Conceptually New 20-*epi*-22-Oxa Sulfone Analogues of the Hormone 1α,25-Dihydroxyvitamin D_3: Synthesis and Biological Evaluation

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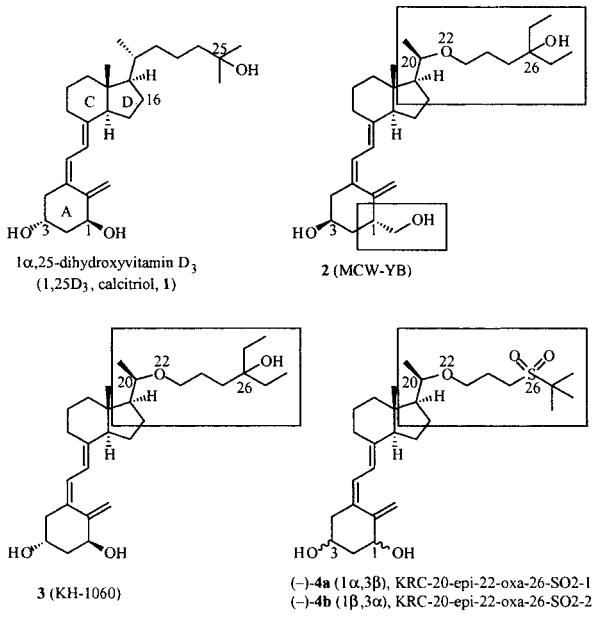
New C,D-ring side-chain-modified sulfone **4a**, with natural 1α,3β-hydroxyl groups but lacking the 25-hydroxyl group characteristic of the natural hormone 1α,25-dihydroxyvitamin D_3 (**1**), has been prepared and characterized. Novel synthetic features include: (1) chemoselective oxidation of only a primary silyl ether in a primary−secondary bis-silyl ether intermediate and (2) smooth reductive etherification without interference by a neighboring sulfonyl group. Sulfone **4a**, but not its 1β,3α-diastereomer **4b**, is powerfully antiproliferative and transcriptionally active in vitro but desirably noncalcemic in vivo. Although sulfone **4a**, designed to resemble Leo Pharmaceutical Co.'s KH-1060 (**3**), is recognized by catabolic enzymes, the selective biological profile of sulfone **4a** is likely not due to its metabolites that are formed in only minor amounts.

Introduction

Conventional wisdom requires the presence of **both** the 1α- and 25-hydroxyl groups for the natural hormone 1α,25-dihydroxyvitamin D_3 (1,25D_3, calcitriol, **1**) to elicit its characteristically potent and diverse physiological responses in humans.[1,2] In 1994, however, we showed for the first time that hybrid analogue **2** bearing an unnatural 1β-hydroxymethyl group and also a potentiating side chain with a tertiary hydroxyl group [cf. Leo Pharmaceutical Co.'s promising drug candidate KH-1060 (**3**)] was able to match the antiproliferative/prodifferentiating potency of the natural hormone but importantly without causing hypercalcemia.[3] Recently, we showed for the first time that some synthetic 16-ene 24- and 25-*tert*-butyl sulfones, with natural 1α,3β-hydroxyl groups but lacking a side-chain tertiary hydroxyl group, also have powerful but selective biological activities in vitro as well as nontoxic properties in vivo.[4] Now we report that the 16-ene functionality, introduced and popularized by Hoffmann-La Roche researchers,[5] is not required for a side-chain sulfone to be therapeutically desirable. Thus, 20-*epi*-22-oxa-26-sulfone **4a** is powerfully antiproliferative and transcriptionally active in vitro but noncalcemic in vivo. The high potency but low toxicity of oxa sulfone **4a** reinforces and broadens



our recent claim[4] that side-chain *tert*-butyl sulfones, even though lacking the traditional side-chain tertiary hydroxyl group, represent a conceptually new class of calcitriol analogues having considerable potential as sensitive molecular probes of ligand−receptor interactions and as pharmacologically valuable new chemical entities. Details of synthesis of sulfone diastereomers **4a** and **4b** and preliminary biological profiles are provided in the following sections.

Results and Discussion

Synthesis of the target sulfone analogues **4** is outlined in Scheme 1, starting with enantiomerically pure C,D-

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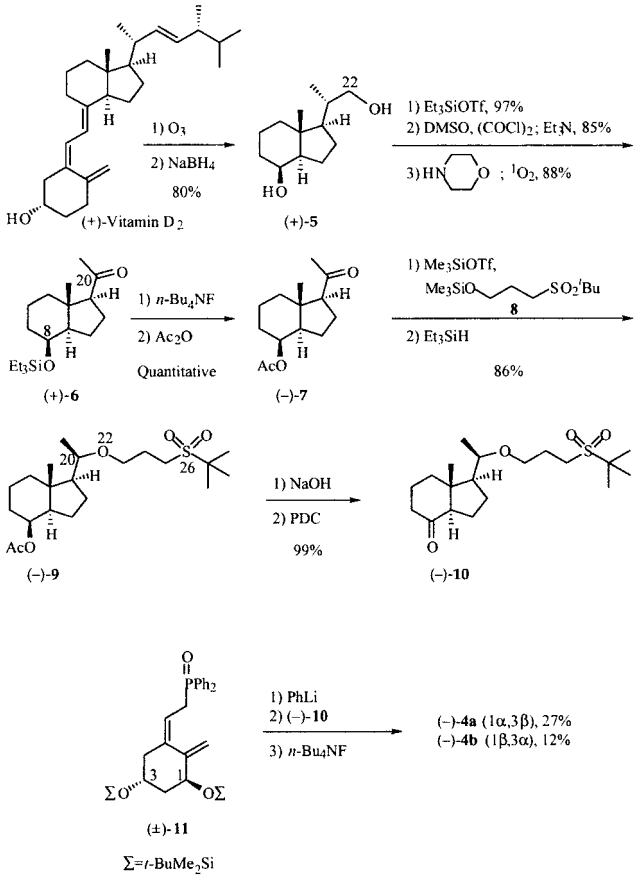
Scheme 1



ring chiron (+)-**5** derived from commercial vitamin D_2.[3] Chemoselective oxidation of only the primary C-22 silyl ether of the bis-silyl ether derived from diol (+)-**5** was achieved using Swern conditions,[6] and oxidative removal of C-22 in chiron (+)-**5** was achieved by treating the morpholine enamine of the C-22 aldehyde with singlet oxygen[7] to produce C-20 ketone (+)-**6**. Quantitative replacement of the C-8 silyl ether group in (+)-**6** by the C-8 acetate group in (−)-**7** allowed application of the Japanese reductive etherification protocol[8] to form the desired 20-*epi*-22-oxa-26-sulfone (−)-**9** in 86% yield; noteworthy is the compatibility of this reductive etherification step with the presence of a neighboring sulfonyl group. Acetate saponification and alcohol oxidation with pyridinium dichromate (PDC) provided C,D-ring ketone (−)-**10**. Coupling of ketone (−)-**10** with racemic A-ring phosphine oxide (±)-**11**[9] and then desilylation produced diastereomers **4a** and **4b** that were separated via semipreparative HPLC and were characterized as we have done previously in closely related cases by high-field ^1H NMR spectroscopy (see Experimental Section).[10]

Sulfone analogue **4a**, with natural 1α,3β-hydroxyl groups but lacking a side-chain tertiary hydroxyl group, has greater antiproliferative efficacy in vitro than 1,25D_3 (**1**) at 7 nM concentration in murine keratinocytes using our previously described protocol (Figure 1).[9] In sharp contrast, sulfone diastereomer **4b**, with unnatural A-ring hydroxyl group stereochemistry, is inactive at this concentration. In the LNCaP human prostate cancer cell line[11] at concentrations above 1 nM, sulfone **4a** is only slightly less effective than 1,25D_3 (**1**) or KH-1060 (**3**) (Figure 2); sulfone **4b** does not inhibit LNCaP cell growth even at concentrations as high as 100 nM.

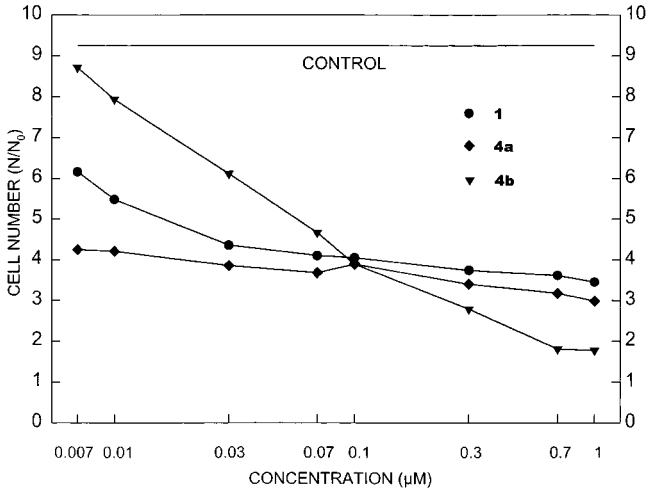


Figure 1. Dose−response effects of analogues on murine keratinocyte proliferation (96 h).

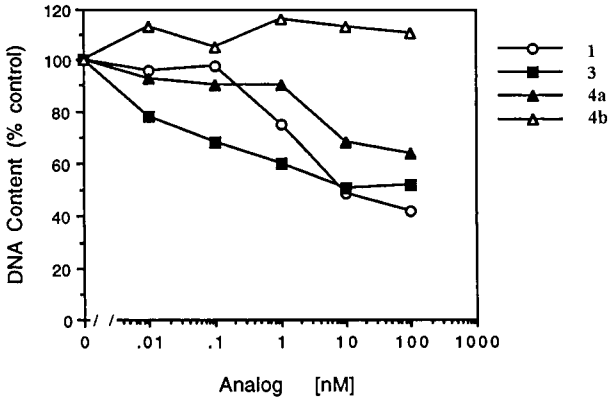


Figure 2. Dose−response effects of vitamin D analogues on LNCaP prostate cancer cell proliferation (120 h). Cells were treated for 5 days with either vehicle alone or the indicated concentrations of vitamin D analogues in RPMI medium containing 5% FBS. The proliferation rate was assessed by determination of DNA content as described in the Experimental Section. Values shown represent the mean of 4−6 experiments at each dose.

In the human breast adenocarcinoma cell line MCF-7,[12] sulfone **4a** is 6 times more antiproliferative than 1,25D_3 (**1**). The ED_{50} values are as follows: 1,25D_3 (**1**), 61 nM; sulfone **4a**, 9.6 nM; and KH-1060 (**3**), 0.27 nM. In the inhibition of phytohemagglutinin-induced stimulation of human peripheral blood mononuclear cells,[13,14] sulfone **4a** is only 3 times less antiproliferative than 1,25D_3 (**1**): sulfone **4a**, ED_{50} = 8.4 nM; and 1,25D_3 (**1**), ED_{50} = 2.7 nM.

The vitamin D receptor (VDR)-mediated transcriptional potencies of sulfones **4a** and **4b** were determined in vitro using a previously described protocol in rat osteosarcoma ROS 17/2.8 cells.[15] The ED_{50} values for transcriptional activity are as follows: 1,25D_3 (**1**), 0.36 nM; natural A-ring sulfone **4a**, 1.6 nM; and unnatural A-ring sulfone **4b**, >100 nM. Thus, although natural A-ring sulfone **4a** lacks a side-chain hydroxyl group, it is only 4-fold less transcriptionally potent than the natural hormone 1,25D_3 (**1**). This significant result emphasizes the ability of atypical sulfone **4a** to bind to the nuclear VDR and thus to initiate genomic responses. Identical results with analogues **4a** and **4b** were seen in VDRE-luciferase reporter transfected LNCaP cells

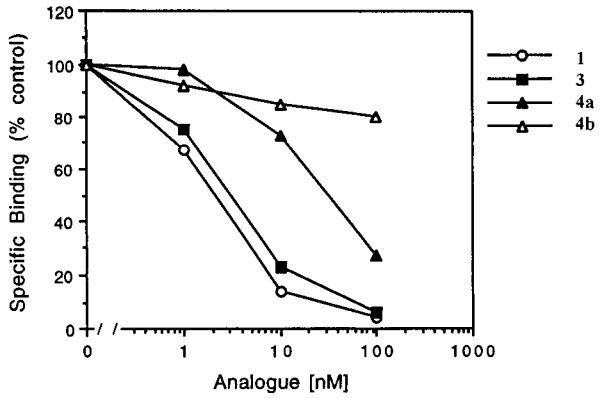


Figure 3. Competition binding analysis of VDR by specific [^{3}H]-1,25D_3 binding in the LNCaP human prostate carcinoma cell line. Cells were grown to near confluence and processed for [^{3}H]-1,25$(OH)_2D_3$ binding as described in the Experimental Section. The data are expressed as the percent of specific binding in the absence of any competing steroid; a level of 100% represents 30 fmol of [^{3}H]-1,25$(OH)_2D_3$/mg of protein. Values shown represent the mean of 3 experiments performed in duplicate.

(data not shown).[16,17] Competitive binding affinities to human VDR for sulfones **4a** and **4b** [relative to 100% binding of 1,25D_3 (**1**)] are as follows: **4a**, 26%; and **4b**, 0.4%. In separate experiments, competitive binding affinities to LNCaP human prostate cell VDR (Figure 3)[11,18] show a similar trend. Competitive binding assays, however, are not able to measure the differential ability of VDR ligands to induce heterodimerization with retinoid X receptors and thus to activate transcription nor are such binding assays able to account for differences in the in vivo rate of metabolism of 1,25D_3 (**1**) and KH-1060 (**3**) in LNCaP cells; both of these factors may explain the greater antiproliferative activity of KH-1060.[19]

A desirable therapeutic ratio of efficacy versus safety of our recently described 16-ene 24- and 25-*tert*-butyl sulfones was demonstrated in a chemoprevention study of mouse skin carcinogenesis.[20] Now we report that sulfone **4a**, administered intraperitoneally to diabetic C57B1/6 mice at a daily dose of 40 μg/kg/day for 13 days, caused a small but significant delay in rejection of allotransplanted Balb/C islets without elevating serum calcium levels. For comparison in this assay, KH-1060 (**3**) administered at 0.3 μg/kg/day produced no delay in islet rejection.[13,14]

For practical chemotherapeutic uses of any new vitamin D analogue, it must be not only efficacious but also safe. Typical toxicity of the vitamin D family of compounds involves dangerously elevated levels of calcium in an animal's blood (hypercalcemia) and urine (hypercalciuria).[1,2] Using our previously reported protocol in which rats are treated orally with 1,25D_3 (**1**) at 0.5 μg/kg body weight and with our new analogues at 10 μg/kg daily for 1 week,[4] sulfones **4a** and **4b** produced no calcium elevation above control under identical treatment regimens (Figure 4). Thus, new chemical entity sulfone **4a** is not only potently antiproliferative and transcriptionally active in vitro but also safe in vivo.[21,22] This result therefore adds a promising 20-*epi*-22-oxa-26-sulfone analogue to the several therapeutically desirable 16-ene side-chain sulfones we reported recently.[4]

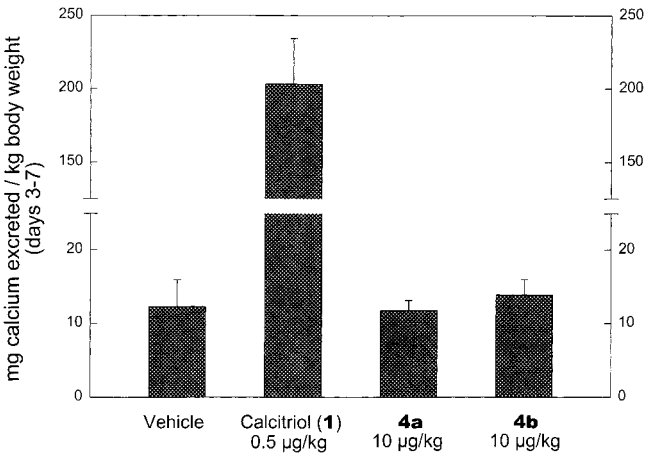
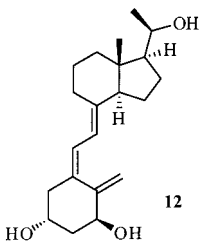


Figure 4. Effects of vitamin D_3 analogues on urinary calcium excretion in rats. Animals were treated with 0.5−10 μg/kg body weight of test compound po for 7 consecutive days, and urinary excretion of calcium was measured during days 3−7. Values are mean ± SE from 3 animals in each group.

Catabolism in vivo of 1,25D_3 (**1**) leads to several metabolites, some of which themselves are biologically active.[23] For example, 3-*epi*-1,25D_3 has been identified as a natural metabolite having considerable antiproliferative but noncalcemic activities.[24] Using the previously described Providence metabolism protocol,[24] both 1,25D_3 (**1**) and the new sulfones **4** were examined concurrently for 24 h at 1 μM concentrations in rat osteosarcoma cells (UMR 106). Relative to 1,25D_3, sulfone **4b** undergoes negligible metabolism, whereas sulfone **4a** undergoes 20% catabolism, compared to the 35% catabolism of 1,25D_3 (Figure 5). Of the four metabolites formed from sulfone **4a**, the two major ones (metabolites 1 and 2) have been identified by GC−MS and UV spectroscopy; the mass spectrum of metabolite 1 is consistent with the structure of side-chain-cleaved compound **12**, and metabolite 2 is 3-(or 1-)*epi*-sulfone **4a**. Metabolite 2 has very low transcriptional potency in ROS 17/2.8 cells (data not shown). Formation of this A-ring *epi* metabolite from sulfone **4a** in an amount (11% of remaining **4a**) similar to that of the 3-*epi* metabolite formed in this assay system from 1,25D_3 (**1**) indicates that the C,D-ring side-chain hydroxyl group of the natural hormone is not required for enzymatically directed hydroxyl group epimerization in the A-ring. Likewise, formation of side-chain-truncated metabolite **12**, likely via C-23 enzymatic hydroxylation followed by hemiacetal hydrolysis into alcohol **12** as in catabolism of KH-1060 (**3**),[25,26] indicates that a side-chain tertiary hydroxyl group is not required for catabolism of the side chain.



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In conclusion, we have shown here for the first time that 20-*epi*-22-oxa-26-*tert*-butyl sulfone **4a**, a rationally

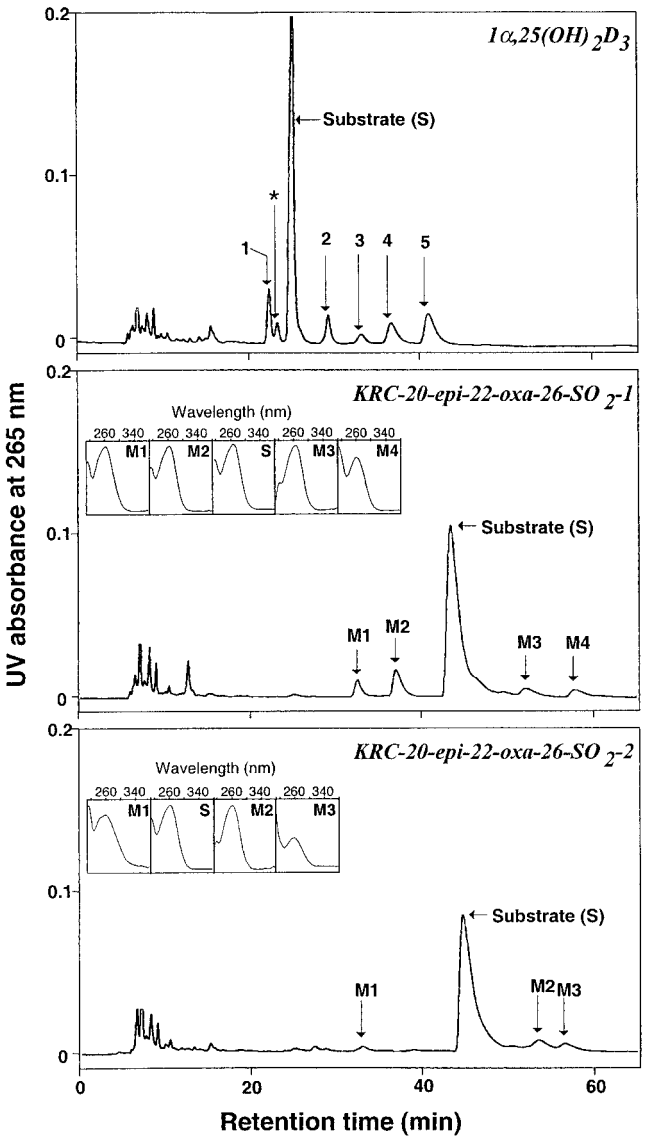

Figure 5. Metabolism of 1α,25(OH)$_2$D$_3$, KRC-20-*epi*-22-oxa-SO$_2$-1 (**4a**), and KRC-20-*epi*-22-oxa-SO$_2$-2 (**4b**) in cultured rat osteosarcoma cells (UMR 106) incubated for 24 h with 1 μM substrate concentrations. Upper panel: HPLC profile of 1α,-25(OH)$_2$D$_3$ and its various metabolites. Metabolite 1 [1α,25-(OH)$_2$-*epi*-D$_3$] is derived from C-1 or C-3 epimerization. [The asterisk indicates the elution position of pre-1α,25(OH)$_2$D$_3$.] Metabolites 2−5 are the well-established metabolites of 1α,-25(OH)$_2$D$_3$ derived from the C-24 oxidation pathway (1α,25-(OH)$_2$-24-oxo-D$_3$, C-23 alcohol, 1α,23(S),25(OH)$_3$-24-oxo-D$_3$, and 1α,24,25(OH)$_3$D$_3$, respectively). Middle panel: HPLC profile and UV spectra of KRC-20-*epi*-22-oxa-SO$_2$-1 (**4a**) and its various metabolites. Metabolite M1 is the side-chain-cleaved compound **12**, and metabolite M2 is the C-1 or C-3 epimer of analogue **4a**. The identity of metabolites M3 and M4 was not established. Lower panel: HPLC profile and UV spectra of KRC-20-*epi*-22-oxa-SO$_2$-2 (**4b**) and its various metabolites. The identity of its metabolites was not established. HPLC analysis was performed using a Zorbax-SIL column eluted with 2-propanol:hexane (10:90 v/v) solvent mixture at a flow rate of 2 mL/min.

designed synthetic analogue of 1,25D$_3$ (**1**) and KH-1060 (**3**), potently simulates the natural hormone's antiproliferative and transcriptional activities in vitro but, in sharp contrast to the natural hormone, without causing undesirable hypercalcemia in vivo. The in vivo allo-islet transplantation results suggest that sulfone **4a** has a potentially desirable therapeutic index in this immunological assay. Thus, conceptually new sulfone **4a** represents a new chemical entity that significantly broadens our recent claim[4] that side-chain *tert*-butyl sulfone analogues of 1,25D$_3$, despite their lacking the classical side-chain tertiary hydroxyl group, are promising new molecular biology probes and are efficacious and safe potential new drug candidates worthy of considerably more preclinical testing.[27]

Experimental Section

Sulfone Analogues (−)-4a and (−)-4b. To a cooled (−78 °C) solution of racemic phosphine oxide (±)-**11**[4] (51 mg, 0.087 mmol) in THF (1.0 mL) was added PhLi (1.69 M in cyclohexane/Et$_2$O, 51 μL, 0.086 mmol) dropwise via syringe. The resulting deep orange solution was stirred for 30 min, at which time a cooled (−78 °C) solution of C,D-ring ketone (−)-**10** (31 mg, 0.082 mmol) in THF (0.7 mL) was added dropwise via cannula. The resulting solution was stirred at −78 °C in the dark for approximately 4 h and then quenched with a 2:1 (v:v) mixture of 2 N aqueous sodium potassium tartrate and 2 N aqueous potassium carbonate (3 mL). Upon warming to room temperature, the reaction mixture was extracted with EtOAc (3 × 20 mL), dried (MgSO$_4$), filtered, concentrated and purified by flash column chromatography (20% EtOAc/1% NEt$_3$/hexanes) to afford 43 mg [72% based on (−)-**10**] of the coupled product as a yellow oil. This oil was immediately dissolved in THF (10 mL) buffered with NEt$_3$ (50 μL) and treated with TBAF (1.0 M in THF, 178 μL, 0.178 mmol) dropwise via syringe. The reaction mixture was stirred for 16 h in the dark, after which the solvent was evaporated and the crude mixture purified by flash column chromatography (1% NEt$_3$/EtOAc) to give a mixture of two diastereomers (−)-**4a** and (−)-**4b** (21 mg, 71%). This diastereomeric mixture was purified by reversed-phase HPLC (C-18 semipreparative column, 40% MeCN/H$_2$O, 3 mL/min) giving 11 mg (27%) of (−)-**4a** (1α,3β, t_R 70.7 min) and 4.7 mg (12%) of (−)-**4b** (1β,3α, t_R 66.6 min). (−)-**4a** (1α,3β): [α]^{25} −50.8 (*c* 4.4, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 6.38 (d, *J* = 11.2 Hz, 1H), 6.00 (d, *J* = 11.2 Hz, 1H), 5.32 (m, 1H), 4.99 (m, 1H), 4.46−4.40 (m, 1H), 4.26−4.19 (m, 1H), 3.70 (dt, *J* = 9.2, 6.0 Hz, 1H), 3.34 (dt, *J* = 9.2, 6.0 Hz, 1H), 3.29 (m, 1H), 3.03 (m, 2H), 2.83 (dd, *J* = 13.2, 4.4 Hz, 1H), 2.60 (dd, *J* = 13.2, 3.6 Hz, 1H), 2.29 (dd, *J* = 13.4, 6.4 Hz, 1H), 1.41(s, 9H) 1.08 (d, *J* = 5.6 Hz, 3H), 0.55 (s, 3H); ^{13}C NMR (CDCl$_3$) δ 147.60, 142.85, 132.99, 124.87, 117.05, 111.75, 78.30, 70.78, 66.82, 65.94, 58.87, 56.73, 55.79, 45.76, 45.21, 43.20, 42.83, 40.43, 29.05, 25.11, 23.40, 23.41, 22.44, 21.67, 18.28, 12.64; IR (neat, cm^{-1}) 3663−3143 (b), 2946, 2872, 1302, 1276, 1113, 1055, 753; UV (MeOH) λ_{max} 263 nm (ε 13679); HRMS calcd for $C_{28}H_{46}O_5S + NH_4^+$ 512.3410, found 512.3422. (−)-**4b** (1β,3α): [α]^{25} −36.2 (*c* 3.0, CHCl$_3$); ^{1}H NMR (CDCl$_3$) δ 6.39 (d, *J* = 11.4 Hz, 1H), 5.99 (d, *J* = 11.4 Hz, 1H), 5.31 (m, 1H), 5.00 (m, 1H), 4.47−4.41 (m, 1H), 4.26−4.19 (m, 1H), 3.71 (dt, *J* = 9.2, 6.0 Hz, 1H), 3.35 (dt, *J* = 9.2, 6.0 Hz, 1H), 3.29 (m, 1H), 3.04 (m, 2H), 2.83 (dd, *J* = 13.2, 4.0 Hz, 1H), 2.61 (dd, *J* = 13.2, 4.0 Hz, 1H), 2.30 (dd, *J* = 13.0, 7.6 Hz, 1H), 1.41 (s, 9H) 1.08 (d, *J* = 5.6 Hz, 3H), 0.55 (s, 3H); ^{13}C NMR (CDCl$_3$) δ 147.27, 142.90, 132.85, 124.90, 117.05, 112.49, 78.29, 71.31, 66.79, 65.95, 58.86, 56.74, 55.80, 45.78, 45.45, 43.20, 42.81, 40.43, 29.03, 25.12, 23.48, 23.42, 22.48, 21.68, 18.28, 12.65; IR (neat, cm^{-1}) 3641−3113 (b), 2940, 2873, 1301, 1276, 1114, 1057, 754; UV (MeOH) λ_{max} 263 nm (ε 10319); HRMS calcd for $C_{28}H_{46}O_5S + NH_4^+$ 512.3410, found 512.3427.

LNCaP Studies. Materials: [^{3}H]-1,25(OH)$_2$D$_3$ (specific activity, 116 Ci/mmol) was purchased from Amersham Chemical Co. (Arlington Heights, IL). Nonradioactive 1,25(OH)$_2$D$_3$ was a gift from Dr. M. Uskokovic (Hoffman-La Roche Co., Nutley, NJ). The 1,25(OH)$_2$D$_3$ analogue KH-1060 was a gift from Dr. L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). RPMI-1640 cell culture medium was purchased from Mediatech (Herndon, VA). Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from GIBCO-BRL (Grand Island, NY).

Cell culture: LNCaP human prostate carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). In all experiments, LNCaP cells were maintained in RPMI-1640 medium supplemented with 5% FBS and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Antiproliferative assays: LNCaP cell proliferation was assessed by measurement of attained cell mass using an assay of DNA content. Cells were seeded with 100 μL of medium at a density of 2000 cells/well in 96-well flat-bottom tissue culture plates (Becton Dickinson & Co., Lincoln Park, NJ). After 24 h, cells were treated with 100 μL of fresh medium containing 5% FBS with vehicle alone (ethanol, final concentration 0.1%) or vitamin D analogues (0.01−100 nM final concentration in 200 μL of medium/well). Medium containing vehicle or vitamin D analogues was subsequently renewed after 3 days. After 5 days of hormone treatment, cell monolayers were solubilized with 0.2 N sodium hydroxide and the DNA content was determined using the diphenylamine assay of Burton.[28] DNA content for each treatment was derived from the mean value of 6 wells in an experiment.

VDR binding analysis: The ability of vitamin D analogues to compete with [^{3}H]-1,25$(OH)_2D_3$ for VDR binding sites in LNCaP cells was performed as previously described.[11] Soluble extracts from LNCaP cells were incubated for 16 h at 4 °C in the presence of 1 nM [^{3}H]-1,25$(OH)_2D_3$ and increasing concentrations of vitamin D analogues (0−100 nM). Bound and free hormones were separated by the hydroxylapatite method.[29] Specific binding was calculated by subtracting nonspecific binding obtained in the presence of a 300-fold excess of radioinert 1,25$(OH)_2D_3$ from the total binding measured in the absence of radioinert steroid and is expressed as femtomoles of ligand bound/mg of protein. The protein concentration of soluble extracts was measured by the method of Bradford.[18]

Metabolism Studies. UMR 106 cells were maintained in McCoy's culture media supplemented with 10% FCS and antibiotics (penicillin, 100 IU/mL) and streptomycin (100 μg/mL). Cell culture medium was changed every 3−4 days. The cells were subcultured when approximately 80% confluent and were not subcultured beyond passage 5. For the metabolism studies, 3×10^6 cells were seeded in T150 tissue culture bottles and grown to confluence. The incubations were carried out at 37 °C in a humidified atmosphere under 5% CO_2.

Confluent UMR 106 cells were incubated with 1 μM concentration of either 1α,25$(OH)_2D_3$, KRC-20-*epi*-22-oxa-SO_2-1 (**4a**) or KRC-20-*epi*-22-oxa-SO_2-2 (**4b**) in 50 mL of media containing 10% FCS. The incubations were stopped after 24 h with 10 mL of methanol and the lipids from both cells and media were extracted for HPLC analysis, using the procedure previously described.[30] The lipid extract from both media and cells were analyzed using HPLC system no. 1.

Control incubations containing only media and the vitamin D compounds, without cells, were performed to ensure that no metabolic conversion occurred in the absence of cells. The control studies indicated that the vitamin D compounds did not undergo chemical change or breakdown either during the 24-h incubation period or during the extraction procedure (data not shown).

HPLC and GC−MS Analyses. HPLC analysis of the lipid extracts from the media and cells was performed with a Waters System Controller (model 600E) equipped with a photodiode array detector (model PDA 996) to monitor UV absorbing material at 265 nm (Waters Associates, Milford, MA). The vitamin D compounds were isolated and purified by HPLC with the use of a Zorbax-SIL column (9 × 250 mm) (DuPont, Wilmington, DE) eluted with two different solvent mixtures at a flow rate of 2 mL/min. The solvent mixtures used were as follows: 10% 2-propanol:hexane (10:90 v/v), HPLC system no. 1; and 6% 2-propanol:methylene chloride (6:94 v/v), HPLC system no. 2.

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Supporting Information Available: Experimental details for preparation of intermediate compounds **5**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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