Noncalcemic, Antiproliferative, Transcriptionally Active, 24-Fluorinated Hybrid Analogues of the Hormone 1α,25-Dihydroxyvitamin D_3. Synthesis and Preliminary Biological Evaluation

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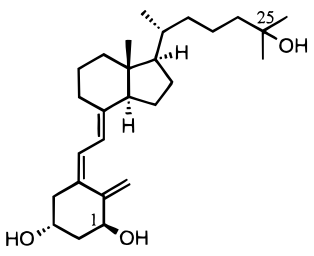
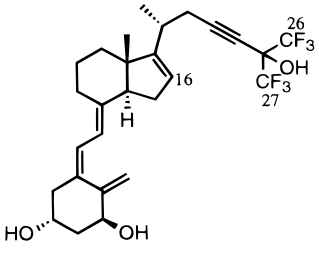
Four new hybrid analogues of 1α,25-dihydroxyvitamin D_3 (**1**) have been synthesized in a convergent manner by joining A-ring and C,D-ring fragments. Each hybrid analogue, having a noncalcemic 1-hydroxymethyl group and a potentiating 16-ene 24,24-difluorinated C,D-ring side chain, was designed to be lipophilic and inert toward 24-hydroxylase enzyme catabolism. Each hybrid analogue with 1*β*,3α-substituent stereochemistry (i.e., analogues **3b** and **4b**) showed a pharmacologically desirable combination of in vitro high antiproliferative activity in two different cell lines and high transcriptional activity with also low calcemic activity in vivo.

Because of its extraordinarily high potency in regulating diverse biochemical events vital to good health in humans,[1] 1α,25-dihydroxyvitamin D_3 (1,25D_3, calcitriol, **1**) has stimulated the interest worldwide of medical researchers, molecular biologists, pharmacologists, and medicinal and organic chemists.[2] A major chemical challenge has been to design and synthesize analogues of 1,25D_3 that retain potent antiproliferative and pro-differentiating activities but that lack hypercalcemic activity.[3−12] Some synthetic analogues exhibiting such selective physiological activities, like Hoffmann-La Roche's 16-ene-26,27-hexafluorinated compound **2**,[13] have been shown to possess very desirable pharmacological properties with good potential as new drug candidates for the treatment of proliferative and immunological diseases.[3−13]



1α,25-dihydroxyvitamin D_3
(1,25D_3, calcitriol, **1**)



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As part of our research program to prepare analogues of 1,25D_3 carrying a 1-hydroxymethyl group to diminish calcemic activity and carrying also a potently prodifferentiating side chain (i.e., hybrid analogues), we have already succeeded in synthesizing several selectively active lead compounds;[14−16] some of these new hybrid analogues are serving also as effective molecular probes to elucidate the mechanistic details of the selective biological responses that they stimulate.[17−19] Now, we report on a new set of 1-hydroxymethyl hybrid analogues **3** and **4** bearing two fluorine atoms at side-chain position 24. These 16-ene-*gem*-difluoro hybrid analogues were designed based mainly on the following four considerations: (1) position 24 is typically the site of side chain metabolic oxygenation,[1] and therefore, replacing C−H by stronger C−F[20] bonds at this position should increase such an analogue's half-life in vivo; (2) the atomic size of a fluorine substituent closely matches that of a hydrogen atom,[20] thereby causing no steric hindrance to receptor binding; (3) the presence of two fluorine atoms should increase the lipophilicity of the hybrid analogue relative to its nonfluorinated counterpart, thereby enhancing rates of absorption and transport in vivo; and (4) a 16-ene carbon−carbon double bond often potentiates antiproliferative activity.[9,11,13] Only a few 24-fluoro and 24,24-difluoro analogues of 1,25D_3, having natural A-ring substituents and stereochemistry, have been synthesized.[21−30] They have been shown, however, to be disappointingly similar to 1,25D_3 in terms of calcemic activity. Although their binding affinity to the vitamin D receptor (VDR) is similar to that of calcitriol, they do have longer plasma half-lives.[21−30] To our knowledge, no hybrid analogue containing both an A-ring modification and also 24-fluorination has been reported previously.[31]

Chemistry

As shown in Scheme 1, the known unprotected hydroxy olefin (+)-**5** reacted with dimethylaluminum chloride and paraformaldehyde via an ene process to give homoallylic diol (+)-**6** stereoselectively, as described

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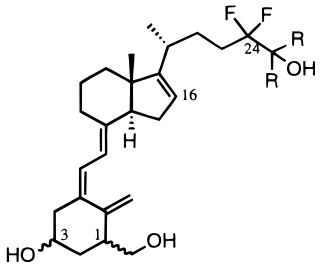

3a (1α,3β), R = Me (JK-1624F_2-1)
3b (1β,3α), R = Me (JK-1624F_2-2)
4a (1α,3β), R = Et (QW-1624F_2-1)
4b (1β,3α), R = Et (QW-1624F_2-2)

previously.[16] After tosylation of the primary hydroxyl group and silylation of the secondary hydroxyl group, tosylate (+)-**7** was converted into the corresponding nitrile that was then reduced to form the one-carbon homologated aldehyde (+)-**8**. A Reformatskii reaction using ethyl bromodifluoroacetate and activated zinc[32] gave *gem*-difluoro ester alcohol **9** as a 1:1 ratio of diastereomers.[30] Barton radical deoxygenation at C-23 proceeded without loss of the adjacent 24-fluorine atoms to give difluoro ester (+)-**10**. Although Grignard addition of a methyl group to this ester proceeded in moderate yield, use of ethylmagnesium chloride caused primarily reduction of the ester functionality. In contrast, both methyllithium and ethyllithium cleanly converted this ester (+)-**10** into the corresponding tertiary alcohols (+)-**11** and (+)-**12**. Fluoride-induced desilylation, C-8 oxidation, and finally C-25 hydroxyl silylation gave enantiomerically pure C,D-ring ketones (+)-**13** and (+)-**14**. Coupling of these C,D-ring chirons with the racemic A-ring allylic phosphine oxide[12] (±)-**15** and desilylation then produced the target hybrid analogues **3** and **4**. Separation of diastereomers by HPLC gave enantiomerically pure hybrid analogues (−)-**3a**, (+)-**3b**, (−)-**4a**, and (+)-**4b**; within each pair of diastereomers, tentative stereochemical assignment was achieved based primarily on characteristic 400 MHz ^{1}H NMR signals (see Table 1) as we have demonstrated previously in similar systems.[31,33]

Biology

Each of these new hybrid analogues (**3** and **4**) was evaluated initially for in vitro antiproliferative activity in murine keratinocytes, using our previously described protocol.[33] As seen previously with 1-(hydroxymethyl)-3-hydroxy diastereomeric pairs of hybrid analogues differing only in relative stereochemistry at the 1- and 3-positions (i.e., 1α,3*β* vs 1*β*,3α), only those diastereomers with the unnatural 1*β*,3α stereochemistry (i.e., **3b** and **4b**) showed significant antiproliferative activities.[16] As shown in Figure 1, the antiproliferative activity of both fluoro analogues **3b** and **4b** was at least equal to that of 1,25D_3 (**1**) even at physiologically relevant 7 nM concentrations. In sharp contrast, diastereomeric analogues **3a** and **4a** were much less potent. Because of their high antiproliferative activity in keratinocytes, fluorinated hybrid analogues **3b** and **4b** were evaluated in vitro in murine malignant melanoma cells also. As shown in Figure 2, even at 1 nM concentration, both of these hybrid analogues were more potent antiproliferative agents than 1,25D_3 (**1**). The average standard deviation of the N/N_0 cell number measurements was 0.21 in Figure 1 and 2.1 in Figure 2.

Scheme 1. Synthesis of 16-Ene-24-difluoro Calcitriol Analogues

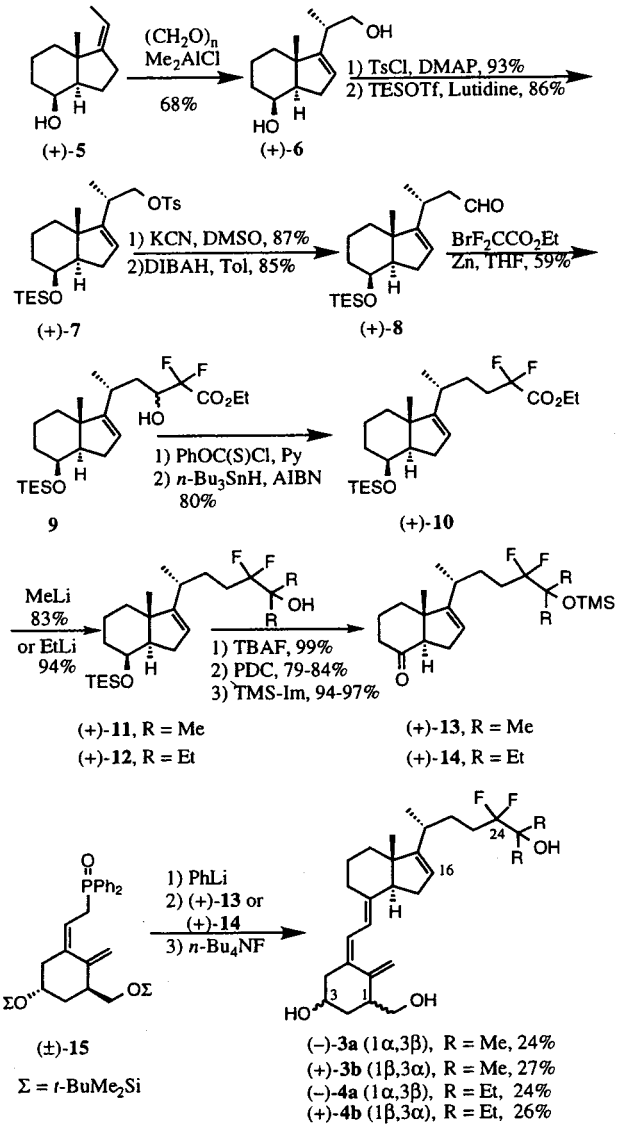

Table 1. ^{1}H NMR (*δ*) and Optical Rotation Characteristics of Hybrid Analogues

analogue	C-18	C-19a	C-19b	$[\alpha]^{25}_D$
3a	0.68	5.18 (d, 1.6 Hz)	5.03 (d, 2.0 Hz)	−14.0
3b	0.66	5.16 (dd, 2.0, 0.8 Hz)	5.00 (d, 2.0 Hz)	+93.0
4a	0.68	5.18 (d, 1.6 Hz)	5.03 (d, 2.0 Hz)	−1.3
4b	0.66	5.15 (d, 0.8 Hz)	5.00 (d, 1.6 Hz)	+78.0

In experiments different from those summarized in Figures 1 and 2, the in vitro vitamin D receptor-mediated transcriptional activities of the two most antiproliferative analogues **3b** and **4b** were tested in rat osteosarcoma ROS 17/2.8 cells. The nonhomologated fluoro hybrid analogue **3b** was found to be slightly more transcriptionally potent ($ED_{50} = 2 \times 10^{-10}$ M) than calcitriol ($ED_{50} = 3 \times 10^{-10}$ M), and 26,27-homologated analogue **4b** was found to be the most potent ($ED_{50} = 5 \times 10^{-11}$ M). The high transcriptional activities of fluoro hybrid analogues **3b** and **4b** are especially noteworthy because they do not have the natural 1α-hydroxyl

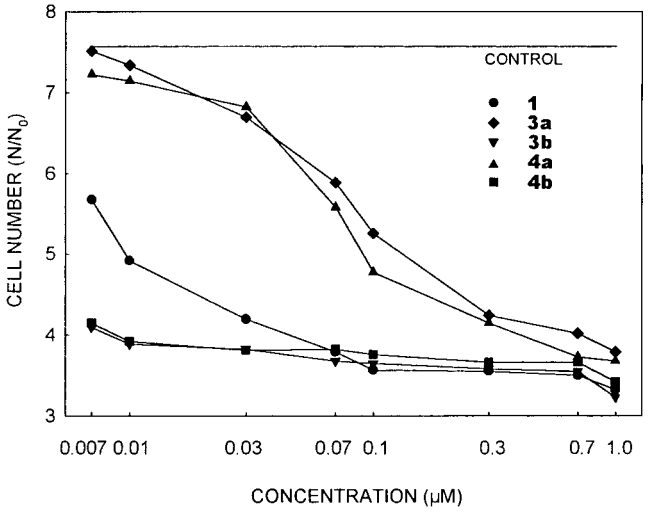

Figure 1. Dose−response effects of analogues on keratinocyte proliferation (96 h).

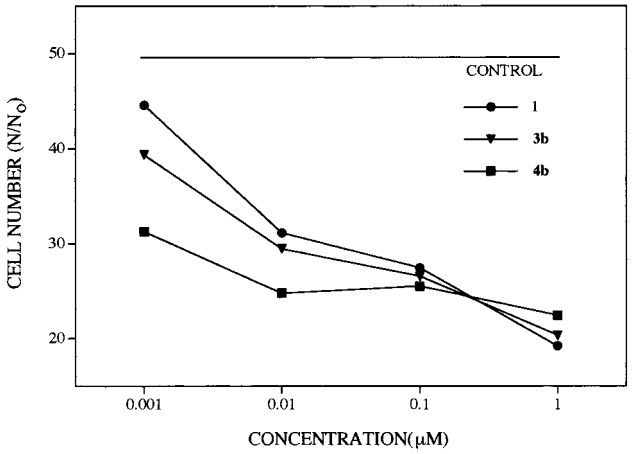

Figure 2. Dose−response effects of analogues on murine B16 malignant melanoma cell proliferation.

substituent on the A ring that for many years was considered to be essential for high biological activity.[11,16−18]

Because of their high antiproliferative and transcriptional activities in vitro, fluorinated hybrid analogues **3b** and **4b** were evaluated for hypercalcemic effects in vivo. In contrast to $1,25D_3$ (**1**), which produced marked excretion of calcium into the urine of rats treated daily for 1 week, the two fluorinated hybrid analogues **3b** and **4b** produced no calcium elevation above control under identical treatment regimens (Figure 3). In addition, suppression of body weight gain seen with $1,25D_3$ was not observed with these hybrid analogues. On the basis of these preliminary biological studies, therefore, fluorinated analogues **3b** and **4b** are promising candidates for further in vivo biological evaluation.

In conclusion, effective chemical syntheses of four new 1-(hydroxylmethyl)-16-ene-24-fluorinated hybrid analogues of $1,25D_3$ have been achieved. Of these new hybrid analogues, being structurally modified on both the A-ring and the C,D-ring side chain, difluoro hybrid analogues **3b** and **4b** stand out as potential drug candidates based on their high antiproliferative and transcriptional potencies and based also on their apparent nontoxicity (noncalcemic activity) when administered orally to rats.

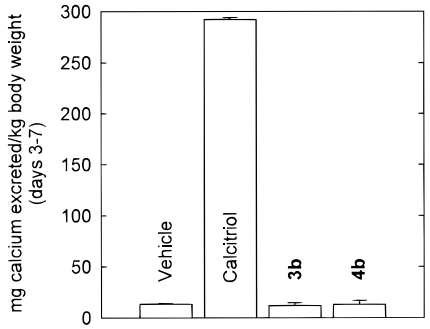

Figure 3. Effects of 24-fluorinated hybrid analogues on urinary calcium excretion in rats. Animals were treated with 1 μg/kg body weight of test compound po for 7 consecutive days, and urinary excretion of calcium was measured during days 3−7. Values are mean ± SE from three animals in each group.

Experimental Section

General. Unless otherwise noted, reactions were run in flame-dried round-bottomed flasks under an atmosphere of ultra high purity (UHP) argon. Diethyl ether (ether) and tetrahydrofuran (THF) were distilled from sodium benzophenone ketyl prior to use. Methylene chloride (CH_2Cl_2) was distilled from calcium hydride prior to use. All other compounds were purchased from Aldrich Chemical Co. and used without further purification. Analytical thin-layer chromatography (TLC) was conducted with silica gel 60 F_{254} plates (250 μm thickness, Merck). Column chromatography was performed using short path silica gel (particle size <230 mesh), flash silica gel (particle size 400−230 mesh), or Florisil (200 mesh). Yields are not optimized. Purity of products was judged to be >95% based on their chromatographic homogeneity. High-performance liquid chromatography (HPLC) was carried out with a Rainin HPLX system equipped with two 25 mL/min preparative pump heads using Rainin Dynamax 10 mm × 250 mm (semipreparative) columns packed with 60 Å silica gel (8 μm pore size), either as bare silica or as C-18-bonded silica. Melting points were measured using a Mel-Temp metal-block apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were obtained either on a Varian XL-400 spectrometer, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, or on a Varian XL-500 spectrometer, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane. Infrared (IR) spectra were obtained using a Perkin-Elmer 1600 FT-IR spectrometer. Resonances are reported in wavenumbers (cm^{-1}). Low- and high-resolution mass spectra (LRMS and HRMS) were obtained with electronic or chemical ionization (EI or CI) either (1) at Johns Hopkins University on a VG Instruments 70-S spectrometer run at 70 eV for EI and run with ammonia (NH_3) as a carrier gas for CI or (2) at the University of Illinois at Champaign−Urbana on a Finnigan-MAT CH5, a Finnigan-MAT 731, or a VG Instruments 70-VSE spectrometer run at 70 eV for EI and run with methane (CH_4) for CI.

(1′*S*,3a*R*,4*S*,7a*S*)-1′-(1′-Methyl-2′-hydroxyethyl)-1-octahydro-7a-methyl-22-hydroxy-1*H*-inden-4-ol [(+)-6]. To a suspension of paraformaldehyde (272 mg, 9.1 mmol) in 50 mL of CH_2Cl_2 was added 13.5 mL (13.5 mmol) of 1 M dimethylaluminum chloride solution in hexanes at −78 °C. After 30 min, a solution of (+)-**5** (503 mg, 2.8 mmol) in 5 mL of CH_2Cl_2 was added into the mixture at −78 °C, and then the reaction mixture was warmed to −40 °C. After being stirred for 16 h at −40 °C, it was quenched with 10% K_2HPO_4 at −40 °C and then warmed to room temperature. The reaction mixture was extracted with EtOAc (2 × 100 mL), washed with 10% HCl, saturated aqueous $NaHCO_3$ solution, and brine, dried, concentrated in vacuo, and then purified by chromatography (50% EtOAc/hexanes) to give 400 mg (68%) of (+)-**6** as a white solid: mp 84−87 °C; $[\alpha]^{25}_D$ +35.0 (*c* 6.8, EtOH).

C,D-Ring TES Tosylate (+)-7. To a solution of the diol (+)-**6** (210 mg, 1.0 mmol) and 4-(dimethylamino)pyridine (DMAP, 210 mg, 1.7 mmol) in 15 mL of CH_2Cl_2 was slowly added the solution of *p*-toluenesulfonyl chloride (210 mg, 1.1 mmol) in 5 mL of CH_2Cl_2 at 0 °C. After being stirred for 12 h at 0 °C, the reaction mixture was quenched with water and diluted with CH_2Cl_2, the organic phase was separated, and the aqueous phase was extracted with CH_2Cl_2. The organic portions were combined, washed with brine, dried, concentrated in vacuo, and then purified by chromatography (25% EtOAc/hexanes) to give 337 mg (93%) of the desired tosylate as a colorless oil: $[\alpha]^{25}_D$ +27.0 (*c* 16.7, $CHCl_3$).

To a solution of the tosylate (337 mg, 0.93 mmol) and 2,6-lutidine (0.34 mL, 2.9 mmol) in 20 mL of CH_2Cl_2 was added triethylsilyl trifluoromethanesulfonate (TESOTf, 0.25 mL, 1.1 mmol) dropwise at −78 °C. After 30 min, the reaction was quenched with water, extracted with pentane, washed with 5% HCl (2 × 30 mL) and brine, dried, and then concentrated in vacuo. Purification by chromatography (5% EtOAc/hexanes) gave 386 mg (86%) of compound (+)-**7** as a colorless oil: $[\alpha]^{25}_D$ +47.9 (*c* 4.7, $CHCl_3$).

TES Aldehyde (+)-8. A mixture of the tosylate (+)-**7** (386 mg, 0.81 mmol) and KCN (184 mg, 2.8 mmol) in 30 mL of anhydrous dimethyl sulfoxide (DMSO) was stirred for 3 h at 65 °C. After being cooled to room temperature, it was quenched with water, extracted with ether, washed with brine, dried, and then concentrated in vacuo. Purification by chromatography (10% ether/hexanes) gave 234 mg (87%) of the desired nitrile as a colorless oil: $[\alpha]^{25}_D$ +43.8 (*c* 8.4, $CHCl_3$).

To a solution of the nitrile (234 mg, 0.70 mmol) in 30 mL of anhydrous toluene was added dropwise 1.4 mL (1 M solution in toluene, 1.4 mmol) of diisobutylaluminum hydride (DIBAH) at 0 °C. After being stirred for an additional 20 min at 0 °C, it was diluted with ether, quenched with 5% HCl, extracted with ether, washed with brine, dried, and then concentrated in vacuo. Purification by chromatography (5% ether/hexanes) gave 200 mg (85%) of compound (+)-**8** as a colorless oil: $[\alpha]^{25}_D$ +38.8 (*c* 7.1, $CHCl_3$).

Difluoro C,D-Ring Ethyl Ester 9. A suspension of activated zinc powder (195 mg, 3.0 mmol) and ethyl bromodifluoroacetate (0.39 mL, 3.0 mmol) in 6 mL of THF was refluxed for 20 min and then cooled to 0 °C. To this was added the solution of the aldehyde (+)-**8** (200 mg, 0.59 mmol) in 5 mL of THF. The reaction mixture was warmed to room temperature followed by refluxing for 20 min and then cooled to room temperature. The reaction mixture was poured into 1 M $KHSO_4$ and extracted with EtOAc (2 × 30 mL). The combined extracts were successively washed with 1 M $KHSO_4$ and brine, dried, and then concentrated. The resulting mixture was purified by column chromatography (10% ether/hexanes) to give 162 mg (59%) of a 1:1 mixture of diastereomers of the desired alcohol **9** as a colorless oil.

Difluoro Ethyl Ester (+)-10. To a solution of ethyl ester **9** (162 mg, 0.34 mmol) and pyridine (0.12 mL, 1.5 mmol) in CH_2Cl_2 (5 mL) was added phenyl chlorothianocarbonate (0.1 mL, 0.72 mmol). After being stirred at room temperature for 20 h, the reaction mixture was quenched with water and then extracted with ether. The organic portions were combined, washed with saturated $NaHCO_3$ solution and brine, dried, concentrated in vacuo, and then purified by chromatography (5% ether/hexanes) to give 186 mg (90%) of the desired phenylthianocarbonate as diastereomeric mixtures.

To the solution of the resulting phenylthianocarbonate (186 mg, 0.31 mmol) in anhydrous benzene (10 mL) were added 2,2′-azobiisobutyronitrile (AIBN, 10 mg) and Bu_3SnH (0.13 mL, 0.48 mmol) at room temperature. After being refluxed for 3 h, the mixture was cooled to 0 °C, quenched with water, and extracted with EtOAc. The combined organic portions were washed with brine, dried, and then purified by column chromatography (5% ether/hexanes) to give 125 mg (90%) of the desired deoxygenated difluoro ester **10** as a colorless oil: $[\alpha]^{25}_D$ +16.9 (*c* 1.5, $CHCl_3$).

16-Ene-24-difluoro Alcohols (+)-11 and (+)-12. A. Using MeLi. A solution of ester **10** (65 mg, 0.15 mmol) in THF (3 mL) was treated with 1.4 M solution of MeLi (0.42 mL, 0.60 mmol) in ether at −78 °C, and then it was warmed to room temperature. The mixture was cooled to 0 °C, diluted with ether, and then quenched with saturated NH_4Cl solution. The mixture was extracted with EtOAc, washed with brine, dried, concentrated in vacuo, and then purified by column chromatography (10% EtOAc/hexanes) to give 52 mg (83%) of (+)-**11** as a colorless oil: $[\alpha]^{25}_D$ +23.8 (*c* 6.5, $CHCl_3$).

B. Using EtLi. A solution of difluorinated C,D-ring ester **10** (68 mg, 0.15 mmol) and 5.0 mL of THF was cooled to −78 °C, and then 0.5 mL (0.75 mmol, 1.5 M solution in THF) of EtLi was added dropwise to the solution. The reaction mixture was warmed to room temperature, quenched with 10% HCl at −78 °C, extracted with EtOAc, washed with brine, dried, concentrated in vacuo, and then purified by column chromatography (10% EtOAc/hexanes) to give 66 mg (94%) of C,D-ring alcohol (+)-**12** as a colorless oil: $[\alpha]^{25}_D$ +18.2 (*c* 3.7, $CHCl_3$).

24-Difluoro C,D-Ring Ketones (+)-13 and (+)-14. A. Ketone (+)-13. A solution of silyl ether **11** (65 mg, 0.15 mmol) in THF (3 mL) and 0.45 mL of 1 M solution of tetra-*n*-butylammonium fluoride (TBAF) in THF was stirred for 16 h at room temperature. The mixture was quenched with water and extracted with EtOAc. The combined organic portions were washed with brine, dried, concentrated in vacuo, and then purified by chromatography (30% EtOAc/hexanes) to give 47 mg (99%) of the desired alcohol as a colorless oil: $[\alpha]^{25}_D$ +8.5 (*c* 4.7, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 (br d, J = 1.6 Hz, 1H), 4.17 (br s, 1H), 2.02−2.10 (m, 1H), 1.70−2.00 (m, 9H), 1.50−1.69 (m, 3H), 1.40 (td, J = 13.2, 3.6 Hz, 1H), 1.27 (s, 6H), 1.02 (s, 3H), 1.01 (d, J = 6.8 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.18, 125.38 (J = 246 Hz), 120.10, 77.20 (J = 27 Hz), 69.10, 54.34, 46.25, 35.37, 33.80, 31.52, 30.21, 28.96 (J = 24 Hz), 27.38 (J = 3.0 Hz), 23.52, 22.37, 18.25, 17.77; IR (neat, cm^{-1}) 3396, 2931, 1454, 1381; MS *m*/*z* (70 eV, EI) 316 (M^+); HRMS *m*/*z* (M^+) calcd 316.2214 for $C_{18}H_{30}F_2O_2$, found 316.2216.

To a solution of this diol (47 mg, 0.15 mmol) in CH_2Cl_2 (5 mL) were added 160 mg of oven-dried Celite and pyridinium dichromate (PDC, 163 mg, 0.43 mmol) at room temperature. After 3.5 h of stirring at room temperature, the mixture was passed through a 2 cm pad of flash silica gel and then washed with EtOAc. The filtrate was concentrated and chromatographed with 30% EtOAc in hexanes to give 39 mg (84%) of ketone as a colorless oil: $[\alpha]^{25}_D$ +20.6 (*c* 3.9, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (t, J = 1.6 Hz, 1H), 2.84 (dd, J = 10.8, 6.4 Hz, 1H), 2.43 (ddt, J = 16.0, 10.8, 1.6 Hz, 1H), 2.30−2.56 (m, 2H), 2.04−2.17 (m, 3H), 1.72−2.02 (m, 6H), 1.59−1.69 (m, 1H), 1.27 (s, 6H), 1.07 (d, J = 6.8 Hz, 3 H), 0.79 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.97, 157.07, 125.27 (J = 246 Hz), 120.72, 73.16 (J = 27 Hz), 63.07, 53.73, 40.46, 34.30, 32.50, 28.65 (J = 24 Hz), 27.30 (J = 3.0 Hz), 27.07, 23.99, 23.50, 21.62, 17.18; IR (neat, cm^{-1}) 3448, 2942, 1711, 1456, 1380; MS *m*/*z* (70 eV, EI) 314 (M^+); HRMS *m*/*z* (M^+) calcd 314.2057 for $C_{18}H_{28}F_2O_2$, found 314.2053.

To a solution of this keto alcohol (39 mg, 0.12 mmol) in CH_2Cl_2 (3 mL) was added trimethylsilyl imidazole (TMS-imidazole, 35 μL, 0.24 mmol) at room temperature. After being stirred for 16 h at room temperature, the mixture was concentrated in vacuo and then chromatographed with 10% EtOAc in hexanes to give 47 mg (97%) of (+)-**13** as a colorless oil: $[\alpha]^{25}_D$ +18.1 (c 4.7, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31 (t, J = 1.6 Hz, 1H), 2.84 (dd, J = 10.8, 6.4 Hz, 1H), 2.44 (ddt, J = 16.0, 10.8, 1.6 Hz, 1H), 2.26−2.31 (m, 2H), 2.05−2.15 (m, 3H), 1.68−2.05 (m, 6H), 1.59−1.66 (m, 1H), 1.26 (s, 6H), 1.07 (d, J = 6.8 Hz, 3 H), 0.80 (s, 3H), 0.10 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.89, 157.11, 125.02 (J = 246 Hz), 120.61, 75.82 (J = 27 Hz), 63.10, 53.74, 40.51, 34.32, 32.59, 28.42 (J = 24 Hz), 27.37 (J = 3.0 Hz), 27.07, 24.42 (J = 2.6 Hz), 24.27 (J = 3.0 Hz), 24.02, 21.89, 17.09, 2.30; IR (neat, cm^{-1}) 2958, 2873, 1721, 1458, 1383; MS *m*/*z* (70 eV, EI) 386 (M^+); HRMS *m*/*z* (M^+) calcd 386.2453 for $C_{21}H_{36}F_2O_2Si$, found 386.2457.

B. Ketone (+)-14. Difluorinated C,D-ring silyl ether **12** (105 mg, 0.23 mmol) was dissolved in 3 mL of THF. To this

solution was added dropwise 1 mL (1.0 M solution in THF, 1.0 mmol) of TBAF. The reaction mixture was stirred overnight at room temperature, quenched with water, and extracted with EtOAc. The combined organic portions were washed with brine, dried, concentrated in vacuo, and purified by column chromatography (20% EtOAc/hexanes) to give 78 mg (99%) of the deprotected alcohol as a colorless oil: $[\alpha]^{25}_D$ +8.7 (*c* 5.4, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 (t, J = 1.6 Hz, 1H), 4.16 (d, J = 2.4 Hz, 1H), 2.21−2.30 (m, 1H), 2.01−2.06 (m, 1H), 1.93−2.00 (m, 1H), 1.50−1.90 (m, 13H), 1.33−1.43 (m, 2H), 1.02 (s, 3H), 1.00 (d, J = 7.2 Hz, 3H), 0.86−0.93 (tt, J = 7.6 Hz, 1.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.47, 126.59 (t, J = 247.8 Hz), 120.47, 77.04, 69.39, 54.60, 46.50, 35.61, 34.10, 31.78, 30.44, 29.87 (t, J = 25.3 Hz), 27.52, 25.58, 25.47, 22.65, 18.49, 18.00, 7.81; IR (neat, cm^{-1}) 3604, 3430, 1460; MS *m*/*z* (70 eV, EI) 344 (M^+); HRMS *m*/*z* (M^+) calcd 344.2527 for $C_{20}H_{34}F_2O_2$, found 344.2533.

To the solution of the deprotected C,D-ring alcohol (67 mg, 0.20 mmol) in 3.0 mL CH_2Cl_2 were added 3 Å molecular sieves (0.6 g) and pyridinium chlorochromate (PCC, 320 mg, 1.50 mmol). The mixture turned dark red and was stirred overnight. The reaction mixture was then passed through a short silica gel pad, washed with ether, concentrated, and then purified by column chromatography (20% EtOAc/hexanes) to give 52 mg (79%) of the desired C,D-ring keto alcohol as a colorless oil: $[\alpha]^{25}_D$ +19.8 (*c* 4.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31 (t, J = 1.2 Hz, 1H), 2.85 (dd, J = 6.4 Hz, 10.4 Hz, 1H), 2.45 (ddt, J = 16.0, 10.8, 1.6 Hz, 1H), 2.25−2.31 (m, 2H), 1.54−2.20 (m, 14H), 1.08 (d, J = 6.8 Hz, 3H), 0.90 (t, J = 7.6 Hz, 6H), 0.80 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.90, 157.06, 126.25 (t, J = 247.4 Hz), 120.75, 76.75 (t, J = 23.6 Hz), 63.10, 53.75, 40.49, 34.33, 32.54, 29.34 (t, J = 24.4 Hz), 27.23 (t, J = 3.8 Hz), 27.09, 25.28 (t, J = 1.9 Hz), 24.02, 21.17, 17.21, 7.57; IR (neat, cm^{-1}) 3448, 2955, 1713, 1455; MS *m*/*z* (70 eV, EI) 342 (M^+); HRMS *m*/*z* (M^+) calcd 342.2370 for $C_{20}H_{32}F_2O_2$, found 342.2368.

To a solution of the C,D-ring keto alcohol (47.4 mg, 0.14 mmol) and 4.0 mL of CH_2Cl_2 was added 41 μL (0.28 mmol) of TMS-imidazole at room temperature. After being stirred for 16 h at room temperature, the reaction mixture was concentrated and purified by column chromatography (17% EtOAc/hexanes) to give 54 mg (94%) of the protected C,D-ring ketone (+)-**14** as a colorless oil: $[\alpha]^{25}_D$ +15.4 (*c* 4.9, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.29 (t, J = 1.6 Hz, 1H), 2.84 (dd, J = 6.4 Hz, 10.4 Hz, 1H), 2.44 (ddt, J = 16.0, 10.8, 1.6 Hz, 1H), 2.20−2.32 (m, 2H), 1.48−2.34 (m, 14H), 1.06 (d, J = 6.8 Hz, 3H), 0.84 (t, J = 7.6 Hz, 6H), 0.78 (s, 3H), 0.08 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.91, 157.05, 126.40 (t, J = 247.4 Hz), 120.65, 80.65 (t, J = 25.4 Hz), 63.11, 53,76, 40.53, 34.34, 32.59, 29.57 (t, J = 24.4 Hz), 27.23 (t, J = 3.8 Hz), 27.08, 26.08, 25.93, 24.04, 21.93, 17.14, 8.17, 8.09, 2.40; IR (neat, cm^{-1}) 2958, 1721; MS *m*/*z* (70 eV, EI) 414; HRMS *m*/*z* (M^+) calcd 414.2766 for $C_{23}H_{40}F_2O_2Si$, found 414.2775.

Synthesis of 16-Ene-24-difluoro Calcitriol Analogues (−)-3a and (+)-3b. A solution of 96 mg (0.16 mmol) of phosphine oxide (±)-**15** in 1.5 mL of anhydrous THF was treated dropwise with 100 μL (0.15 mmol) of a 1.5 M solution of phenyllithium in THF under argon at −78 °C. The resulting reddish orange solution was stirred for 30 min at −78 °C. To the solution was added dropwise a solution of 43 mg (0.11 mmol) of C,D-ring ketone (+)-**13** in 1 mL of anhydrous THF. The reaction mixture was stirred until the reddish orange color turned to pale yellow, and then it was quenched with 3 mL of a 1:1 mixture of 2 N sodium potassium tartrate and 2 N K_2CO_3 solution, extracted with EtOAc (50 mL × 2), and washed with brine. The combined organic portions were dried, concentrated in vacuo, and then purified by chromatography (3% EtOAc/hexanes) to afford 55 mg (66%) of the coupled product as a colorless oil. The silyl ethers were dissolved in 3 mL of anhydrous THF. To the solution were added 0.44 mL (0.44 mmol) of 1 M TBAF solution in THF and 43 μL (0.31 mmol) of triethylamine. After 16 h at room temperature, the mixture was quenched with water, extracted with EtOAc (2 × 50 mL), and washed with brine. The combined organic portions were dried, concentrated in vacuo, and then purified by chromatography (EtOAc/hexanes/NEt_3 = 90/10/1) to afford 33 mg (98%) of a mixture of two diastereomers as a white solid. The diastereomers were separated by reverse phase HPLC (C-18 semipreparative column, 60% MeCN/H_2O, 3 mL/min) to afford 12.5 mg (24%) of (−)-**3a** (1α,3β, t_R 24.4 min) as a foaming solid and 13.6 mg (27%) of (+)-**3b** (1β,3α, t_R 29.9 min) as a viscous oil. (−)-**3a** (1α,3β): $[\alpha]^{25}_D$ −14.0 (*c* 0.4, EtOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.32 (d, J = 11.2 Hz, 1H), 6.04 (d, J = 11.2 Hz, 1H), 5.32 (t, J = 1.2 Hz, 1H), 5.18 (d, J = 1.6 Hz, 1H), 5.03 (d, J = 2.0 Hz, 1H), 3.93−4.00 (m, 1H), 3.52−3.59 (m, 2H), 2.78−2.83 (m, 1H), 2.59−2.67 (m, 2H), 2.37 (dd, J = 9.6, 6.4 Hz, 1H), 2.12−2.30 (m, 3H), 1.97−2.02 (m, 2H), 1.50−1.90 (m, 10H), 1.29 (s, 6H), 1.06 (d, J = 6.8 Hz, 3H), 0.68 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 160.44, 147.54, 141.95, 136.22, 126.67 (t, J = 245 Hz), 123.92, 121.89, 118.88, 114.14, 73.62 (t, J = 27 Hz), 67.39, 64.68, 59.77, 51.13, 47.38, 46.55, 37.64, 36.52, 33.89, 30.41, 29.93 (t, J = 25 Hz), 29.74, 28.65(t, J = 3 Hz), 24.73, 23.88 (t, J = 21 Hz), 22.27, 17.25; UV (MeOH) λ_{max} 262 nm (ϵ 21 400); IR (neat, cm^{-1}) 3350, 2930, 1378, 1043; MS *m*/*z* (70 eV, CI) 482 (M + NH_4^+); HRMS *m*/*z* (M^+) calcd 464.3102 for $C_{28}H_{42}F_2O_3$, found 464.3102. (+)-**3b** (1β,3α): $[\alpha]^{25}_D$ +93.0 (*c* 0.5, EtOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.31 (d, J = 11.2 Hz, 1H), 6.04 (d, J = 11.2 Hz, 1H), 5.28 (t, J = 1.2 Hz, 1H), 5.16 (dd, J = 2.0, 0.8 Hz, 1H), 5.00 (d, J = 2.0 Hz, 1H), 4.00 (septet, J = 4.0 Hz, 1H), 3.57−3.65 (m, 2H), 2.79−2.83 (m, 1H), 2.58−2.67 (m, 2H), 2.37 (dd, J = 11.2, 6.4 Hz, 1H), 2.29 (dd, J = 12.4, 6.4 Hz, 1H), 2.13−2.23 (m, 2H), 2.00 (dddd, J = 14.8, 9.6, 6.4, 3.2 Hz, 1H), 1.50−1.88 (m, 11H), 1.28 (s, 6H), 1.06 (d, J = 6.8 Hz, 3H), 0.66 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.90, 145.30, 142.41, 134.30, 125.38 (t, J = 245 Hz), 123.58, 120.72, 116.83, 113.77, 73.29 (t, J = 27 Hz), 67.12, 64.34, 58.31, 59.96, 46.19, 44.32, 37.38, 35.23, 32.56, 29.33, 28.77, 28.74 (t, J = 25 Hz), 27.28 (t, J = 3 Hz), 23.58 (2C), 21.71, 16.72; UV (MeOH) λ_{max} 262 nm (ϵ 16 100); IR (neat, cm^{-1}) 3366, 2930, 2874, 1369, 1178, 1040; MS *m*/*z* (70 eV, CI) 482 (M + NH_4^+), 446 (M + NH_4^+); HRMS *m*/*z* (M^+) calcd 464.3102 for $C_{28}H_{42}F_2O_3$, found 464.3107.

Synthesis of 16-Ene-24-difluoro Calcitriol Analogues (−)-4a and (+)-4b. A solution of 69.4 mg (0.12 mmol) of phosphine oxide (±)-**15** in 2.0 mL of anhydrous THF was cooled to −78 °C and treated with 81 μL (0.12 mmol, 1.5 M solution in THF) of phenyllithium under argon atmosphere. The mixture turned reddish orange and was stirred for 30 min at −78 °C. To the solution was added dropwise a solution of 47.9 mg (0.12 mmol) of the C,D-ring ketone (+)-**14** in 1.0 mL of anhydrous THF. The reaction kept going on until the reddish orange color faded to yellow (about 6 h). The reaction was quenched by adding 3.0 mL of a 1:1 mixture of 2 N sodium potassium tartrate and 2 N K_2CO_3 solution. The reaction mixture was extracted with EtOAc, washed with brine, dried, concentrated in vacuo, and then purified by column chromatography (97% hexanes/ether) to afford 68.0 mg (74%) of the coupled product as a colorless oil. The silyl ethers were dissolved in 3.0 mL of anhydrous THF, and to this solution were added TBAF (0.52 mL, 0.52 mmol, 1.0 M solution in THF) and 52 μL (0.39 mmol) of Et_3N. The reaction was run in darkness overnight, quenched with water, and extracted with EtOAc. The combined organic portions were washed with brine, dried, concentrated in vacuo, and then purified by column chromatography (90% EtOAc/hexanes) to give 38.7 mg (92%) of a mixture of two diastereomers as a white solid. The diastereomers were separated by reverse phase HPLC (C-18 semipreparative column, 60% MeCN/H_2O, 3.0 mL/min) to afford 14.0 mg (24%) of (−)-**4a** (1α,3β, t_R 48.5 min) as a colorless oil and 15.5 mg (26%) of (+)-**4b** (1β,3α, t_R 57.3 min) as a foaming solid. (−)-**4a:** $[\alpha]^{25}_D$ −1.3 (*c* 1.4, EtOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.32 (d, J = 11.2 Hz, 1H), 6.04 (d, J = 11.2 Hz, 1H), 5.32 (t, J = 1.6 Hz, 1H), 5.18 (d, J = 1.6 Hz, 1H), 5.03 (d, J = 2.0 Hz, 1H), 3.97 (septet, J = 4.0 Hz, 1H), 3.50−3.60 (m, 2H), 2.77−2.85 (m, 1H), 2.57−2.69 (m, 2H), 2.10−2.40 (m, 4H), 1.44−2.02 (m, 16H), 1.06 (d, J = 6.8 Hz, 3H), 0.91 (t, J = 7.6 Hz, 6H), 0.68 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 160.37, 147.52, 141.91, 136.61, 127.57 (t, J = 247.3

Hz), 123.88, 121.91, 118.88, 114.12, 77.56 (t, J = 24.7 Hz), 67.37, 64.67, 59.76, 51.12, 47.38, 46.52, 37.64, 36.51, 33.92, 30.60 (t, J = 24.2 Hz), 30.40, 29.73, 28.53, 25.87 (d, J = 6.8 Hz), 24.73, 22.32, 17.26, 7.99; IR (neat, cm^{-1}) 3350, 2919, 1607, 1449; UV (EtOH) λ_{max} 263 nm (ϵ 17 500); MS m/z (70 eV, EI) 492 (M^+); HRMS m/z (M^+) calcd 492.3415 for $C_{30}H_{46}F_2O_3$, found 492.3412. (+)-**4b**: $[\alpha]^{25}_D$ +78.0 (*c* 1.6, EtOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.31 (d, J = 11.2 Hz, 1H), 6.04 (d, J = 11.2 Hz, 1H), 5.32 (t, J = 1.4 Hz, 1H), 5.15 (d, J = 0.8 Hz, 1H), 5.00 (d, J = 1.6 Hz, 1H), 4.02 (septet, J = 4.0 Hz, 1H), 3.58−3.64 (m, 2H), 2.75−2.85 (m, 1H), 2.55−2.68 (m, 2H), 2.10−2.40 (m, 4H), 1.44−2.03 (m, 16H), 1.05 (d, J = 6.8 Hz, 3H), 0.91 (t, J = 7.6 Hz, 6H), 0.66 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 160.43, 147.63, 142.06, 136.72, 127.57 (t, J = 242.6 Hz), 123.83 (d, J = 3.8 Hz), 121.89 (d, J = 8.3 Hz), 118.83, 113.79, 77.56 (t, J = 24.7 Hz), 67.43, 64.62, 59.71, 51.07, 47.36, 46.32, 37.60, 36.57, 33.90, 30.60 (t, J = 24.2 Hz), 30.23, 29.74, 28.53 (t, J = 3.8 Hz), 25.86 (d, J = 6.8 Hz), 24.64, 22.33, 17.25, 7.99; IR (neat, cm^{-1}) 3342, 2931, 1648, 1625, 1455; UV (EtOH) λ_{max} 262 nm (ϵ 18 000); MS m/z (70 eV, EI) 492 (M^+); HRMS m/z (M^+) calcd 492.3415 for $C_{30}H_{46}F_2O_3$, found 492.3417.

Antiproliferative Assays Using Malignant Melanoma Cells. Murine B16 malignant melanoma cells (generously provided by Dr. Drew Pardoll of Johns Hopkins Medical Institutions) were grown and propagated in RPMI medium supplemented with 10% fetal bovine serum, l-glutamine, penicillin, and streptomycin and incubated at 37 °C in 5% CO_2. For proliferation studies, cells were washed with PBS, trypsinized, and suspended in 8 mL of supplemented RPMI medium. The cell density was then determined using a hemacytometer, and cells were resuspended in RPMI at 10 000 cells/cm^3. One milliliter of cell suspension (10 000 cells) was added to each well of a Falcon 24 well flat bottom tissue culture plate (Becton-Dickinson, Lincoln Park, NJ). Plates were incubated for 24 h to allow for cell attachment. The medium was then removed and replaced with fresh RPMI medium containing either 0.4% solvent (2-propanol) or drug at concentrations ranging from 1 to 1000 nM in triplicate. When control wells neared confluence, cells were washed with PBS, trypsinized, and suspended in 10 mL of Isoton II Coulter balanced electrolyte solution in FISHERbrand Dilu-Vial cuvettes. Cell number was then determined for each well as an average of two readings on a ZM Coulter counter. Results are expressed as the average cell number for each drug treatment group divided by the intital cell number (N/N_0).

Transfections and Transcriptional Activity[17] of the Analogues. Rat osteosarcoma ROS 17/2.8 cells were maintained in 50% Dulbecco's modified Eagle medium (DMEM) and 50% F12 nutrient mixture supplemented with 10% fetal bovine serum. Forty-eight hours before transfections, the cells were plated in 35 mm dishes at a density of 10^5/dish in DMEM and 10% fetal bovine serum. ROS 17/2.8 cells were transfected with 2 μg of plasmid containing the vitamin D responsive element from the human osteocalcin gene (GGTGACTCAC-CGGGTGAACGGGGGCATT)[34] attached to the thymidine kinase promoter/growth hormone fusion gene. All transfections were performed by the diethylaminoethyl dextran method,[35] and the cells were then treated for 1 min with 10% dimethyl sulfoxide, washed twice in phosphate-buffered saline, incubated in DMEM supplemented with 10% fetal bovine serum without or with graded concentrations of the analogues. Medium samples for measurements of reporter gene expression (growth hormone) were collected 2 days after transfection. Growth hormone was measured by a radioimmunoassay as described by the manufacturer (Nichols Institute, San Juan Capistrano, CA).

Determination of Urinary Calcium Levels. Male F344 rats (150 g) were housed individually in glass metabolism cages and received food and water ad libitum. After several days acclimation, rats received 1 μg/kg of body weight of test compound per os for 7 consecutive days in 150 μL of propylene glycol/0.05 M Na_2HPO_4 (80:20). Urine samples, which were collected on ice, were centrifuged at 650g for 10 min, adjusted to pH 6.0 as necessary, and assayed for calcium content spectrophotometrically at 575 nm using reagents and standards from Sigma calcium kit no. 587.

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Supporting Information Available: ^{1}H and ^{13}C NMR, IR, and mass spectral data for compounds **6**−**12** (4 pages). Ordering information is given on any current masthead page.

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