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Synthesis and biological properties of 2-methylene-19-nor-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactones—weak agonists

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ABSTRACT

In a continuing effort to explore the 2-methylene-1α-hydroxy-19-norvitamin D_3 class of pharmacologically important vitamin D compounds, two novel 2-methylene-19-nor-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactones, **GC-3** and **HLV**, were synthesized and biologically tested. Based on reports of similarly structured molecules, it was hypothesized that these compounds might act as antagonists, at least in vitro. The pathway designed to synthesize these compounds was based on two key steps: first, the Lythgoe-type Wittig–Horner coupling of Windaus–Grundmann-type ketone **18**, with phosphine oxide **15**, followed, later in the synthesis, by the Zn-mediated Reformasky-type allylation of aldehyde **20** with methylbromomethylacrylate **8**. Our biological data show that neither compound has antagonistic activity but acts as weak agonists in vitro and in vivo.

1. Introduction

1α,25-Dihydroxyvitamin D_3, 1α,25$(OH)_2D_3$ (**1**) (Fig. 1), regulates various biological events including calcium and phosphorus homeostasis and cell differentiation.[1,2] The biological responses of 1α,25$(OH)_2D_3$ (**1**) are mediated by a specific receptor, vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily and acts as a ligand-dependent gene transcription factor with coactivators.[3,4] 1α,25$(OH)_2D_3$ (**1**) has significant therapeutic potential in the treatment of osteoporosis, various types of rickets, secondary hyperparathyroidism, psoriasis, autoimmune diseases, and cancer. However, therapy using 1α,25$(OH)_2D_3$ (**1**) is limited because it easily causes hypercalcemia. A large number of 1α,25$(OH)_2D_3$ (**1**) analogs have been synthesized in an attempt to dissociate its various physiological activities.[5] In particular, our group has recently focused on a class of vitamin D compounds modified at the 2-carbon position of the A-ring that is highly potent and selective for actions on osteoblasts, particularly the anabolic or bone-forming actions pertinent to this cell type. Among these compounds, the most promising is 2-methylene-19-nor-20*S*-1α,25-dihydroxyvitamin D_3 (**2MD**) (Fig. 1).[6,7]

Understanding the specific interaction of ligands with the ligand-binding domain (LBD) of VDR has recently progressed, since the X-ray crystal structure of a deletion mutant of VDR complexed with the natural ligand (**1**) was solved in 2000.[8] These and subsequent studies with selective ligands have failed to reveal a structural basis for selective activity.[9,10] It is suggested that a change in the AF-2 domain (helix 12) 3D structure would likely determine whether a ligand acts as an agonist or as an antagonist. Approximately 3000 analogs of 1α,25$(OH)_2D_3$ (**1**) have been synthesized as candidate ligands for VDR and to-date, a vitamin D analog with clinically significant in vivo antagonism remains to be discovered. In the late 1970s and early 1980s, a few studies were reported showing vitamin D compounds with antagonistic activity.[11–15] To our knowledge, most of these compounds were not tested beyond the original report or upon further testing, found to act as agonists. More recently, three types of in vitro VDR antagonists have been reported.[16–18] These compounds are the 25-carboxylic esters, ZK168281 (**2**) and its analogs, the 26,23 lactones, TEI-9647 (**3**) and TEI-9648 (**4**) and its analogs and an analog with an adamantane ring in the side-chain AD47 (**5**) (Fig. 1).[16–25] The development of potent VDR antagonists is important, since these compounds may find application in the treatment of sarcoidosis or certain disease states characterized by hypersensitivity to 1α,25$(OH)_2D_3$ (**1**), as observed in patients with Paget's disease of bone.[26] Furthermore, VDR antagonists are valuable tools for the elucidation of VDR function as well as the mode of action of 1α,25$(OH)_2D_3$ (**1**). In this paper, we present the synthesis, chemical, and biological characterization of two novel 2-methylene-19-nor-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactones **GC-3** and **HLV** (Fig. 1) that, similar to TEI-9647 (**3**) and TEI-9648 (**4**), have an α-methylene-γ-butyrolactone moiety on the side chain. Surprisingly, both **GC-3** and **HLV** have no antagonistic activity but instead act as weak agonists.[27] These results are in contrast with two reports describing these exactly same compounds that came out during completion of the testing of **GC-3** and **HLV**.[25,28] Possible reasons for the discrepant results is included in the discussion of this report.

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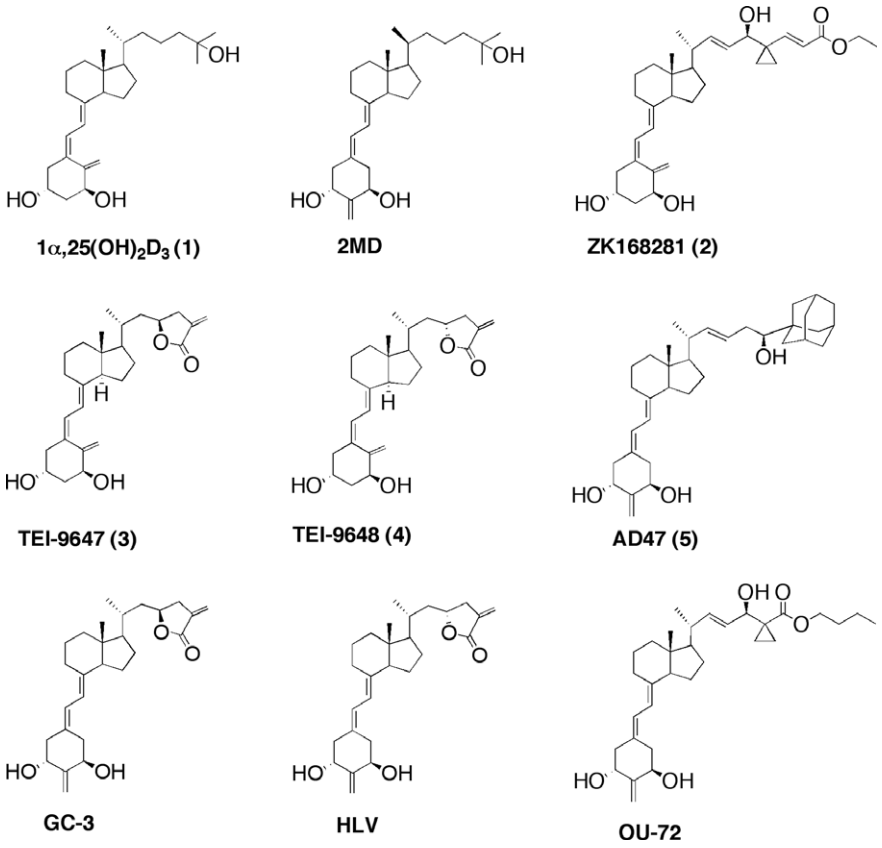


Figure 1. Chemical structures of 1α,25-$(OH)_2D_3$ (calcitriol, **1**) and its analogs.

2. Results and discussion

2.1. Chemistry

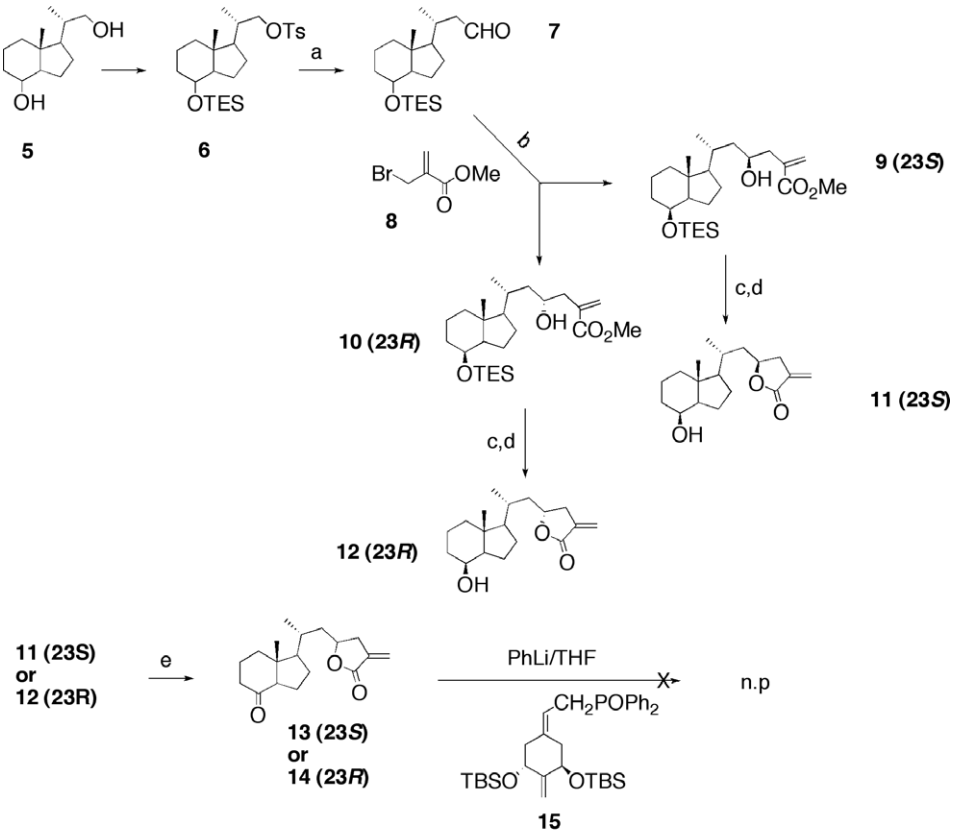
In order to synthesize the 2-methylene-19-nor-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactones, **GC-3** and **HLV**, we have taken advantage of the Lythgoe-type Wittig–Horner coupling approach,[29] which we have previously used in the preparation of other 2-substituted 19-norvitamins.[30]

Following this approach, our first attempt at making the desired protected 19-norvitamin D_3 analog, was to prepare the Windaus–Grundmann-type ketones **13** and **14** (Scheme 1), with the α-methylene-γ-lactone moiety-side chain already installed at the proper position, followed by condensation with the allylic phosphine oxide **15**, which was prepared according to a procedure already published.[30] The ketones **13** and **14** were prepared starting from the known Inhoffen–Lythgoe diol **5** (Scheme 1), obtained from commercially available vitamin D_2.[31] After protection of the diol groups, the substitution reaction of tosylate **6**[32] with potassium cyanide in DMSO followed by DIBAL-H reduction of the resulting cyano group afforded the aldehyde compound **7**, which was then employed in a zinc-mediated Reformasky-type allylation with methyl bromomethylacrylate **8** to give a mixture of two C_{23}-epimeric γ-hydroxyesters **9** and **10.** In accordance with Saito and co-workers's previous reports,[22] chromatographic separation afforded the two isolated isomers **9** and **10**. Treatment of the γ-hydroxyesters **9** and **10** with NaH in THF first, followed by deprotection of silyl groups using HF/CH_3CN, provided the CD-ring precursors **11** and **12**. The absolute configuration at the C_{23} position (based on steroidal numbering) on the side chain of **11** and **12** was determined by comparison with the reported data of ^1H NMR described by Saito and co-workers for similar CD-ring precursors having the same lactone moiety.[22] In addition, the X-ray analysis of a single-crystal of isomer **12** obtained from a mixture of ethyl acetate–hexane (Fig. 2) confirmed the stereochemistry at C_{23} as 23*R*.

Although the oxidation of the alcohols **11** or **12** with PDC afforded the desired Grundmann-type ketones **13** or **14** in good yields, the Wittig–Horner reaction of ketones **13** or **14** with the anion generated from the phosphine oxide **15** and phenyllithium did not provide the expected protected 19-norvitamin D analogs, resulting instead in the complete degradation of the α-methylene-γ-lactone ring (Scheme 1).

Thus, since the presence of the α-methylene-γ-lactone side chain in the Grundmann ketone molecule proved to be unsuitable for the Wittig–Horner olefination reaction, we envisioned an alternative synthetic pathway.[33] As shown in Scheme 2, starting from the Inhoffen–Lythgoe diol **5**, the short side-chain ketone **18** was obtained in five steps. Next, by condensing the ketone **18**, with the allylic phosphine oxide **15,** the 19-norvitamin D_3 compound **19** was obtained in good yield (76%). Then, TES removal followed by Swern oxidation[34] provided the key aldehyde **20**, which was employed in a zinc-mediated Reformasky-type allylation with methyl bromomethylacrylate **8** to give two C_{23}-epimeric γ-hydroxyesters **21** and **22** (Scheme 2). Consistent with our previous results, chromatographic separation afforded the two isolated isomers, **21** and **22**. Treatment of **21** with NaH in THF first, followed by deprotection of silyl groups using HF/CH_3CN, provided the desired (23*S*)-25-dehydro-2-methylene-19-nor-1α-hydroxyvitamin D_3-26,23-lactone **GC-3** (40%, in two steps) (Scheme 2). Similarly, the desired (23*R*) lactone **HLV** was synthesized from γ-hydroxyester **22** (66%, two steps) (Scheme 2).

The absolute configuration at the C_{23} position on the side chain was confirmed by comparison of the ^1H NMR spectra of the present



Scheme 1. Reagents and conditions: (a) 1—KCN/DMSO, 2—DIBALH/toluene, **7** (67% in two steps); (b) **8**/Zn/THF/NH_4Cl aq, **9** (33%) and **10** (48%); (c) NaH/THF; (d) HF/CH_3CN, **11** (63% in two steps) and **12** (70% in two steps); (e) PDC/PPTS/CH_2Cl_2, **13** (71%) and **14** (79%).

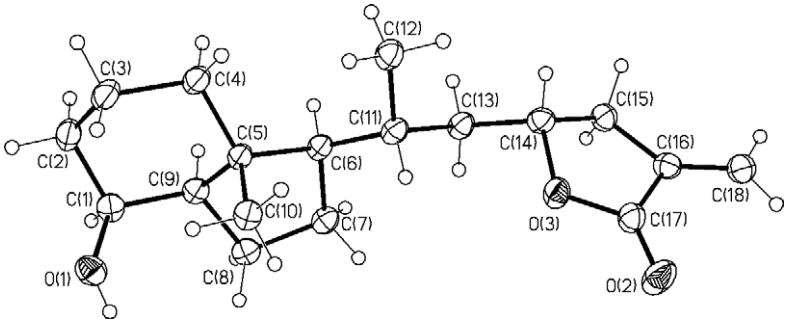


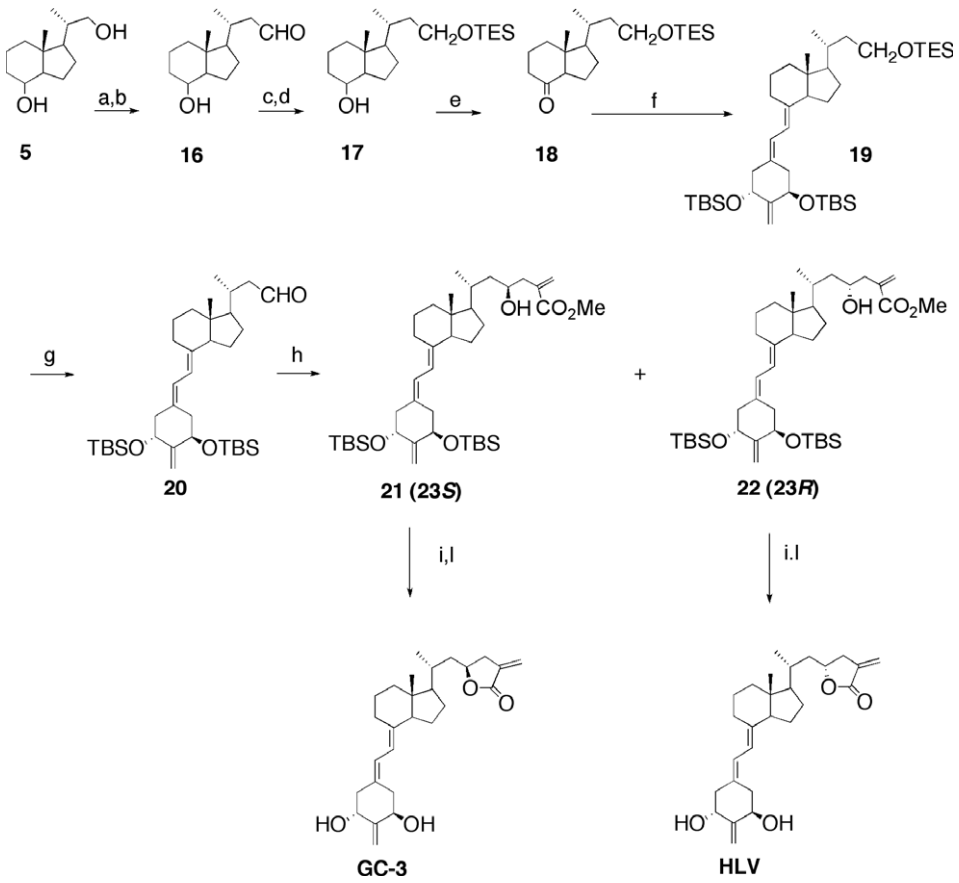
Figure 2. X-ray crystal structure of (8S,20*R*)-des-A,B-20-{[(5′*R*)-3′-methylene-dihydrofuran-2′-one-5′-yl]methyl}-pregnan-8-ol **12** (Scheme 1).

new two lactones **GC-3** and **HLV**, with those of lactones **11** and **12**, respectively (Scheme 1). In addition, the synthesis and biological properties of two lactones **LAC67a** and **LAC67b** structurally identical to our **HLV** and **GC-3** have been recently reported by a different group.[28] A perfect chemical shift overlapping of diagnostic NMR signals between **LAC67a** and **HLV** and between **LAC67b** and **GC-3** was observed, yielding further evidence of the correct assignment of the stereochemistry at C_{23} for compounds **HLV** and **GC-3**.

2.2. Biology

Both **GC-3** and **HLV** compete for binding to the rat VDR, but are approximately one and a half logs less potent compared to the native hormone as shown in Figure 3. In cell differentiation assays and in vitro transcription assays, both **GC-3** and **HLV** show lower efficacy and potency compared to the native hormone (Figs. 4 and 5).

Because of the reports that closely related compounds, TEI-9647 and TEI-9648, act as antagonists in vitro, we tested our compounds for antagonistic activity in vivo. Figure 6 depicts the results of the intestinal calcium transport assay. **GC-3** and **HLV** act as agonists in the intestine, albeit they are less potent than the native hormone. In combination with 1α,25-$(OH)_2D_3$ at 10 (panel A) or 30 (panel B) times excess, no diminution in the response was seen. No additive effects were seen because the combined doses result in responses outside the linear range of the assay. Figure 7 shows the bone calcium mobilization results in rats given 10 (panel A) or 30 times (panel B) excess **GC-3** or **HLV**. **GC-3** and **HLV** have little or no activity on bone and do not antagonize the activity of 1α,25-$(OH)_2D_3$ in this regard. Since no antagonistic activity of **GC-3** and **HLV** could be detected in vivo, we attempted to determine if they possessed any in vitro antagonistic activity. In the in vitro reporter cell assay, significant plate-to-plate variation was noticed. When comparisons were made on the same plate, clearly no antagonism was found.



Scheme 2. Reagents and conditions: (a) TsCl/py, (b) 1—KCN/DMSO, 2—DIBALH/toluene, **16** (50% in three steps); (c) $NaBH_4$/EtOH; (d) $TESCl/CH_2Cl_2/NEt_3$, **17** (71% in two steps); (e) $PDC/PPTS/CH_2Cl_2$, **18** (76%); (f) **15**/PhLi/THF, **19** (76%); (g) 1—AcOH/THF/H_2O, 2—$C_2O_2Cl_2$/DMSO/CH_2Cl_2, **20** (63% in two steps); (h) **8**/Zn/THF/NH_4Cl aq, **21** (13%) and **22** (17%); (i) NaH/THF; (l) HF/CH_3CN, **GC-3** (40% in two steps), **HLV** (66% in two steps).

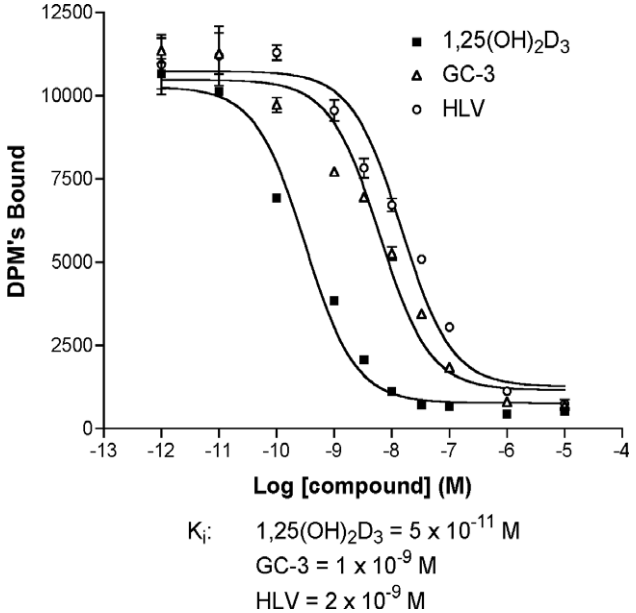


Figure 3. Competitive binding of $1\alpha,25\text{-}(OH)_2D_3$ (**1**) and the synthesized 2-methylene-19-norvitamin D lactone analogs **GC-3** and **HLV** to the full-length recombinant rat vitamin D receptor.

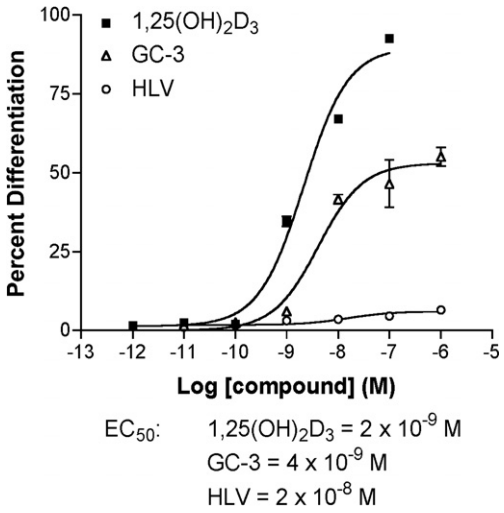


Figure 4. Induction of differentiation of HL-60 promyelocytes to monocytes by $1\alpha,25\text{-}(OH)_2D_3$ (**1**) and the synthesized 2-methylene-19-norvitamin D lactone analogs **GC-3** and **HLV**.

However, if data from one plate are compared to data gathered from a different plate, incorrect conclusions are possible. Figure 8 shows three graphs, each graph representing one 96-well plate. These data clearly indicate that there is no antagonism present, but there is weak agonist activity noted at 10^{-6} M and 10^{-7} M.

Studies were carried out with a different agonist, **2MD** (Fig. 1), which is more structurally similar to **GC-3** and **HLV**. **2MD** is nearly 2 logs more potent than the native hormone, therefore permitting a much larger excess of putative antagonist to be used (Fig. 9). Once again, only weak agonist activity with no evidence of

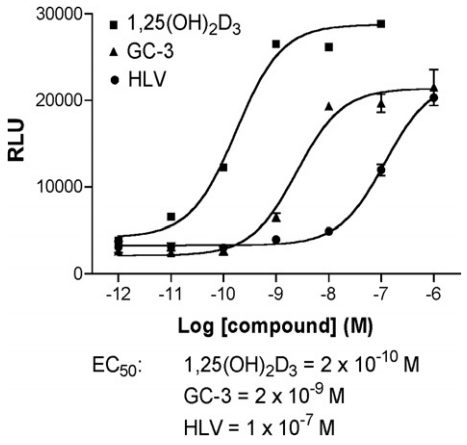


Figure 5. Dose–response effects of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized 2-methylene-19-norvitamin D lactone analogs **GC-3** and **HLV** on transcriptional activity.

antagonist function was observed. Another 2-methylene 19-nor analog (**OU-72**) (Fig. 1) structurally similar to the previously reported 25-carboxylic esters clearly shows antagonistic activity in this reporter cell assay[35] providing evidence that this cell system is suitable for screening for antagonistic function of vitamin D ligands (Fig. 10).

More recent publications[36,37] indicate that 1α,25-$(OH)_2D_3$ analogs containing 26,23-lactones only act as antagonists in human cells, not in rat cells. Since ROS cells are rat osteosarcoma cells, **GC-3** and **HLV** were tested for antagonistic activity in the human cell line HL-60. **GC-3** and **HLV** were unable to antagonize 1α,25-$(OH)_2D_3$-mediated cell differentiation (Fig. 11). This assay was repeated for four separate times with identical results.

Based on the experiments conducted in this laboratory and presented in this report, **GC-3** and **HLV** are weak agonists with no ability to function as antagonists. This was shown to be true regardless of the cell type (human promyelocytic leukemia cells or rat bone cells), regardless of assay endpoint (differentiation or transcription), regardless of the agonist (1α,25-$(OH)_2D_3$ or 2MD), and regardless of whether the testing was done in vitro or in vivo. These results are somewhat surprising given that these two compounds differ in only the A-ring when compared to the previously reported antagonists **TEI-9647** and **9648**.

Furthermore, during completion of the biological testing and the preparation of this manuscript two reports became available describing the in vitro biological activity of the same compounds presented in this manuscript[25,28]: **LAC67b (GC-3)** and **LAC67a (HLV)**. In the second of these two reports, the investigators stated that these compounds acted as antagonists in Cos7 and HEK293 cells. Interestingly, the 20*S* derivatives functioned only as antagonists in the monkey kidney cells, not in the human cells. Upon careful scrutiny of the data presented in that report, it seems likely that the antagonism described is because of assay variation. In Figure 4 of that report, panel A has a range of 0–3000, panel B 0–50 and panel C 0–1.2. Additional assay variation is highlighted in Figure 3C. The second bar in this graph represents the response when cells were given 10^{-8} M 1α,25-$(OH)_2D_3$. The third and fourth bars show the cellular activity at 10^{-10} and 10^{-9} M 1α,25-$(OH)_2D_3$, and the response is higher than that shown for the cells given 10^{-8} M. Thus, considerable variation is noted in that report. It is also possible that the discrepancy between the results shown in this report versus those presented by that group may be because of differing cell lines used.[28] However, the first report using **LAC67b** and **LAC67a** were tested for antagonistic activity in a large number of different cell lines, and antagonism was observed in all six cell lines. It is unclear of how many independent assays were conducted. Interestingly, **LAC67b** shows antagonism in HEK293 cells when the effect on endogenous CYP24A1 gene expression is the endpoint (Fig. 8 of the Inaba et al. report), but not when an exogenous reporter is used (Fig. 2D of the Inaba et al. report).

3. Conclusions

The results of studies presented in this report indicate that both **GC-3** (**LAC67b**) and **HLV** (**LAC67a**) act as weak agonists in rat bone cells or in human promyelocytic cells. These two compounds also

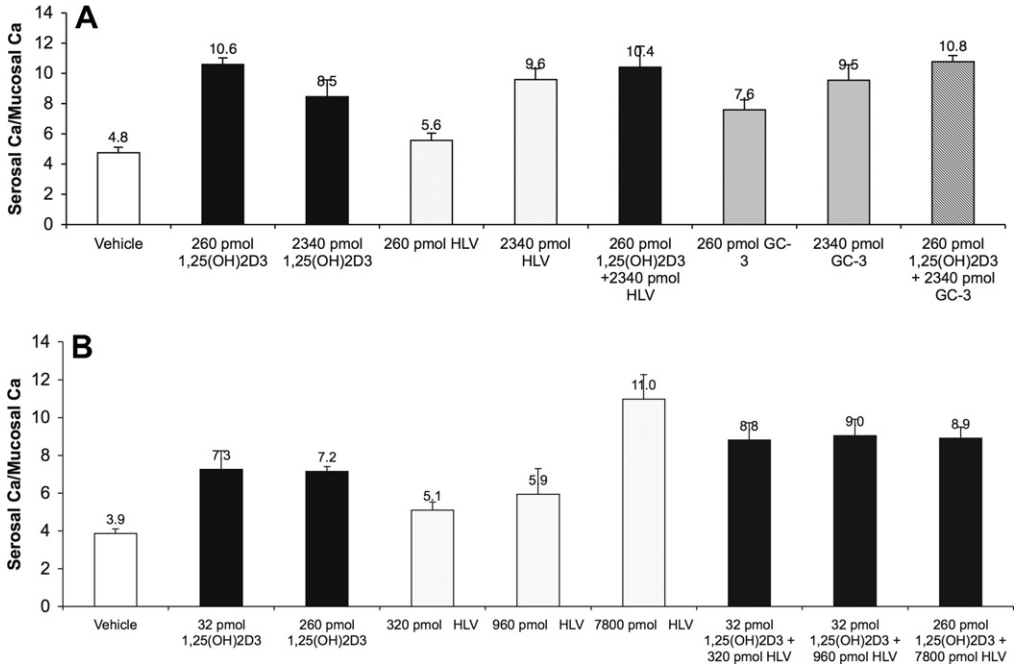


Figure 6. Intestinal calcium transport of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized 2-methylene-19-norvitamin D lactone analogs **GC-3** and **HLV**. Rats were made vitamin D-deficient and placed on a diet nearly devoid of calcium. Duodena were collected 24 h following the last of four consecutive daily ip doses and calcium transport measured. (A) Results of one experiment conducted using the analogs at 10× excess the native hormone. (B) Done using **HLV** at 30× excess the native hormone.

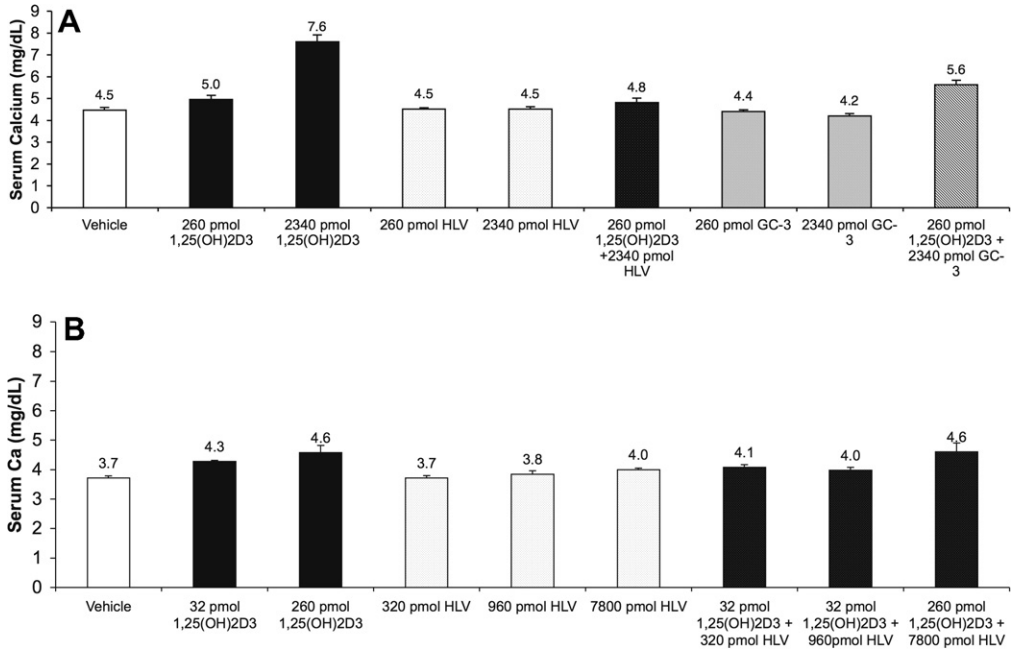


Figure 7. Bone calcium mobilization of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized 2-methylene-19-norvitamin D lactone analogs **GC-3** and **HLV**. Rats were made vitamin D-deficient and placed on a diet nearly devoid of calcium. Blood was collected 24 h following the last of four consecutive daily ip doses, and total serum calcium levels were determined. (A) Results of one experiment conducted using the analogs at 10× excess the native hormone. (B) Done using **HLV** at 30× excess the native hormone.

function as weak agonists in vivo, at least in the bone and intestine. Whether or not these compounds might function as antagonists in other cell types or on endogenous gene expression either in vitro and/or in vivo requires further investigation to help resolve the disparate results present in multiple reports.

4. Experimental

4.1. Chemical procedures

Optical rotations were measured in chloroform using a Perkin-Elmer Model 343 polarimeter at 22 °C. Ultraviolet (UV) absorption spectra were recorded with a Perkin-Elmer Lambda 3B UV–VIS spectrophotometer. ^1H and ^{13}C NMR spectra were recorded in deuterochloroform on Bruker Instruments DMX-400 Avance console, on Bruker Instruments DMX-500 Avance console, and on Varian Unity Inova spectrometers 600, 800, and 900 MHz, equipped with a cryogenic probe. Chemical shifts (δ) in parts per million are quoted relative to internal Me_4Si (δ 0.00). Electron impact (EI) MS were obtained with a Micromass AutoSpec (Beverly, MA) instrument. High-performance liquid chromatography (HPLC) was performed on a Waters Associates liquid chromatograph equipped with a Model 6000A solvent delivery system, Model U6K Universal injector, and a Model 486 tunable absorbance detector. All reactions were monitored by thin-layer chromatography using 0.2 mm E. Merck silica gel 60 F_{254} plates. Merck silica gel (230–400 mesh) was used for column chromatography. THF was freshly distilled before use from sodium benzophenone ketyl under argon.

4.1.1. Synthesis of (8*S*,20*R*)-des-A,B-8-[(triethylsilyl)oxy]-20-(formylmethyl)-pregnane (7)

To a solution of tosylate **6**[32] (300 mg, 0.62 mmol) in DMSO (2 mL) was added KCN (81 mg, 1.25 mmol), and the mixture was stirred at 70 °C for 1.5 h. The mixture was diluted with Et_2O, and the organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was dissolved in CH_2Cl_2 (3 mL). To the mixture was added a solution of DIBAL-H in toluene (1 M, 0.62 mL, 0.62 mmol) at 0 °C. The reaction mixture was stirred at the same temperature for 1.5 h, then it was quenched with 10% potassium sodium tartrate aq solution, and the aqueous layer was extracted with Et_2O. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (3% EtOAc/hexane) to give **7** (140 mg, 0.42 mmol, 67% in two steps). α_D^{20} +18 (*c* 1.41, $CHCl_3$). ^1H NMR ($CDCl_3$, 400 MHz) δ 9.75 (d, *J* = 2.4 Hz, 1H), 4.03 (s, 1H), 2.45 (dm, *J* = 15.7 Hz, 1H), 2.15 (m, 1H), 0.99 (d, *J* = 6.5 Hz, 3H), 0.95 (t, *J* = 7.9 Hz, 9H) 0.55 (q, *J* = 7.9 Hz, 6H) 0.52 (s, 3H). ^{13}C NMR ($CDCl_3$, 400 MHz) δ 203.46, 69.22, 56.52, 53.03, 50.77, 42.26, 40.60, 34.50, 31.25, 27.55, 22.90, 19.91, 17.59, 13.50, 6.92, 4.90; exact mass calculated for $C_{18}H_{34}O_2Si[M-C_2H_5]^+$ 309.2250, found 309.2237.

4.1.2. Synthesis of (8*S*,20*R*)-des-A,B-8-[(triethylsilyl)oxy]-20-[(2′*S*)-hydroxy-4′-methoxycarbonyl-4′-penten-1′-yl]-pregnane (9); (8*S*,20*R*)-des-A,B-8-[(triethylsilyl)oxy]-20-[(2′*R*)-hydroxy-4′-methoxycarbonyl-4′-penten-1′-yl]-pregnane (10)

To a mixture of **7** (140 mg, 0.42 mmol) in saturated aq NH_4Cl solution and THF (5:1, 6 mL) were added methylbromomethylacrylate **8** (106 μL, 0.86 mmol) and activated Zn dust (113 mg, 1.7 mmol) at 0 °C. The mixture was stirred at the same temperature for 1.5 h, then it was diluted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (5% EtOAc/hexane) to give **9** (62 mg, 0.14 mmol, 33%) and **10** (86 mg, 0.20 mmol, 48%) as colorless oils, respectively. **9**, ^1H NMR ($CDCl_3$, 400 MHz) δ 6.26 (s, 1H), 5.68 (s, 1H), 4.02 (s, 1H), 3.84 (m, 1H), 3.77 (s, 3H), 2.68 (dd, *J* = 14.0, 1.8 Hz, 1H), 2.17 (dd, *J* = 14.0, 8.9 Hz, 1H), 1.96 (dm, *J* = 13.0 Hz, 1H), 0.97 (d, *J* = 6.1 Hz, 3H), 0.94 (t, *J* = 7.9 Hz, 9H), 0.92 (s, 3H), 0.54 (q, *J* = 7.9 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 168.11, 137.59, 127.84, 69.37, 69.22, 57.53, 53.03, 52.05, 43.77, 42.21, 40.79, 39.94, 34.60, 33.30, 27.54, 22.98, 19.08, 17.66, 13.51, 6.93, 4.93; exact mass calculated for $C_{25}H_{46}O_4Si$ $[M]^+$ 438.3165, found 438.3163. **10**, ^1H NMR ($CDCl_3$, 400 MHz) δ 6.25 (s, 1H), 5.67 (s, 1H), 4.02 (s, 1H), 3.84 (m, 1H), 3.76 (s, 3H), 2.50 (dd, *J* = 14.0, 3.6 Hz, 1H), 2.36 (dd, *J* = 14.0, 8.2 Hz, 1H), 1.98 (dm, *J* = 13.0 Hz, 1H), 0.93 (t, *J* = 7.9 Hz, 9H), 0.93 (d, 3H), 0.92 (s, 3H),

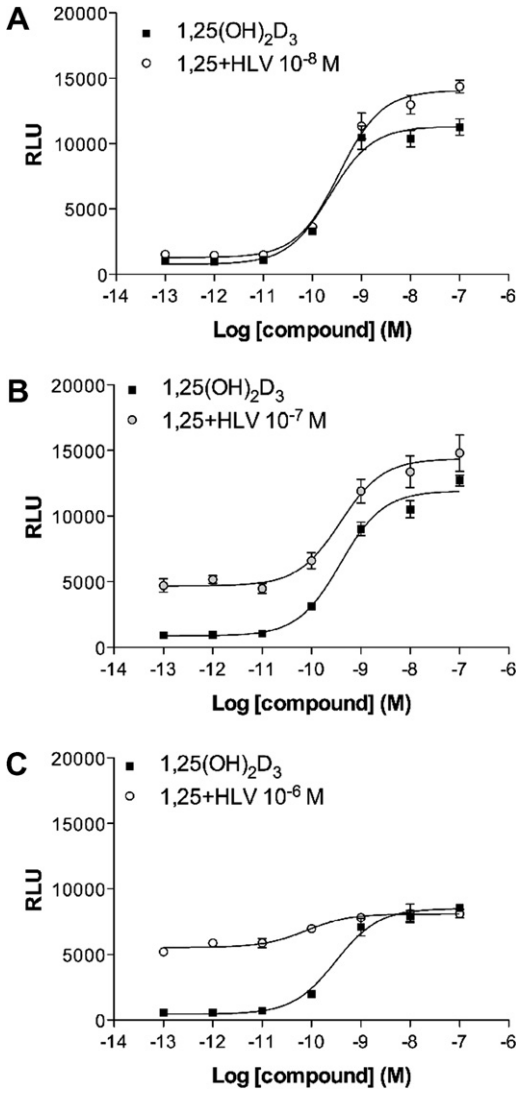


Figure 8. Transcriptional activity of 1α,25-$(OH)_2D_3$ (**1**) and **HLV**. In this experiment, each plate of cells had a dose–response curve generated with 1α,25$(OH)_2D_3$ alone to compare directly with those cells given both native hormone and the putative antagonist at 10^{-8} M (A), 10^{-7} M (B), or 10^{-6} M (C).

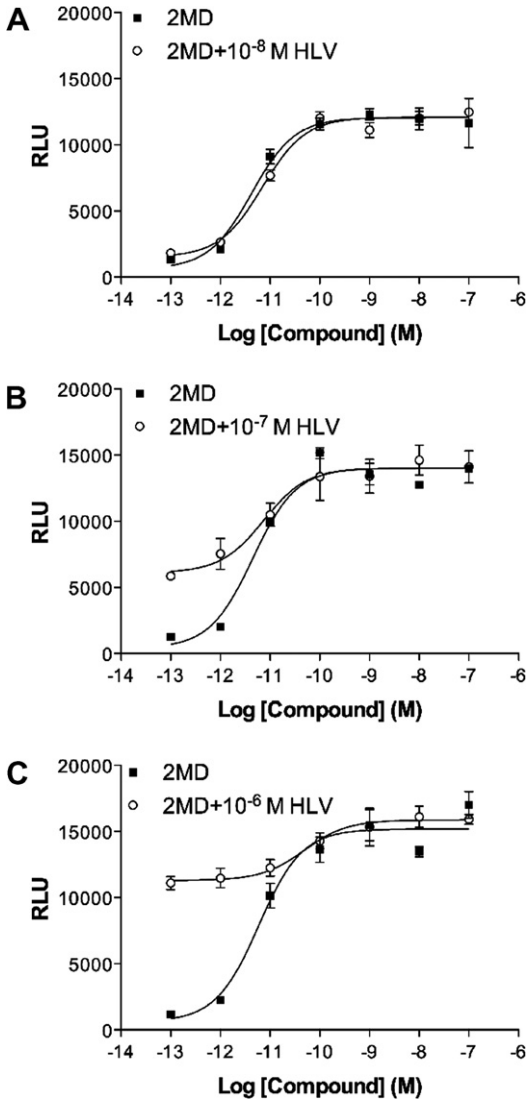


Figure 9. Transcriptional activity of 2-methylene-19-nor-(20S)-1α,25$(OH)_2D_3$ (**2MD**) and the synthesized 2-methylene-19-norvitamin D lactone analog **HLV**. In this experiment, each plate of cells had a dose–response curve generated with **2MD** alone to compare directly with those cells given both **2MD** and the putative antagonist at 10^{-8} M (A), 10^{-7} M (B) or 10^{6} M (C).

0.54 (q, J = 7.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 168.31, 137.58, 127.86, 69.38, 67.64, 57.43, 53.13, 52.00, 43.65, 42.27, 41.49, 40.84, 34.63, 32.05, 27.46, 23.00, 18.50, 17.67, 13.55, 6.91, 4.92; exact mass calculated for $C_{25}H_{46}O_4Si$ $[M]^+$ 438.3165, found 438.3162.

4.1.3. Synthesis of (8*S*,20*R*)-des-A,B-20-{[(5′*S*)-3′-methylene-dihydrofuran-2′-one-5′-yl]methyl}-pregnan-8-ol (11)

To a suspension of NaH (60% oil dispersion, 40 mg, 1.0 mmol) was added a solution of **9** (62 mg, 0.14 mmol) in THF (10 mL) at 0 °C. The reaction mixture was stirred at the same temperature for 30 min, then it was quenched with saturated aq NH_4Cl solution, and the aqueous layer was extracted with Et_2O. Combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (5% EtOAc/hexane) to give the protected lactone (54 mg, 0.13 mmol, 93%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.21 (br s, 1H), 5.61 (br s, 1H), 4.58 (m, 1H) 4.02 (s, 1H), 3.04 (dddd, J = 16.9, 7.4, 2.3, 2.3 Hz, 1H), 2.54 (dddd, J = 16.9, 6.4, 2.9, 2.9 Hz, 1H), 1.95 (dm, J = 12.5 Hz, 1H), 0.98 (d, J = 6.5 Hz, 3H), 0.94 (t, J = 7.9 Hz, 9H), 0.92 (s, 3H), 0.55 (d, J = 7.9 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 170.25, 134.79, 121.83, 76.75, 69.29, 56.82, 52.98, 42.21, 42.12, 40.69, 34.52, 33.97, 32.77, 27.65, 22.90, 19.00, 17.61, 13.46, 6.92, 4.90; exact mass calculated for $C_{22}H_{37}O_3Si$ $[M-CH_2CH_3]^+$ 377.2512, found 377.2518.

To a solution of the protected lactone (49 mg, 0.12 mmol) in MeCN (5 mL) was added a mixture of HF/MeCN (1:9, 1 mL) at 0 °C. The reaction mixture was stirred at the same temperature for 30 min, then it was quenched with saturated aq $NaHCO_3$ solution, and the aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (10% EtOAc/hexane) to give **11** (24 mg, 82 μmol, 68%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.21 (br s, 1H), 5.61 (br s, 1H), 4.58 (m, 1H), 4.08 (s, 1H), 3.05 (dddd, J = 16.9, 7.4, 2.3, 2.3 Hz, 1H), 2.54 (dddd, J = 16.9, 6.5, 2.9, 2.9 Hz, 1H), 1.98 (dm, J = 12.9 Hz, 1H), 1.00 (d, J = 6.5 Hz, 3H), 0.94 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 170.38, 134.67, 121.88, 76.58, 69.29, 56.62, 52.46, 42.11, 41.92, 40.30, 34.00, 33.54, 32.84, 27.50, 22.43, 18.92, 17.35, 13.44; exact mass calculated for $C_{18}H_{28}O_3$ $[M]^+$ 292.4174, found 292.4179.

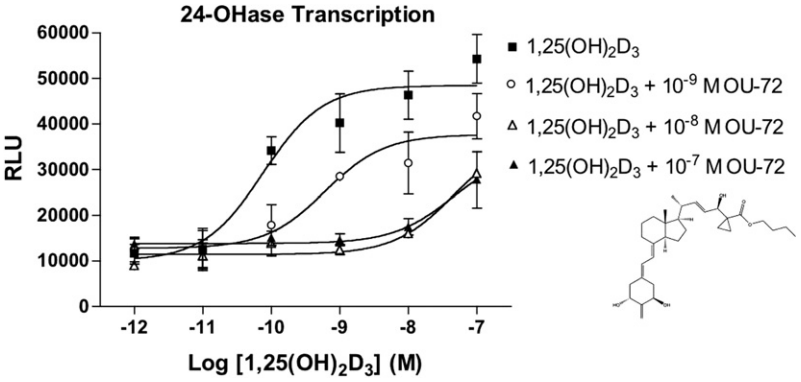


Figure 10. Antagonism of $1,25(OH)_2D_3$-induced transcription in ROS reporter cells. Another 2-methylene 19-nor analog similar to the previously described carboxylic ester antagonists was prepared and given in combination with the native hormone to rat osteosarcoma cells stably transfected with a luciferase reporter construct containing three repeats of the 24-OHase VDRE upstream. Three independent assays were conducted by three separate individuals resulting in similar findings.

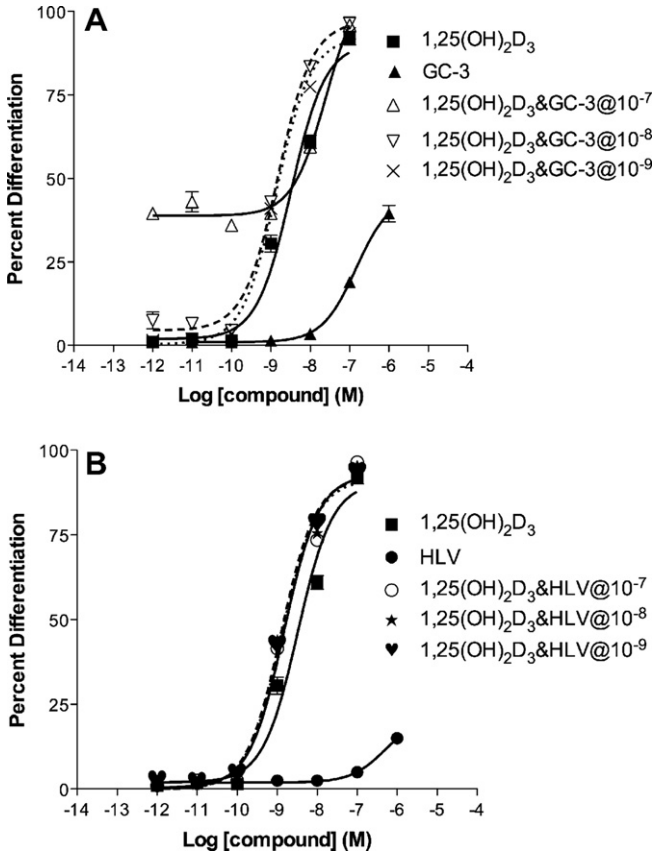


Figure 11. Testing for **GC-3** or **HLV** antagonism in human promyelocytic leukemia cells.

4.1.4. Synthesis of (8*S*,20*R*)-des-A,B-20-{[(5′*R*)-3′-methylene-dihydrofuran-2′-one-5′-yl]methyl}-pregnan-8-ol (12)

Compound **12** was obtained from **10** by the same procedure as described for **11** (yield 70%, in two steps) as a colorless oil.

12 ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.22 (br s, 1H), 5.62 (br s, 1H), 4.64 (m, 1H), 4.07 (s, 1H), 3.06 (dddd, J = 16.9, 7.5, 2.4, 2.4 Hz, 1H), 2.52 (dddd, J = 16.9, 6.3, 3.0, 3.0 Hz, 1H), 2.01 (dm, J = 12.9 Hz, 1H), 0.99 (d, J = 6.5 Hz, 3H), 0.95 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 170.44, 134.81, 121.92, 75.15, 69.28, 56.99, 52.60, 43.33, 42.03, 40.40, 34.50, 33.52, 32.26, 27.18, 22.47, 18.35, 17.39, 13.56; exact mass calculated for $C_{18}H_{28}O_3$ $[M]^+$ 292.4174, found 292.4168.

Crystallization of **12** in a mixture of ethyl acetate–hexane (2:1) gave white crystals, whose structure was determined by a single-crystal X-ray analysis (Fig. 2).[38]

4.1.5. Synthesis of (20*R*)-des-A,B-20-{[(5′*S*)-3′-methylene-dihydrofuran-2′-one-5′-yl]methyl}-pregnan-8-one (13)

To a solution of alcohol **11** (20 mg, 0.07 mmol) in CH_2Cl_2 (5 mL) were added PPTS (5 mg, 0.01 mmol) and PDC (0.128 g, 0.34 mmol). After being stirred for 6 h at room temperature, the mixture was passed through 2 cm of flash silica gel pad and washed with EtOAc. The filtrate was concentrated and purified by column chromatography (20% EtOAc/hexane) to give desired ketone **13** (15 mg, 0.05 mmol, 71%) as a colorless oil. For analytical purpose, a sample of ketone **13** was further purified by HPLC (Zorbax Silica 250/9.4 mm, 30% EtOAc/hexane, 4 mL/min, Rv = 14.70 mL). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.23 (br s, 1H), 5.64 (br s, 1H), 4.60 (m, 1H), 3.06 (m, 1H), 2.54 (m, 1H), 2.49 (dd, J = 11.7, 7.5 Hz, 1H), 1.08 (d, J = 6.1 Hz, 3H), 0.66 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 211.71, 170.29, 134.59, 122.11, 74.67, 61.79, 56.75, 49.81, 43.28, 40.63, 38.97, 34.44, 32.29, 27.43,24.01, 19.13, 18.64, 12.44; exact mass calculated for $C_{18}H_{26}O_3$ $[M]^+$ 290.1882, found 290.1888.

4.1.6. Synthesis of (20*R*)-des-A,B-20-{[(5′*R*)-3′-methylene-dihydrofuran-2′-one-5′-yl]methyl}-pregnan-8-one (14)

Compound **14** was obtained from **12** by the same procedure described for **13** (yield 79%) as a colorless oil. For analytical purpose, a sample of ketone **14** was further purified by HPLC (Zorbax Silica 250/9.4 mm, 30% EtOAc/hexane, 4 mL/min, Rv = 14.59 mL). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.23 (br s, 1H), 5.63 (br s, 1H), 4.64 (m, 1H), 3.07 (m, 1H), 2.53 (m, 1H), 2.44 (dd, J = 11.6, 7.5 Hz, 1H), 1.05 (d, J = 6.5 Hz, 3H), 0.66 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 211.68, 170.29, 134.61, 122.11, 74.91, 61.89, 56.94, 49.91, 43.22, 40.89, 38.97, 34.44, 32.38, 27.41, 23.95, 19.08, 18.55, 12.58; exact mass calculated for $C_{18}H_{26}O_3$ $[M]^+$ 290.1882, found 290.1890.

The following experimental procedure, applied for obtaining the desired final products **GC-3** and **HLV**, was previously reported by us in a US Patent.[33]

4.1.7. Synthesis of (8*S*,20*R*)-des-A,B-20-(formylmethyl)-pregnan-8-ol (16)

A solution of diol **5** (500 mg, 2.35 mmol) in anhydrous pyridine (5 mL) was cooled to −25 °C. A precooled solution of tosyl chloride (553 mg, 2.9 mmol) in anhydrous pyridine (1 mL) was added dropwise to the diol solution via cannula. Upon stirring for 3.5 h at −25 °C, the reaction was warmed up to 0 °C and allowed to stir for an additional 20 h. The mixture was extracted with CH_2Cl_2, washed with saturated $CuSO_4$ aq solution, dried over $MgSO_4$, fil-

tered, and concentrated. The residue was purified by column chromatography (20% EtOAc/hexane) to afford 600 mg (1.64 mmol) of the corresponding tosylate in 70% yield. To a solution of that tosylate (300 mg, 0.82 mmol) in DMSO (2 mL) was added KCN (106 mg, 1.64 mmol), and the mixture was stirred at 70 °C for 1.5 h. The reaction mixture was diluted with Et_2O and the organic layer was washed with H_2O and brine, dried over $MgSO_4$ and concentrated. The residue was dissolved in CH_2Cl_2 (3 mL). To the solution was added a solution of DIBAL-H in toluene (1 M, 0.9 mL, 0.90 mmol) at 0 °C. The reaction mixture was stirred at the same temperature for 1.5 h, then it was quenched with 10% potassium sodium tartrate aq solution, and the aqueous layer was extracted with Et_2O. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (3% EtOAc/hexane) to give **16** (133 mg, 0.59 mmol, 72% in two steps). α_D^{20} +19 (*c* 1.21, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.75 (d, *J* = 2.4 Hz, 1H), 4.08 (s, 1H), 2.45 (dm, *J* = 15.7 Hz, 1H), 2.15 (m, 1H), 1.00 (d, *J* = 6.6 Hz, 3H), 0.98 (s, 3H). ^{13}C NMR ($CDCl_3$, 400 MHz) δ 203.46, 69.17, 56.34, 52.54, 50.68, 41.99, 40.22, 33.54, 31.22, 27.40, 22.44, 19.85, 17.34, 13.50; exact mass calculated for $C_{14}H_{24}O_2$ $[M]^+$ 224.1776, found 224.1771.

4.1.8. Synthesis of (8*S*,20*R*)-des-A,B-20-{2′-[(triethylsilyl)oxy]-ethyl}-pregnan-8-ol (17)

To a solution of **16** (100 mg, 0.44 mmol) in anhydrous EtOH (10 mL) was added $NaBH_4$ (85 mg, 2.22 mmol) at 0 °C, and the mixture was stirred at the same temperature for 1.5 h. The reaction mixture was diluted with EtOAc, washed with water, 1 N aq HCl solution, saturated aq $NaHCO_3$ solution, brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (10% EtOAc/hexane) to give the desired diol (75 mg, 0.33 mmol, 75%). ^{1}H NMR ($CDCl_3$, 800 MHz) δ 4.06 (d, *J* = 2.4 Hz, 1H), 3.69 (ddd, *J* = 10.4, 8.8, 4.8 Hz 1H), 3.62 (ddd, *J* = 10.4, 7.2, 7.2 Hz, 1H), 1.98 (dm, *J* = 12.8 Hz, 1H), 0.92 (d, *J* = 6.6 Hz, 3H), 0.91 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 69.55, 61.05, 57.11, 52.82, 42.16, 40.61, 39.00, 33.78, 32.67, 27.49, 22.73, 18.93, 17.64, 13.70; exact mass calculated for $C_{14}H_{26}O_2$ $[M]^+$ 226.1933, found 226.1945.

To a solution of diol (50 mg, 0.22 mmol) in anhydrous CH_2Cl_2 (5 mL) at 0 °C was added triethylamine (95 μL, 0.67 mmol) followed by triethylsilylchloride (40 μL, 0.22 mmol). The solution was stirred at 0 °C for 30 min and then it was quenched with water. The mixture was extracted with CH_2Cl_2, and combined organic phases were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography (30% EtOAc/hexane) to afford the *O*-silylated compound **17** (68 mg, 0.21 mmol, 95%). α_D^{20} +31 (*c* 1.45, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.08 (s, 1H), 3.68 (m, 1H), 3.59 (m, 1H), 1.99 (m, 1H), 0.97 (t, *J* = 7.9 Hz, 9H), 0.96 (d, 3H), 0.95 (s, 3H), 0.60 (q, *J* = 7.9 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 69.45, 60.95, 56.84, 52.60, 41.89, 40.35, 38.86, 33.53, 32.47, 27.19, 22.52, 18.91, 17.41, 13.41, 6.56, 5.78; exact mass calculated for $C_{20}H_{40}O_2Si$ $[M]^+$ 340.2798, found 340.2803.

4.1.9. Synthesis of (20*R*)-des-A,B-20-{2′-[(triethylsilyl)oxy]-ethyl}-pregnan-8-one (18)

To a solution of alcohol **17** (41 mg, 127 μmol) in CH_2Cl_2 (10 mL) were added PPTS (10 mg, 0.02 mmol) and PDC (231 mg, 0.60 mmol) at room temperature. After being stirred for 6 h, the mixture was passed through 2 cm of flash silica gel pad and washed with EtOAc. The filtrate was concentrated and purified by column chromatography (20% EtOAc/hexane) to give desired ketone **18** (31 mg, 96 μmol, 76%) as a colorless oil. For analytical purpose, a sample of ketone **18** was further purified by HPLC (Zorbax Silica 250/9.4 mm, 10% EtOAc/hexane, 5 mL/min, Rv = 23 mL). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.67 (m, 1H), 3.59 (m, 1H), 2.43 (dd, *J* = 12.0, 7.2 Hz, 1H), 0.95 (d, *J* = 6.6 Hz, 3H), 0.94 (t, *J* = 7.8 Hz, 9H), 0.62 (s, 3H), 0.57 (q, *J* = 7.8 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 212.09, 61.99, 60.72, 56.89, 49.94, 40.97, 38.95, 38.79, 32.64, 27.51, 24.04, 19.08, 19.03, 12.41, 6.80, 4.42; exact mass calculated for $C_{18}H_{33}O_2Si$ $[M-C_2H_5]^+$ 309.2250, found 309.2252.

4.1.10. Synthesis of (1*R*,3*R*,7*E*,20*R*)-1,3-di-[(*tert*-butyldimethylsilyl)oxy)]-2-methylene-20-{2′-[(triethylsilyl)oxy]-ethyl}-9,10-seco-19-nor-pregnan-5,7-diene (19)

To a stirred solution of phosphine oxide **15** (89 mg, 0.15 mmol) in anhydrous THF (600 μL) at −20 °C was slowly added phenyllithium (1.5 M in hexane, 130 μL, 0.15 mmol) under argon. The solution turned deep orange. The mixture was cooled to −78 °C, and a precooled (−78 °C) solution of ketone **18** (29 mg, 90 μmol) in anhydrous THF (400 μL) was slowly added. The mixture was stirred under argon at −78 °C for 1 h and at 0 °C for 18 h. Ethyl acetate was added, and the organic phase was washed with brine, dried ($MgSO_4$), and evaporated. The residue was dissolved in hexane, applied on a silica Sep-Pack cartridge, and washed with 0.5% EtOAc/hexane to give the 19-norvitamin derivative **19** (48 mg, 76%). The Sep-Pack was then washed with ethyl acetate to recover diphenylphosphine oxide (20 mg). ^{1}H NMR ($CDCl_3$, 900 MHz) δ 6.21 (d, *J* = 11.2 Hz, 1H), 5.83 (d, *J* = 11.2 Hz, 1H), 4.97 (s, 1H), 4.92 (s, 1H), 4.42 (m, 2H), 3.68 (ddd, *J* = 10.4, 8.1, 4.5 Hz, 1H), 3.59 (ddd, *J* = 10.4, 7.2, 7.2 Hz, 1H), 2.82 (br d, *J* = 12.6 Hz, 1H), 2.51 (dd, *J* = 13.5, 5.4 Hz, 1H), 2.46 (dd, *J* = 12.6, 4.5 Hz, 1H), 2.33 (dd, *J* = 13.5, 3.6 Hz, 1H), 2.18 (dd, *J* = 11.7, 8.1 Hz, 1H), 0.96 (t, *J* = 8.1 Hz, 9H), 0.94 (d, *J* = 6.3 Hz, 3H), 0.89 (s, 9H), 0.86 (s, 9H), 0.60 (q, *J* = 8.1 Hz, 6H), 0.54 (s, 3H), 0.08, 0.06, 0.04, and 0.02 (each s, each 3H); ^{13}C NMR ($CDCl_3$) δ 153.22, 141.37, 132.98, 122.63, 116.37, 106.48, 72.73, 71.88, 61.24, 56.96, 56.52, 47.83, 45.95, 40.80, 39.27, 38.80, 33.50, 28.97, 27.93, 26.06, 26.00, 23.65, 22.47, 19.47, 18.47, 18.39, 12.22, 7.03, 5.00, −4.63, −4.68, −4.87; exact mass calculated for $C_{41}H_{78}O_3Si_3$ $[M]^+$ 702.5259, found 702.5286.

4.1.11. Synthesis of (1R,3*R*,7*E*,20*R*)-1,3-di-[(*tert*-butyldimethylsilyl)oxy)]-2-methylene-20-(formylmethyl)-9,10-seco-19-nor-pregnan-5,7-diene (20)

To a solution of **19** (48 mg, 68 μmol) in benzene (2 mL) was added a mixture of AcOH/THF/H_2O (8:8:1, 8 mL) at 0 °C. The reaction mixture was stirred at the same temperature for 3 h, then it was quenched with saturated aq $NaHCO_3$ solution, and the aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (5% EtOAc/hexane) to give the primary alcohol (32 mg, 54 μmol, 79%). α_D^{20} +29 (*c* 1.11, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 800 MHz) δ 6.19 (d, *J* = 11.2 Hz, 1H), 5.82 (d, *J* = 11.2 Hz, 1H), 4.95 (s, 1H), 4.90 (s, 1H), 4.40 (m, 2H), 3.71 (m, 1H), 3.63 (m, 1H), 2.82 (br d, *J* = 12.8 Hz, 1H), 2.50 (dd, *J* = 13.6, 6.4 Hz, 1H), 2.44 (dd, *J* = 12.8, 4.0 Hz, 1H), 2.30 (dm, *J* = 10.4 Hz, 1H), 2.16 (dd, *J* = 12.8, 8.8 Hz, 1H), 0.95 (d, *J* = 7.2 Hz, 3H), 0.88 (s, 9H), 0.84 (s, 9H), 0.54 (s, 3H), 0.059, 0.044, 0.028, and 0.003 (each s, each 3H). ^{13}C NMR ($CDCl_3$) δ 141.01, 132.85, 125.51, 122.37, 116.21, 106.26, 72.54, 71.61, 60.93, 56.78, 56.27, 47.62, 45.72, 40.59, 38.95, 38.55, 33.25, 28.72, 27.81, 25.84, 25.78, 23.40, 22.22, 18.99, 18.25, 18.16, 12.04, −4.86, −4.91, −5.10; exact mass calculated for $C_{35}H_{65}O_3Si_2$ $[MH]^+$ 589.4472, found 589.4472.

To a solution of DMSO (100 μL, 1.35 mmol) in CH_2Cl_2 (5 mL) at −60 °C, oxalyl chloride (65 μL, 0.71 mmol) was added. After 2 min, a precooled (−60 °C) solution of the primary alcohol (32 mg, 54 μmol) in CH_2Cl_2 (3 mL) was added via cannula. The resulting mixture was stirred at −60 °C for 1 h, quenched with Et_3N (0.400 mL, 2.82 mmol), and warmed up to room temperature. Upon dilution with H_2O, the mixture was extracted with CH_2Cl_2. Combined organic extracts were dried ($MgSO_4$), filtered, and concentrated. The residue was purified by column chromatography

(2–5% EtOAc/hexane) to give the desired aldehyde **20** (25 mg, 43 μmol, 80%). α_D^{20} −8 (*c* 1.25, $CHCl_3$). ^1H NMR ($CDCl_3$, 600 MHz) δ 9.75 (dd, *J* = 3.6, 1.8 Hz, 1H), 6.20 (d, *J* = 11.4 Hz, 1H), 5.83 (d, *J* = 11.4 Hz, 1H), 4.96 (s, 1H), 4.91 (s, 1H), 4.41 (m, 2H), 2.81 (dd, *J* = 12.6, 4.2 Hz, 1H), 2.50 (dd, *J* = 13.2, 6.0 Hz, 1H), 2.47 (m, 2H), 2.45 (dd, *J* = 12.6, 4.8 Hz, 1H), 2.31 (dd, *J* = 13.2, 3.0 Hz, 1H), 1.02 (d, *J* = 6.6 Hz, 3H), 0.88 (s, 9H), 0.85 (s, 9H), 0.58 (s, 3H), 0.07, 0.05, 0.03, and 0.01 (each s, each 3H). ^{13}C NMR ($CDCl_3$) δ 203.58, 153.13, 140.80, 133.32, 122.50, 116.61, 106.54, 72.74, 71.81, 56.45, 51.06, 47.84, 45.94, 40.64, 38.78, 32.17, 32.04, 28.86, 28.14, 26.05, 26.00, 23.54, 22.38, 20.35, 18.47, 18.39, 12.29, −4.63, −4.89; exact mass calculated for $C_{35}H_{62}O_3Si_2$ $[M]^+$ 586.4238, found 586.4247.

4.1.12. Synthesis of (1*R*,3*R*,7*E*,20*R*)-1,3-di-[(*tert*-butyldimethylsilyl)oxy)]-2-methylene-20-[(2′*S*)-hydroxy-4′-methoxycarbonyl-4′-penten-1′-yl]-9,10-seco-19-nor-pregnan-5,7-diene (21), (1*R*,3*R*,7*E*,20*R*)-1,3-di-[(*tert*-butyldimethylsilyl)oxy)]-2-methylene-20-[(2′*R*)-hydroxy-4′-methoxycarbonyl-4′-penten-1′-yl]-9,10-seco-19-nor-pregnan-5,7-diene (22)

To a mixture of the aldehyde **20** (25 mg, 43 μmol) in saturated aq NH_4Cl solution and THF (5:1, 3 mL) were added methylbromomethylacrylate **8** (10 μL, 85 μmol) and activated Zn dust (11 mg, 0.17 mmol) at 0 °C. The reaction mixture was stirred at the same temperature for 1.5 h, then it was diluted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (5% EtOAc/hexane) to give **21** (4 mg, 5.8 μmol, 13%) and **22** (5 mg, 7.3 μmol, 17%), as colorless oils, respectively. **21**, ^1H NMR ($CDCl_3$, 400 MHz) δ 6.29 (s, 1H), 6.22 (d, *J* = 11.1 Hz, 1H), 5.84 (d, *J* = 11.1 Hz, 1H), 5.70 (s, 1H), 4.98 (s, 1H), 4.93 (s, 1H), 4.43 (m, 2H), 3.90 (m, 1H), 3.79 (s, 3H), 2.84 (br d, *J* = 12.1 Hz, 1H), 2.71 (dd, *J* = 13.8, 1.7 Hz, 1H), 2.56-2.42 (m, 2H), 2.34 (m, 1H), 1.03 (d, *J* = 6.4 Hz, 3H), 0.90 (s, 9H), 0.87 (s, 9H), 0.57 (s, 3H), 0.09, 0.08, 0.06, and 0.04 (each s, each 3H). ^{13}C NMR ($CDCl_3$) 168.30, 153.17, 141.23, 137.77, 133.04, 128.07, 122.58, 116.41, 106.46, 72.72, 71.83, 69.45, 57.47, 56.43, 52.28, 47.80, 45.91, 44.13, 40.81, 40.27, 38.77, 34.39, 28.93, 28.12, 26.03, 25.98, 23.61, 22.42, 19.55, 18.74, 18.37, 12.29, −4.67, −4.88; exact mass calculated for $C_{40}H_{70}O_5Si_2$ $[M]^+$ 686.4762, found 686.4789.

22, ^1H NMR ($CDCl_3$, 400 MHz) δ 6.25 (s, 1H), 6.21 (d, *J* = 11.2 Hz, 1H), 5.84 (d, *J* = 11.2 Hz, 1H), 5.67 (s, 1H), 4.97 (s, 1H), 4.91 (s, 1H), 4.42 (m, 2H), 3.87 (m, 1H), 3.77 (s, 3H), 2.82 (br d, *J* = 11.3, 1H), 2.56–2.44 (m, 3H), 2.40–2.25 (m, 2H), 2.18 (m, 1H), 0.97 (d, *J* = 6.6 Hz, 3H), 0.90 (s, 9H), 0.85 (s, 9H), 0.57 (s, 3H), 0.08, 0.06, 0.05, and 0.02 (each s, each 3H); ^{13}C NMR ($CDCl_3$) δ 168.31, 153.19, 141.28, 137.76, 133.03, 127.92, 122.58, 116.41, 106.44, 72.79, 71.78, 67.85, 57.35, 56.54, 52.28, 47.85, 46.00, 43.93, 41.78, 40.86, 38.72, 33.09, 28.94, 28.08, 26.04, 25.97, 23.62, 22.44, 18.87, 18.45, 18.35, 12.35, −4.67, −4.90; exact mass calculated for $C_{40}H_{70}O_5Si_2Na$ $[M+Na]^+$ 709.4660, found 709.4680.

4.1.13. Synthesis of (23*S*)-25-dehydro-2-methylene-19-nor-1α-hydroxyvitamin D_3-26,23-lactone (GC-3) (LAC67b)

To a suspension of NaH (60% oil dispersion, 4 mg, 100 μmol) was added a solution of **21** (4 mg, 5.8 μmol) in THF (3 mL) at 0 °C, and the mixture was stirred at the same temperature for 30 min. The reaction mixture was quenched with saturated aq NH_4Cl solution, and the aqueous layer was extracted with Et_2O. The organic layers were combined and washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (5% EtOAc/hexane) to give protected vitamin (2 mg, 3 μmol, 52%) as a colorless oil. ^1H NMR ($CDCl_3$, 900 MHz) δ 6.21 (t, *J* = 2.7 Hz, 1H), 6.20 (d, *J* = 10.8 Hz, 1H), 5.82 (d, *J* = 10.8 Hz, 1H), 5.61 (t, *J* = 2.7 Hz, 1H), 4.96 (s, 1H), 4.91 (s, 1 H,), 4.59 (m, 1H), 4.41 (m, 2H), 3.04 (dddd, *J* = 16.7, 7.2, 2.7, 1.8 Hz, 1H), 2.81 (dm, *J* = 12.6 Hz, 1H), 2.54 (dddd, *J* = 16.7, 6.3, 3.6, 2.7 Hz, 1H), 2.49 (dd, *J* = 13.5, 6.3 Hz, 1H), 2.45 (dd, *J* = 13.5, 4.5 Hz, 1H), 2.32 (dd, *J* = 13.5, 3.6 Hz, 1H), 2.17 (dd, *J* = 13.5, 9.0 Hz, 1H), 1.02 (d, *J* = 7.2 Hz, 3H), 0.88 (s, 9H), 0.85 (s, 9H), 0.55 (s, 3H), 0.07, 0.05, 0.03, and 0.01 (each s, each 3H). ^{13}C NMR ($CDCl_3$) δ 170.57, 153.12, 140.92, 134.94, 133.21, 122.52, 122.07, 116.55, 106.51, 76.89, 72.71, 71.83, 56.80, 56.35, 47.80, 45.87, 42.54, 40.72, 38.79, 34.27, 33.90, 28.87, 28.24, 26.06, 26.02, 23.54, 22.38, 19.48, 18.45, 18.37, 12.23, −4.66, −4.90; exact mass calculated for $C_{39}H_{66}O_4Si_2Na$ $[M+Na]^+$ 677.4397, found 677.4407.

To a solution of the protected vitamin (2 mg, 3 μmol) in MeCN (1 mL) was added a mixture of HF/MeCN (1:9, 1 mL) at 0 °C. The reaction mixture was stirred at the same temperature for 30 min, then it was quenched with saturated aq $NaHCO_3$ solution, and the aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (20% EtOAc/hexane) to give **GC-3** (1 mg, 2.3 μmol, 77%) as a colorless oil. For analytical purpose, a sample of the final product **GC-3** was further purified by HPLC (Zorbax Eclipse XDB-C18, 250/9.4 mm, 15% MeOH/H_2O, 3 mL/min, Rv = 22.6 mL). The purity of **GC-3** was proved to be about 100% by HPLC. UV (in ethanol) λ_{max} 243, 251, 261 nm. ^1H NMR ($CDCl_3$, 800 MHz) δ 6.35 (d, *J* = 11.2 Hz, 1H), 6.22 (t, *J* = 2.4 Hz, 1H), 5.88 (d, *J* = 11.2 Hz, 1H), 5.62 (t, *J* = 2.4 Hz, 1H), 5.12 (s, 1H), 5.09 (s, 1H), 4.59 (m, 1H), 4.49 (m, 1H), 4.47 (m, 1H), 3.05 (dddd, *J* = 16.8, 7.2, 2.4, 2.4 Hz, 1H), 2.84 (dd, *J* = 13.6, 4.0 Hz, 1H), 2.81 (dm, *J* = 15.2 Hz, 1H), 2.58 (dd, *J* = 13.6, 4.0 Hz, 1H), 2.55 (dddd, *J* = 16.8, 6.4, 3.2, 3.2 Hz, 1H), 2.33 (dd, *J* = 13.6, 6.4 Hz, 1H), 2.30 (dd, *J* = 13.6, 8.8 Hz, 1H), 1.03 (d, *J* = 6.4 Hz, 3H), 0.57 (s, 3H); exact mass calculated for $C_{27}H_{38}O_4Na$ $[M+Na]^+$ 449.2668, found 449.2688.

4.1.14. Synthesis of (23*R*)-25-dehydro-2-methylene-19-nor-1α-hydroxyvitamin D_3-26,23-lactone (HLV) (LAC67a)

HLV was obtained from **22** by the same procedure as described for **GC-3** (yield 66%, in two steps) as a colorless oil. For analytical purpose, a sample of the final product **HLV** was further purified by HPLC (Zorbax Eclipse XDB-C18 250/9.4 mm, 15% MeOH/H_2O, 3 mL/min, Rv = 21.8 mL). The purity of **HLV** was proved to be about 100% by HPLC. UV (in ethanol) λ_{max} 243, 251, 261 nm. ^1H NMR ($CDCl_3$, 800 MHz) δ 6.34 (d, *J* = 11.2 Hz, 1H), 6.23 (t, *J* = 2.4 Hz, 1H), 5.88 (d, *J* = 11.2 Hz, 1H), 5.62 (t, *J* = 2.4 Hz, 1H), 5.11 (s, 1H), 5.09 (s, 1H), 4.65 (m, 1H), 4.49 (m, 1H), 4.46 (m, 1H), 3.07 (dddd, *J* = 16.8, 8.0, 2.4, 2.4 Hz, 1H), 2.87 (dd, *J* = 13.6, 4.8 Hz, 1H), 2.81 (dm, *J* = 12.8 Hz, 1H), 2.57 (dd, *J* = 13.6, 4.0 Hz, 1H), 2.53 (dddd, *J* = 16.8, 5.6, 3.2, 3.2 Hz, 1H), 2.33 (dd, 13.6, 5.6 Hz, 1H), 2.27 (dd, *J* = 12.8, 8.0 Hz, 1H), 2.01 (m, 2H), 1.02 (d, *J* = 6.4 Hz, 3H), 0.57 (s, 3H); exact mass calculated for $C_{27}H_{38}O_4Na$ $[M+Na]^+$ 449.2668, found 449.2666.

4.2. Biological studies

All the biological assays were performed as previously described.[39] Brief descriptions of the assay methods follow.

Measurements of intestinal calcium transport and bone calcium mobilization were conducted in vitamin D-deficient male Sprague–Dawley rats. Dose administration began during the last week the rats were fed a 0.02% calcium containing diet. The animals were given four consecutive intraperitoneal doses in 0.1 mL of (95:5) 1,2-propanediol/ethanol approximately 24 h apart. Twenty-four hours after the last dose, blood was collected from the severed neck and the concentration of serum calcium was determined as a measure of bone calcium mobilization. The first 10 cm of the intestine was also collected for intestinal calcium transport analysis using the everted gut sac method. Calcium was measured in the presence

of 0.1% lanthanum chloride by means of a Perkin-Elmer atomic absorption spectrometer Model 3110. Intestinal calcium transport is expressed as the serosal:mucosal ratio of calcium in the sac to the calcium in the final incubation medium. Bone calcium mobilization represents the rise in serum calcium of the rats maintained on a very low calcium diet.

Measurement of binding to the full-length rat recombinant vitamin D receptor (VDR) was done using purified full-length rat recombinant receptor. The protein was diluted in $TEDK_{50}$ (50 mM Tris, 1.5 mM EDTA, pH 7.4, 5 mM DTT, 150 mM KCl) with 0.1% Chaps detergent. Radiolabeled ligand (^{3}H-1α,25(OH)$_2$D$_3$, ~159 Ci/mmol) was added in ethanol at a final concentration of 1 nM. Radiolabeled and unlabeled ligands were added to 100 μL of the diluted protein at a final ethanol concentration of $\leqslant$10%, mixed and incubated overnight on ice to reach binding equilibrium. The following day, 100 μL of hydroxylapatite slurry (50%) was added to each tube and mixed at 10-min intervals for 30 min. The hydroxylapatite was collected by centrifugation and then washed three times with Tris–EDTA buffer (50 mM Tris, 1.5 mM EDTA, pH 7.4) containing 0.5% Triton X-100. After the final wash, the pellets were transferred to scintillation vials containing 4 mL of Biosafe II scintillation cocktail, mixed and placed in a scintillation counter. Total binding was determined from the tubes containing only radiolabeled ligand.

Measurement of cellular differentiation was performed in human promyelocytic leukemia (HL-60) cells. HL-60 cells were plated at 1.2×10^5 cells/plate. Eighteen hours after plating, cells in duplicate were treated with the compound or compounds tested so that the final concentration of ethanol was less than 0.2%. Four days later, the cells were harvested, and a nitro blue tetrazolium (NBT) reduction assay was performed. The percentage of differentiated cells was determined by counting a total of 200 cells and recording the number that contained intracellular black-blue formazan deposits.

Transcriptional activity was measured in ROS 17/2.8 (bone) cells that were stably transfected with a 24-hydroxylase (24-OHase) gene promoter upstream of a luciferase reporter gene. Cells were given a range of doses. Sixteen hours after dosing the cells were harvested and luciferase activities were measured using a luminometer.

When antagonism was tested, a combination of 1α,25(OH)$_2$D$_3$ or 2MD and the potential antagonist were added so that the final ethanol concentration was the same as that for the cells or animals receiving only one compound.

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38. The authors have deposited crystallographic data (excluding structure factors) for compound **12** with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC 685259. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
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