9} M; 25-(OH)-D_3, 3.77×10^{-9} M; 25*S*,26-$(OH)_2$-D_3, 4.03×10^{-9} M; **16**, 2.58×10^{-8} M; 1,25-$(OH)_2$-D_3, 4.53×10^{-8} M; vitamin D_3, 3.38×10^{-7} M; 1α-(OH)-D_3, 8.48×10^{-7} M; and **21**, 2.20×10^{-6} M.

The ability of **16**, **21**, and other analogues to bind to chick intestinal cytosol receptor for 1,25-$(OH)_2$-D_3 are shown in Figure 6. The B_{50} values are as follows: 1,25-$(OH)_2$-D_3, 7.25×10^{-11} M; **21**, 4.95×10^{-10} M; **16**, 5.28×10^{-8} M; 1α-(OH)-D_3, 1.39×10^{-7} M; 25-(OH)-D_3, 2.82×10^{-7} M; 24*R*,25-$(OH)_2$-D_3, 8.04×10^{-7} M; and 25*S*,26-$(OH)_2$-D_3, 4.30×10^{-6} M.

Discussion

We report here synthesis of two novel vitamin D analogues, 24,24-difluoro-25-hydroxy-26,27-dimethylvitamin D_3 (**16**) and 24,24-difluoro-1α,25-dihydroxy-26,27-dimethylvitamin D_3 (**21**) (Schemes I–III). These compounds are blocked at the 24-position in order to decrease the rate of metabolic degradation of the molecule. The synthetic scheme involves key intermediate **8**, which was synthesized by Reformatsky condensation[53] of aldehyde **5** with readily available ethyl bromodifluoroacetate. The functionality at C-24 was easily removed by treating the product **6** with 1,1′-thiocarbonyldiimidazole and subsequent treatment of **7** with tributyltin hydride.[54] This scheme for introduction of fluorines at the C-24 position seems to be better than the alternative approach[60] where the α-keto ester is treated with (diethylamido)sulfur trifluoride (DAST). DAST is moisture-sensitive and is not a pleasant reagent to work with. Additionally, the present scheme provides a new functionality at C-23 in the molecule, which is not possible by the other approach.

From a biological standpoint, **16** was equipotent with 25-hydroxyvitamin D_3 in its ability to mobilize bone calcium and stimulate intestinal-calcium transport. While differences in the interaction between time and concentration were observed, these differences were small. It also had similar properties with respect to its ability to bind a vitamin D binding protein and the intestinal receptor for 1,25-dihydroxyvitamin D_3. Compound **16** has a biological activity similar to that of 25-hydroxy-26,27-dimethylvitamin D_3.[40] Compound **21** had a biological activity similar to that of 1,25-dihydroxyvitamin D_3 in vivo. Intestinal-calcium transport and bone-calcium mobilization responses were similar. Once again, although small but statistically significant differences in the interaction between time and concentration were observed for the two compounds, the differences were small and biologically irrelevant. The binding properties of **21** and 1,25-dihydroxyvitamin D_3 were also similar in most respects.

In the duodenal organ culture model, **16** was about equipotent to 24,24-difluoro-25-hydroxyvitamin D_3 in the induction of CaBP synthesis;[20] this was approximately 3 times as effective as its nonfluorinated analogue, 25-hydroxyvitamin D_3,[48] but still only $^1/_2$ as active as 1,25-dihydroxyvitamin D_3. On the other hand, **21** was about 3 times as potent as 1,25-dihydroxyvitamin D_3, similar to the superior biopotency of 24,24-difluoro-1,25-dihydroxyvitamin D_3, which was 4–5-fold more potent than 1,25-dihydroxyvitamin D_3.[20] Consistent with these in vitro results, this latter compound, 24,24-difluoro-1,25-dihydroxyvitamin D_3, also exhibited an approximate 5-fold increase in biological activity over the native hormone 1,25-dihydroxyvitamin D_3 in several in vivo assays: stimulation of intestinal-calcium transport, bone calcium mobilization, calcification of epiphyseal plate cartilage, and elevation of blood calcium and phosphate.[60] Interestingly, 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3 was also about 5 times more potent than the native hormone in these in vivo assays.[16] Recent work demonstrated that this hexafluoro analogue, while binding only about $^1/_3$ as avidly to the intestinal 1,25-dihydroxyvitamin D_3 receptor, results in a receptor complex which binds more tightly to DNA.[61] It was suggested that this property might account for the overall greater biological activity of this analogue. It is possible that the same phenomenon might partially explain the greater potency of the present fluoro analogues in duodenal organ culture than in vivo. In addition, degradative pathways may exist in the intact animal that are not present in vitro. This could possibly contribute to a prolonged biological half-life in the organ-cultured duodenum.

Registry No. 1, 1474-14-2; 1 acid chloride derivative, 67711-02-8; 1 diazoketone derivative, 23976-71-8; **2**, 33168-65-9; **2** *o*-deacetyl derivative, 69454-96-2; **3**, 123835-97-2; **4**, 123835-98-3;

(60) Yamada, S.; Ohmori, M.; Takayama, H. *Tetrahedron Lett.* **1979**, *21*, 1859.
(61) Inaba, M.; Okuno, S.; Inoue, A.; Nishizawa, Y.; Morii, H.; DeLuca, H. F. *Arch. Biochem. Biophys.* **1989**, *268*, 35.

5, 111478-66-1; **6** (isomer 1), 123835-99-4; **6** (isomer 2), 123836-00-0; **7** (isomer 1), 123836-01-1; **7** (isomer 2), 123836-02-2; **8**, 123836-03-3; **9**, 123836-04-4; **10**, 123836-05-5; **11**, 123836-06-6; **12**, 106647-58-9; **12** 4,6-diene derivative, 123836-07-7; **12** 5,7-diene derivative, 106647-60-3; **13**, 123836-08-8; **14**, 123930-01-8; **15**, 123836-09-9; **16**, 106647-61-4; **17**, 123836-10-2; **18**, 123836-11-3; **19**, 123836-12-4; **20**, 123836-13-5; **20a**, 123836-14-6; **21**, 106647-71-6; **21a**, 124018-42-4; PTAD, 4233-33-4; $ClCH_2OCH_3$, 107-30-2; Ca, 7440-70-2; ethyl bromodifluoroacetate, 667-27-6; 1,1′-thiocarbonyldiimidazole, 6160-65-2.

Antitumor Properties of Tetrahydrobenz[*a*]anthraquinone Derivatives[1]

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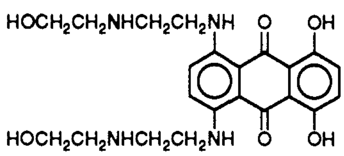
The compound 8,11-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-6-methoxy-1,2,3,4-tetrahydro-7,12-benz[*a*]-anthraquinone (**7**) was synthesized from 3,6-dimethoxyphthalic anhydride and 6-methoxy-1,2,3,4-tetrahydronaphthalene by a Friedel–Crafts reaction, cyclization to form a dihydroxyanthraquinone, and conversion into the amino-substituted derivative by reaction with 2-[(2-hydroxyethyl)amino]ethylamine. The new compound, a ring D analogue of mitoxantrone, showed growth inhibition, at micromolar concentrations, of murine leukemia 1210, human lung H125, human breast MCF7, human ovary 121, and human colon WiDr and increased the life span of leukemic mice by 38%.

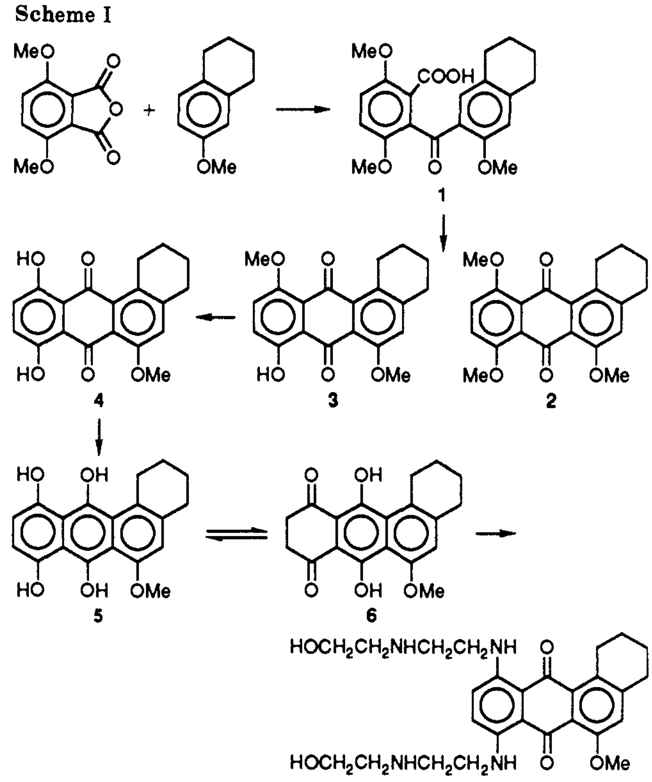
The compound 5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-1,4-dihydroxyanthraquinone (DHAQ, mitoxantrone) is used clinically to treat a variety of human cancers, particularly lung carcinoma,[2,3] leukemia,[4–6] melanoma and lymphoma,[7–9] Hodgkins disease,[7,10] and breast cancer.[7,8,11,12]



DHAQ

Drugs in this class are loosely related to the antineoplastic anthracyclines such as doxorubucin and daunorubicin, which are also anthracenediones; these compounds, as well as DHAQ and its many derivatives, have been shown to bind strongly to DNA[13–16] and are reputed to exercise their antitumor activity by this route.

Scheme I



The DHAQ series of compounds was developed by mimicking the type and stereochemistry of the pertinent functional groups in the hydroxyquinone chromophore of the anthracyclines. Thus, as a structural unit, the unique features of DHAQ eloquently represent an effective arrangement of functional features, i.e. a quinone with a side

(1) Preliminary results were presented at the Medicinal Chemistry Section of the American Chemical Society Meeting, Los Angeles, September 1988, Abstract 13.

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