Supporting Information for:

Novel Alkynylphosphonate Analogue of Calcitriol with Potent Antiproliferative Effects in Cancer Cells and Lack of Calcemic Activity

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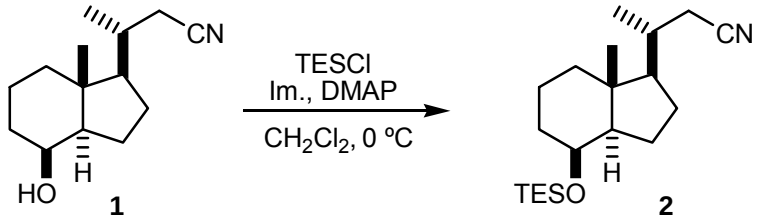
Contents

1. Synthetic procedures

All the common chemicals were analytical grade. THF was distilled over sodium/benzophenone and CH_2Cl_2 over P_2O_5 prior to use. The processes involving moisture sensitive were conducted under the inert atmosphere of dry nitrogen using oven dried glassware. Organic extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure using a Büchi rotary evaporator. TLC analyses were performed on Merck 60 F254 silica gel plates. Purification of crude materials was performed by flash chromatography on silica gel (200–400 mesh, Merck) using ethyl acetate/hexane as eluents. Final purification of compound 10 for biological evaluation was performed by preparative chromatography using Macherey-Nagel TLC plates Sil G 25-UV254. NMR spectra were recorded on a Bruker AVANCE DPX 400 (400 MHz ^{1}H, 100 MHz ^{13}C, 161 MHz ^{31}P) as $CDCl_3$ solutions using TMS or residual $CHCl_3$ as internal standard in ^{1}H and ^{13}C NMR, and 85% H_3PO_4 as external standard in ^{31}P NMR. Chemical shifts (δ) are reported in ppm (integration, multiplicity and coupling constants in Hz). The high-resolution mass spectra were performed on a Bruker Daltonics Apex-Qe instruments at 300.0 V using electron spray ionization (ESI) technique. Ultraviolet spectra were recorded with a Merck Spectroquant Pharo 300 spectrophotometer. Reverse-Phase HPLC analyses were performed using a Waters 600E, equipped with a Spherisorb ODS 2 (C18) column (250 x 4.6 mm, 5µm) and a Waters 2998 PDA detector sets at 263 nm.

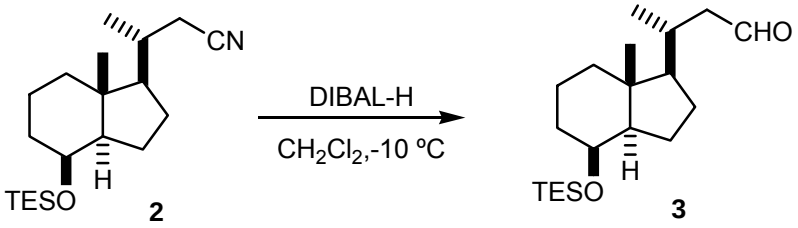
Des-*A,B*-8β-triethylsililoxy-23,24-dinor-22-cianocholane (2)



To a solution of nitrile **1**[1] (0.90 g, 4.07 mmol) in CH_2Cl_2 (15 mL) was added imidazole (0.83 g, 12.21 mmol) and a catalytic amount of DMAP. The mixture was cooled to 0 ºC and TESCl (1.36 ml, 8.14 mmol) was added. After stirring the mixture for 1 h, the reaction was quenched with water (20 mL) and extracted with CH_2Cl_2. The organic phase was washed with brine, dried and concentrated. The residue was purified by flash chromatography (10% EtOAc/hexanes) to afford nitrile **2** (1.32 g, 97%) as a colorless liquid: R_f = 0.76 (30 % EtOAc/Hexanes); **^{1}H-NMR** ($CDCl_3$, δ): 4.02 (1H, br s, H-8), 2.31 (1H, dd J = 16.6 Hz, J = 3.8 Hz, H-22), 2.20 (1H, dd J = 16.6 Hz, J = 7.0 Hz, H-22), 1.11 (3H, d J = 6.6 Hz, H-21), 0.91 (3H, s, H-18), 0.93 (9H, t J = 8.0 Hz, CH_3CH_2Si-), 0.54 (6H, c J = 7.9 Hz -CH_2Si-); **^{13}C-NMR** ($CDCl_3$, δ): 118.93 (CN), 68.96 (CH-8), 55.11 (CH-17), 52.32 (CH-14), 41.87 (C-13), 39.96

(CH_2), 33.47 (CH_2), 33.01 (CH-20), 27.02 (CH_2), 24.65 (CH_2), 22.35 (CH_2), 19.15 (CH_3-18), 17.26 (CH_2), 13.61 (CH_3-21).

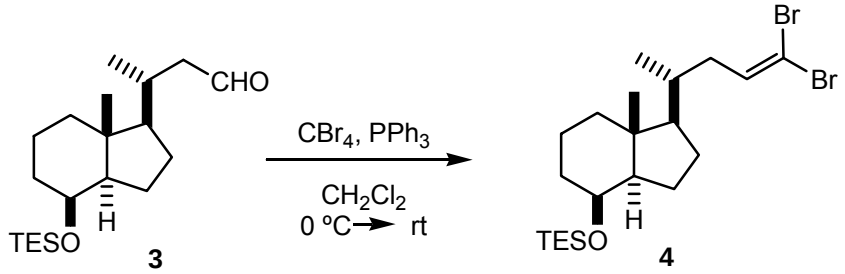
Des-*A,B*-8β-triethylsililoxy-24-norcholan-23-al (3).



2 **3**

To a solution of nitrile **2** (1.32 g, 3.94 mmol) in CH_2Cl_2 (50 mL) a -10 ºC, was added dropwise DIBAL-H (7.8 mL, 1M in hexanes). After 30 minutes the reaction was quenched with aqueous solution of HCl 10 % (60 mL) and Et_2O (30 mL). The mixture was stirred for 15 minutes and was extracted with CH_2Cl_2. The organic phase was dried, filtered and concentrated to afford **3** (1.28 g) as a colorless oil. This product was used directly in the next step without further purification: R_f = 0.64 (10% EtOAc/Hexanes); **^{1}H-NMR** ($CDCl_3$, δ): 9.7 (1H, br s, -CHO), 4.02 (1H, br s, H-8), 2.45-2.41 (1H, m, H-22), 2.16-2.09 (1H, m, H-22), 0.98 (3H, d *J* = 6.5 Hz, H-21), 0.96-0.92 (3H, s, H-18 and 9H, t, CH_3CH_2Si-), 0.54 (6H, q *J* = 7.8 Hz, CH_3CH_2Si-); **^{13}C-NMR** ($CDCl_3$, δ): 203.66 (COH), 69.20 (CH-8), 56.49 (CH-17), 53.00 (CH-14), 50.74 (CH_2-22), 42.22 (C-13), 40.57 (CH_2), 34.47 (CH_2), 31.21 (CH-20), 27.53 (CH_2), 22.87 (CH_2), 19.87 (CH_3-18), 17.57 (CH_2), 13.46 (CH_3-21), 6.89 (CH_3CH_2Si-), 4.87 (-CH_2Si-).

Des-*A,B*-8β-triethylsililoxy-24,24-dibromo-23-cholene (4)[2].

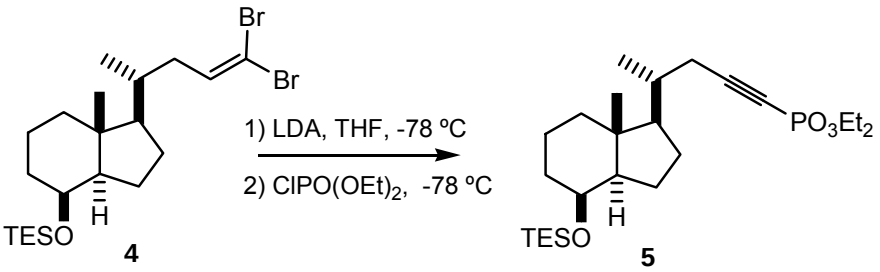


3 **4**

A solution of crude aldehyde **3** (1,28 g, 3,77 mmol) in dry CH_2Cl_2 (40 mL) was cooled to 0 ºC and CBr_4 (1,86 g; 5,62 mmol) and PPh_3 (3,09 g, 11,77 mmol in six parts) were added. The mixture was stirred at room temperature for 40 minutes and then was diluted with hexane (50 mL). The white suspension was filtered through silica gel and the solids were washed with hexane. The filtrate was concentrated under reduced pressure to afford **4** (1.49 g) as a colorless oil. This product was used directly in the next step without further purification. R_f = 0.8 (5% EtOAc/Hexanes); **^{1}H-NMR** ($CDCl_3$, δ): 6.39 (1H, dd *J* = 8.0 Hz, *J* = 6.6, H-23), 4.03 (1H, m, H-8), 2.17 (1H, ddd *J* = 3.4 Hz, *J*= 6.5 Hz, *J* = 14.7 Hz, H-22), 0.97-0.95 (6H, d H-21

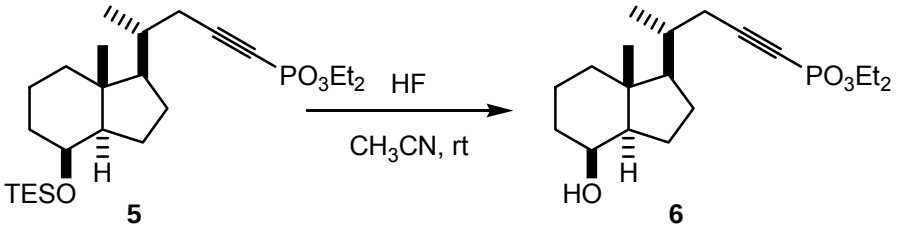
and s H-18), 0.93 (9H, t *J* =7.7 Hz, *CH*$_3$CH$_2$Si-), 0.56 (6H, q *J* = 7.9 Hz, -CH$_2$Si-); **^{13}C-NMR** (CDCl$_3$, δ): 137.84 (CH-23), 88.58 (C-24), 69.32 (CH-8), 56.49 (CH-17), 53.05 (CH-14), 42.26 (C-13), 40.65 (CH$_2$), 39.56 (CH$_2$), 35.22 (CH-20), 34.59 (CH$_2$), 27.41 (CH$_2$), 23.00 (CH$_2$), 18.92 (CH$_3$-18), 17.65 (CH$_2$), 13.53 (CH$_3$-21), 6.94 (*CH*$_3$CH$_2$Si-), 4.95 (-CH$_2$Si-).

Diethyl [Des-*A,B*-8β-triethylsililoxy-chol-23-yn-24-yl] phosphonate (5)[3]**.**



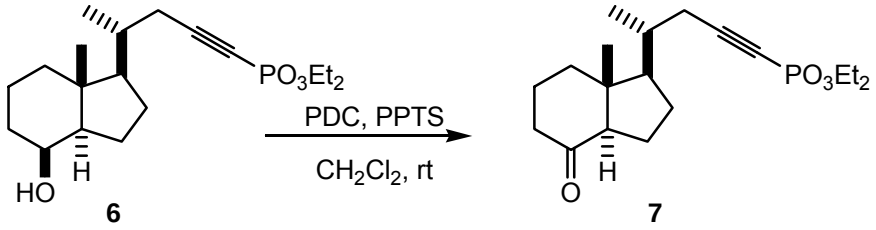
A solution of the above dibromide **4** (1.49 g, 3.01 mmol) in THF (20 mL) was slowly added to a solution of LDA (9.03 mmol) in THF (20 mL) at -78 ºC. After stirring for 1 h was added ClPO(OEt)$_2$ (1.0 mL, 6.63 mmol) slowly at -78 ºC. The reaction mixture was allowed to reach room temperature slowly. The reaction was quenched by addition of an aqueous solution of HCl 5 % (20 mL). The mixture was extracted with Et$_2$O (3 x 50 ml), and the resulting organic phase was dried, filtered and concentrated. The residue was purified by flash chromatography (30% EtOAc/Hexanes) to afford **5** (0.93 g, 50% three steps from **2**) as a colorless oil: ***R$_f$*** = 0.27 (30% EtOAc/Hexanes); **^{1}H-NMR** (CDCl$_3$, δ): 4.08 (4H, m, CH$_3$*CH*$_2$OP), 3.96 (1H, br s, H-8), 2.32 (1H, m, H-22), 2.12 (1H, ddd, *J* = 4.4 Hz, *J* = 7.5 Hz, *J* = 17.2 Hz, H-22), 1.3 (6H, t *J* = 7.0 Hz, *CH*$_3$CH$_2$OP), 1.00 (3H, d *J* = 6.6 Hz, H-21), 0.88 (9H, t *J* =7.9 Hz, *CH*$_3$CH$_2$Si-), 0.84 (3H, s, H-18), 0.48 (6H, q, J = 8.0 Hz, CH$_3$*CH*$_2$Si-); **^{13}C-NMR** (CDCl$_3$, δ): 102.23 (d, *J*= 52.9 Hz, C-23), 71.25 (d, *J*= 302.6 Hz, C-24), 69.0 (CH-8), 62.66 (d, *J*= 5.5 Hz, CH$_3$*CH*$_2$OP), 55.49 (CH-17), 52.80 (CH-14), 41.96 (C-13), 40.34 (CH$_2$), 34.54 (d, *J* = 2.4 Hz, CH-20), 34.32 (CH$_2$), 27.04 (CH$_2$), 26.19 (d, J= 4.3 Hz, CH$_2$), 22.70 (CH$_2$), 18.94 (CH$_3$-18), 17.40 (CH$_2$), 15.93 (d, *J*= 7.0 Hz, *CH*$_3$CH$_2$OP), 13.43 (CH$_3$-21), 6.74 (*CH*$_3$CH$_2$Si), 4.72 (CH$_3$*CH*$_2$Si), **^{31}P-NMR** (CDCl$_3$, δ): -6.09, **HRMS** (ESI): calcd for C$_{25}$H$_{48}$O$_4$PSi (M+H): 471.3059, found: 471.3054.

Diethyl [Des-*A,B*-8β-hidroxy-chol-23-yn-24-yl] phosphonate (6).



An aqueous solution of HF (48 %, 2 droops) was added slowly to a solution of **5** (0.23 g, 0.48 mmol) in CH_3CN (6 mL). After stirring at room temperature for 1 h, a saturated solution of $NaHCO_3$ (5 mL) was added. The resulting mixture was extracted with CH_2Cl_2. The organic phase was dried, filtered and concentrated to afford **6** (158 mg, 92%) as a colorless oil: R_f = 0.2 (50% EtOAc/Hexanes); **^{1}H-NMR** ($CDCl_3$, δ): 4.08 a 4.11 (4H, m, CH_3CH_2OP), 4.00 (1H, br s, H-8), 2.33 (1H, dt *J* = 3.9 Hz, *J* = 17.3 Hz, H-22), 2.13 (1H, ddd *J*= 4.4 Hz, *J* = 7.5 Hz, *J* = 17.1 Hz, H-22), 1.3 (6H, t *J* = 7.0 Hz, CH_3CH_2OP), 1.00 (3H, d *J* = 6.6 Hz, H-21), 0.87 (3H, s H-18); **^{13}C-NMR** ($CDCl_3$, δ): 102.07 (d, *J* = 53.0 Hz, C-23), 71.09 (d, *J* = 304.4 Hz, C-24), 68.59 (CH-8), 62.56 (d, *J* = 5.49 Hz, CH_3CH_2OP), 55.22 (CH-17), 52.24 (CH-14), 41.59 (C-13), 39.89 (CH_2), 34.27 (CH-20), 33.27 (CH_2), 26.79 (CH_2), 26.03 (d, *J* = 4.3 Hz, CH_2-22), 22.14 (CH_2), 18.76 (CH_3-18), 17.06 (CH_2), 15.79 (d, *J* = 6.94 Hz, CH_3CH_2OP), 13.31 (CH_3-21).

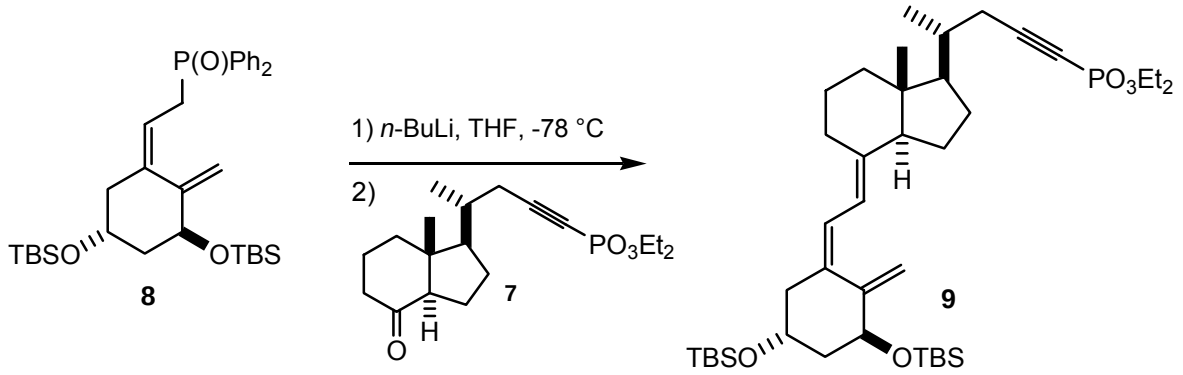
Diethyl [des-*A,B*-8-oxochol-23-yn-24-yl] phosphonate (7)[4].



6 **7**

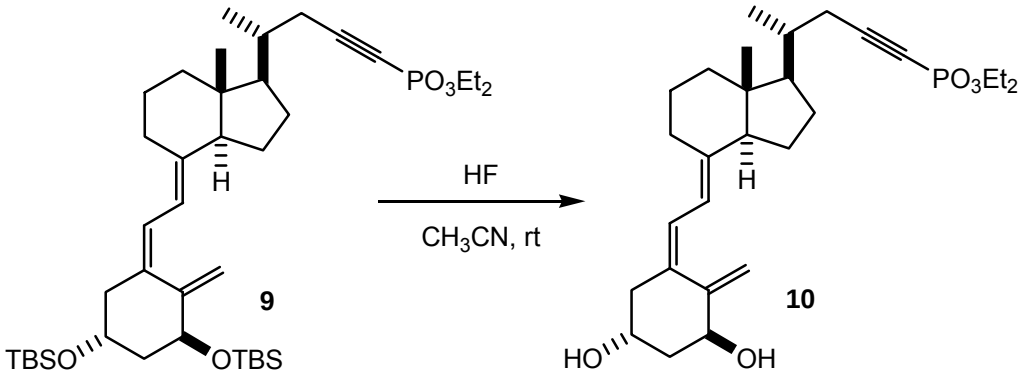
PDC (0.54 g, 1.43 mmol) and a catalytic amount of PPTS were added to a solution of alcohol **6** (0.184 g, 0.52 mmol) in CH_2Cl_2 (9 mL). After stirring the reaction for 3 h at room temperature, 25% EtOAc/Hexanes (5 mL) was added and the resulting mixture was filtered through Celite. Concentration after washing the solids with 50 % EtOAc/Hexanes gave a residue which was purified by flash chromatography (10% EtOAc/hexanes) to afford ketone **7** (178 mg, 97%) as a colorless oil: R_f = 0.13 (40 % EtOAc/Hexanes); **^{1}H-NMR** ($CDCl_3$, δ):4.09 (4H, m, CH_3CH_2OP), 1.31 (6H, t J = 7.0 Hz, CH_3CH_2OP), 1.08 (3H, dd *J* = 6.5 Hz, *J* = 1.0 Hz, H-21), 0.59 (3H, s, H-18); **^{13}C-NMR** ($CDCl_3$, δ): 210.95 (C-8), 101.10 (d, *J*= 52.7 Hz, C-23), 71.38 (d, *J*= 303.9 Hz, C-24), 62.47 (d, *J*= 4.0 Hz, CH_3CH_2OP), 61.15 (CH-14), 54.83 (CH-17), 49.21 (C-13), 40.34 (CH_2), 38.15 (CH_2), 34.19, 26.92 (CH_2), 25.87 (d, *J* = 4.4 Hz, CH_2-22), 23.44(CH_2), 18.73 (CH_3-18), 18.53 (CH_2), 15.65 (d, *J*= 6.9 Hz, CH_3CH_2OP), 12.08 (CH_3-21).

Diethyl [(5Z,7E)-(1S,3R)-1,3bis-[(1,1dimethylethyl)dimethylsililoxy]-9,10-sechocola-5,7,10(19)-trien-23-yn-24-yl] phosphonate (9).



A solution of *n*-butyllithium in hexane (0.61 mmol, 2.5 M) was added drop wise to a – 78 ºC cooled solution of phosphine oxide **8** (0.36 g, 0.61 mmol) in THF (8 mL). The resulting red solution was stirred at the same temperature 1 h. A solution of ketone **7** (0.11 g, 0.30 mmol) en THF (5 mL) was then added and the resulting solution was stirred for 3 h at -78 ºC. The reaction mixture was allowed to come slowly to -40 ºC (2 h) and was quenched by the addition of saturated aqueous solution of NH_4Cl (10 mL). The resulting mixture was extracted with AcOEt. The organic phases were washed with saturated aqueous solution of $NaHCO_3$ (10 mL), dried, filtered and concentrated to afford **9** (120 mg, 60%) as a colorless oil: $\boldsymbol{R_f}$ = 0.48 (50% EtOAc/Hexanes); **^{1}H-NMR** ($CDCl_3$, δ): 6.21 (1H, d *J* = 11.2 Hz, H-6), 5.99 (1H, d *J* = 11.4 Hz, H-7), 5.16 (1H, br s, H-19), 4.83 (1H, br s, H-19), 4.35 (1H, dd *J* = 3.5 Hz y *J* = 6.6 Hz, H-1), 4.20 – 4.10 (1H, c H-3 y 4H, m, CH_3CH_2OP), 2.8 (1H, dd *J* = 12.2 Hz y *J* = 3.5 Hz, H-22), 2.40 (2H, m, H-22 y H-21), 1.35 (6H, t *J* = 7.1 Hz, CH_3CH_2OP), 1.24 (3H, m), 1.09 (3H, d *J* = 6.6 Hz, H-21), 0.85 (18H, s $(CH_3)_3CSi$-), 0.52 (3H, s H-18), 0.03 (12H, m CH_3Si); **^{13}C-NMR** ($CDCl_3$, δ): 148.18 (C-10), 140.26 (C-8), 135.24 (C-5), 122.93 (CH-6), 118.06 (CH-7), 111.16 (CH_2-19), 102.16 (d, *J*= 53.1 Hz, C-23), 71.94 (CH-1), 71.60 (d, *J* = 302.8 Hz, C-24), 67.41 (CH-3), 62.80 (d, *J* = 5.3 Hz, CH_3CH_2OP), 56.09 (CH-17), 55.34 (CH-14), 45.92 (CH_2), 45,62 (C-13),44.69 (CH_2), 40.25 (CH_2), 35.37 (CH-20), 28.68 (CH_2), 27.52 (CH_2), 25.75 (d, *J* = 4.7 Hz, CH_2), 23.27 (CH_2), 21.98 (CH_2), 19.29 (CH_3-18), 18.15 ($(CH_3)_3C$-Si), 18.07 ($(CH_3)_3C$-Si), 16.07 (d, *J* = 7.2 Hz, CH_3CH_2OP), 11.96 (CH_3-21), -4.76 (CH_3-Si), -4.87 (CH_3-Si), -5.15 (CH_3-Si),; **^{31}P-NMR** ($CDCl_3$, δ): -6.18; **HRMS** (ESI): calcd for $C_{40}H_{72}O_5PSi_2$ (M+1): 719.4656, found: 719.4650.

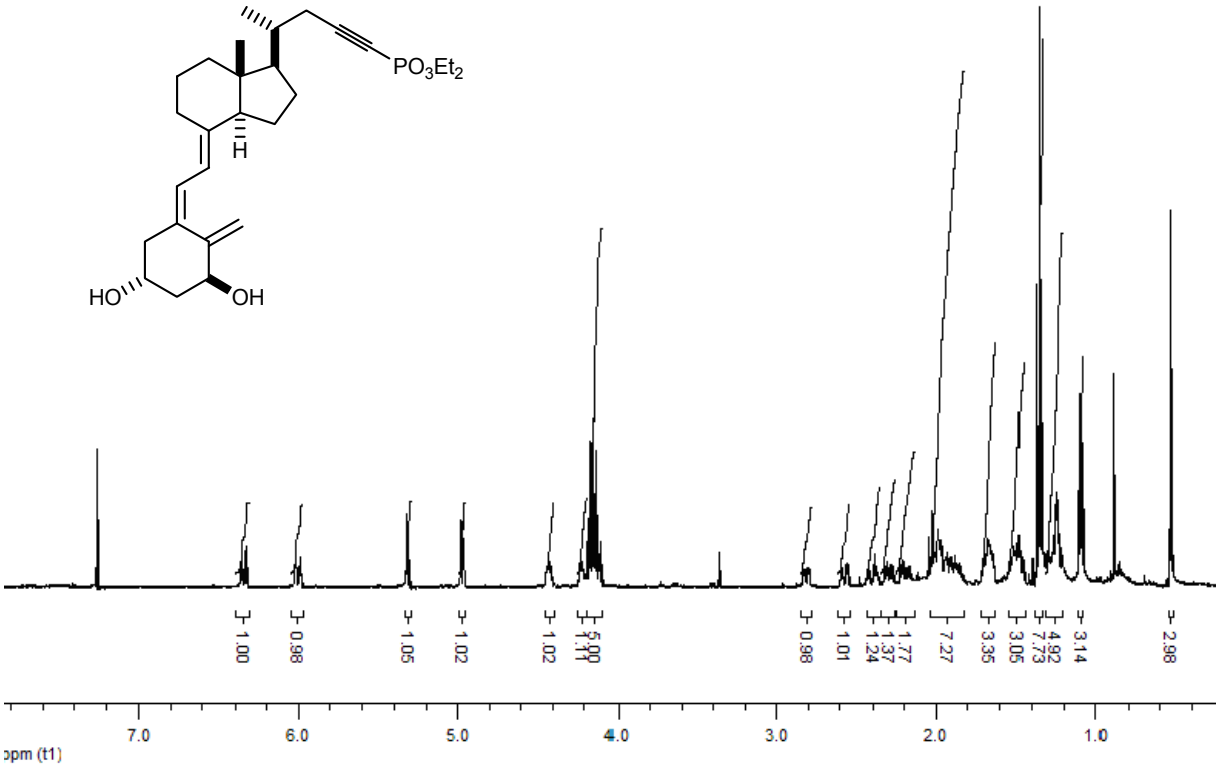
Diethyl [(5Z,7E)-(1S,3R)-1,3-dihidroxi-9,10-secocola-5,7,10(19)-trien-23-in-24-il] phosphonate (10).



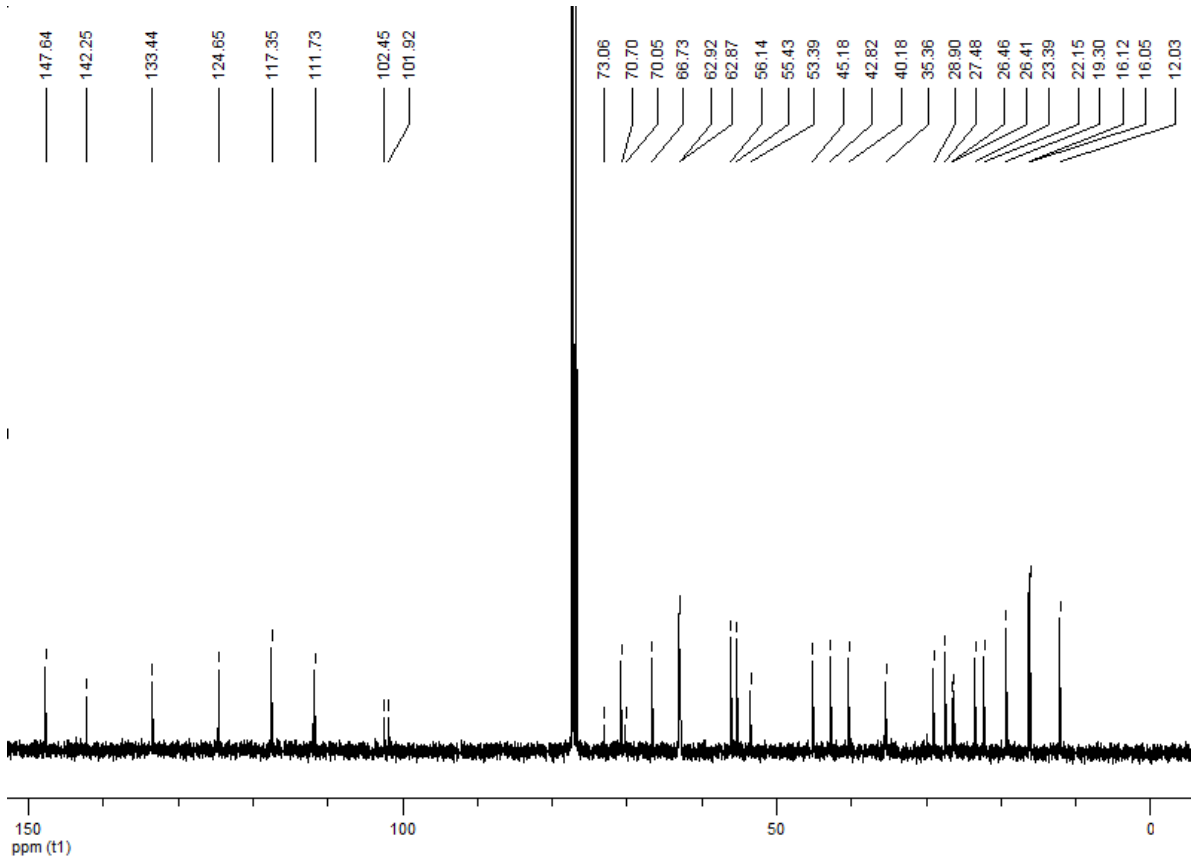
An aqueous solution of HF (48 %, 2 droops) was added slowly to a solution of **9** (0.04 g, 0.06 mmol) in CH_3CN (3 mL). After stirring at room temperature for 35 minutes, a saturated solution of $NaHCO_3$ (5 mL) was added. The resulting mixture was extracted with AcOEt. The organic phase was washed with brine, dried, filtered and concentrated. The residue was purified by flash chromatography (EtOAc) to afford **10** (23 mg, 84%) as a colorless solid: $\boldsymbol{R_f}$ = 0.13 (80% EtOAc/Hexanes); **UV** (*i*-PrOH): λ_{max} 265 nm, λ_{min} 212 nm; **^{1}H-NMR** ($CDCl_3$, δ): 6.21 (1H, d *J* = 11.3 Hz, H-6), 6.00 (1H, d *J* = 11.2 Hz, H-7), 5.31 (1H, br s, H-19), 4.97 (1H, br s, H-19), 4.41 (1H, dd *J* = 4.3 Hz, *J* = 7.7 Hz, H-1), 4.21 (1H, m, H-3), 4.14 (4H, m, CH_3CH_2OP), 2.83 (1H, dd *J* = 12.2 Hz y *J* = 3.9 Hz, H-22), 2.57 (1H, dd *J* = 13.4 Hz, *J* = 3.3 Hz, H-22), 1.35 (6H, t *J* = 7.1 Hz, CH_3CH_2OP), 1.09 (3H, d *J* = 6.6 Hz, H-21), 0.89 (3H, s H-18); **^{13}C-NMR** ($CDCl_3$, δ): 147.64 (C-10), 142.25 (C-8), 133.44 (C-5), 124.65 (CH-6), 117.35 (CH-7), 111.73 (CH_2-19), 102.19 (d, *J* = 53.02 Hz, C-23), 71.56 (d, *J* = 303.3 Hz, C-24), 70.70 (CH-1), 66.73 (CH-3), 62.80 (d, *J* = 5.5 Hz, CH_3CH_2OP), 56.14 (CH-17), 55.43 (CH-14), 53.39 (C-13), 45.18 (CH_2), 42.83 (CH_2), 40.18 (CH_2), 35.36 (d, *J* = 1.2 Hz, CH-20), 28.91 (CH_2), 27.48 (CH_2), 26.44 (d, *J* = 4.4 Hz; CH_2-22), 23.39 (CH_2), 22.15 (CH_2), 19.30 (CH_3-18), 16.08 (d, *J* = 7.1 Hz, CH_3CH_2OP), 12.04 (CH_3-21); **^{31}P-NMR** ($CDCl_3$, δ): -6,18 ppm; **HRMS** (ESI): calcd for $C_{28}H_{43}O_5P$ (M+H): 491.2926, found: 491.2921.

Further purification for biological evaluation was conducted by silica gel preparative TLC (HPLC-grade ethyl acetate). The purity of compound **10** was 98% as determined by analytical reverse-phase HPLC (gradient of 5-95% acetonitrile in water over 30 min at a flow rate of 1 mL/min).

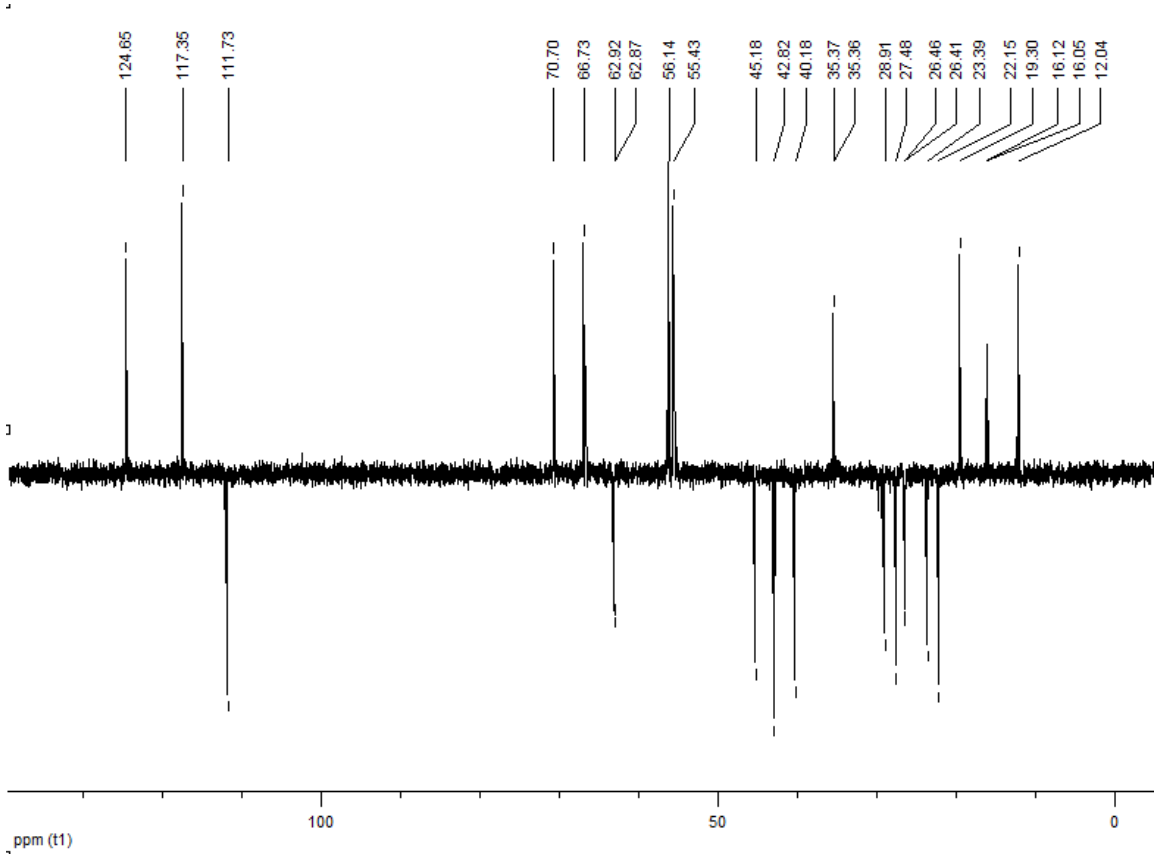
2. ^{1}H–NMR of 10 (400 MHz, $CDCl_3$)



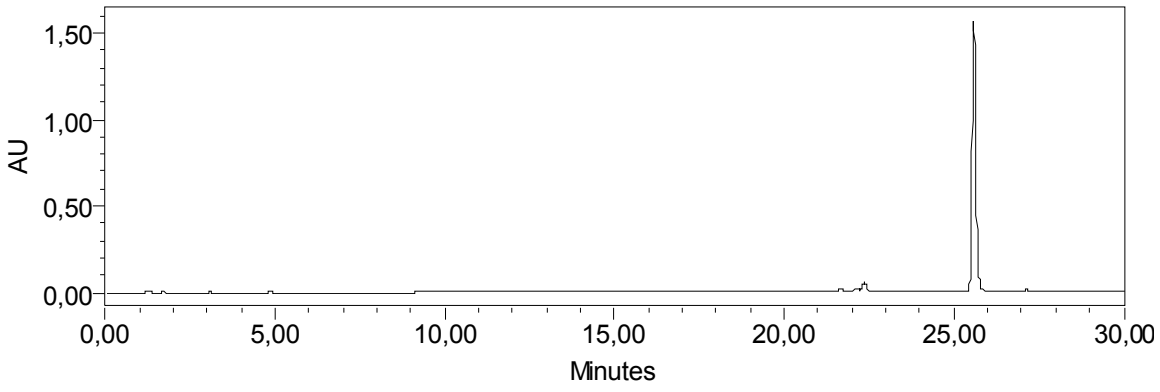
^{13}C–NMR of 10 (100 MHz, $CDCl_3$)



^{13}C–DEPT NMR of 10 (100 MHz, $CDCl_3$)



3. HPLC Chromatogram of 10 (t_R = 25.59)



t_R (min)	Area (µV*sec)	% Area
22.37	245926	2.00
25.59	12037276	98.00

4. Biological procedures

Chemicals and Reagents

1α,25-dihyroxyvitamin D_3 and compound **10** were reconstituted in 100% HPLC-grade isopropanol and stored protected from light at –20° C. The amount of $1\alpha,25(OH)_2D_3$ and its analogue was determined by UV spectrophotometry (Spectroquant Pharo 300, Merck) between 200 and 300 nm. Both drugs were dissolved in isopropanol to the concentration of 10^{-4} M and subsequently diluted in the culture medium to reach the required concentrations (ranging from 0.01 to 100 nM).

Cell lines

Biological evaluation of compound **10** was performed in 8 different cell lines. A murine cellular model of Kaposi sarcoma was employed that involved a parental SV40 large T-antigen immortalized murine endothelial cell (SVEC) and vGPCR-expressing SVEC (SVEC vGPCR) that were kindly donated by Silvio Gutkind (NIH, USA). Also, a human glioma T98G, colorectal carcinoma HCT116 and head and neck squamous cell carcinoma HN12 cell lines were kindly donated by Silvio Gutkind. Additionally, the murine LM3[5] and LM05e[6] and the human T47-D mammary adenocarcinoma cell lines were used. All the cell lines were maintained at 37°C, 5% CO_2 in DMEM supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 4mM glutamine and 10% fetal calf serum (FCS) except for the LM3 that was 5% FCS. The cells were passed every 3−4 days.

Effects of compound 10 on cellular survival

The cell lines were plated at a density of 500 through 2000 cells/well into 96 multi-well dishes in complete medium. They were treated with 0.01 to 100 nM of $1\alpha,25(OH)_2D_3$, compound **10** or vehicle (isopropanol), resulting in a maximum amount of 0.1% v/v alcohol in the assay dishes. We did not find that this amount or lower quantities of alcohol had any significant effect on these cell lines (medium was changed every 2 days). We first performed a time-

course analysis of analogue effect on cellular proliferation, choosing 72 h time points for subsequent experiments. For the dose-response experiments, cells were incubated for 72 h. They were washed with PBS 1X, trypsinized and resuspended in 100 µl complete medium. They were counted manually using a hemocytometer. Additionally, cellular survival was assessed by the WST colorimetric assay (Roche, Argentina) for some cell lines. For this purpose, following analogue treatment cells were incubated for 1 h with the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) and the formazan product determined by reading the absorbance at 440 nM. Data was analyzed using two-way analysis of variance (ANOVA) followed by one-way ANOVA, to determine the effects of increasing concentrations of $1\alpha,25(OH)_2D_3$, compound **10** and vehicle on cellular survival. Bonferroni post test was used to determine statistical significance between sample sets. Graphs were plotted using the Prism 5.0 (GraphPad Prism Software).

Effects of compound 10 on blood calcium levels

Inbred normal CF1 mice aged 8 –10 weeks and weighing 40 g were obtained from the animal facility of the Biology, Biochemistry and Pharmacy Department of the Universidad Nacional del Sur (Bahía Blanca, Argentina). The mice were treated in accordance with the institutional animal care and use committee guidelines. Calcium level studies were performed following daily intraperitoneal injection of 5 µg/Kg body weight or 20 µg/Kg body weight of either $1\alpha,25(OH)_2D_3$, compound **10** or vehicle (isopropanol), during five consecutive days. Blood samples were collected from mice (basal levels as well as at 24, 48, 72 and 96 h). Animals were anesthetized with Acedan® (Holliday Scott, Argentina) 0.22 mg/Kg body weight and heparinized capillary tubes were used to collect blood from the retro orbital sinus. Samples were held on ice, protected from light and processed at 4° C. Plasma was separated by centrifugation at 10 x g and stored at –20°C until assayed. Approximately 10–15 ul of plasma/mouse was obtained each time. Calcium concentration was determined using Ca-Color Arsenazo III AA kit (Wiener Lab, Argentina), measuring the absorbance at 650 nM using a spectrophotometer. The calcium concentration was calculated from calcium

standards provided by the manufacturer. To adjust for differences in hemolysis among samples, blanks were prepared and the absorbance reading was subtracted from the test reading. We also measured the hematocrite for each mouse before and following daily treatments to determine if they were healthy. Mice were observed for 15 days following analogue treatments in order to test compound toxicity. The data was analyzed with Prism 5.0 (GraphPad Prism Software) and statistical significance was evaluated using the Student's t-test. A p value of less than 0.05 was considered significant.

Study of the involvement of VDR on compound 10 effects on cellular survival.

In order to study the involvement of VDR on the effects of compound **10** on cellular survival we performed the silencing of VDR by transfection of cells with different amounts (2-8 μg) of pFIV-SH1-Puro shVDR or scrambled shRNA-expressing plasmid. Cells were seeded in plates with complete medium and transfected by using Lipoafectamine (Invitrogen). After 48 h transfection, cells were treated with 10 nM of compound **10** or vehicle (isopropanol) and 72 h later they were counted as described above. Additionally, protein lysates were prepared according to previously described methods.[7] Briefly, lysates prepared in Laemmli sample buffer were electrophoresed on tris-glicyne SDS polyacrylamide gels and transfered onto PVDF membranes for subsequent blotting. The membranes were blocked with 0,5% semi-skim milk diluted in PBS buffer for 1 h at room temperature. The blots were then incubated overnight with the primary antibody (VDR C20, Santa Cruz Biotechnology). After washing with TBS 1X tween 0,01%, the blots were incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody and processed for chemiluminesence detection using ECL-plus detection kit, according to the manufacturers' directions (Amersham, Sweeden).

5. References

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