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Synthesis, Structure, and Biological Activity of des-Side Chain Analogues of 1α,25-Dihydroxyvitamin D_3 with Substituents at C18

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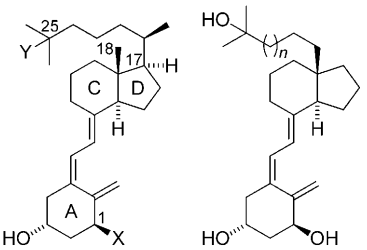
Dedicated to Argentinean Professors Rita H. Rossi, Julio C. Podestá, Manuel González Sierra, and Oscar S. Giordano for their commitment and contribution to organic chemistry.

An improved synthetic route to 1α,25-dihydroxyvitamin D_3 des-side chain analogues **2a** and **2b** with substituents at C18 is reported, along with their biological activity. These analogues display significant antiproliferative effects toward MCF-7 breast cancer cells and prodifferentiation activity toward SW480-ADH colon cancer cells; they are also characterized by a greatly decreased calcemic profile. The crystal structure of the human vitamin D receptor (hVDR) complexed to one of these analogues, 20(17→18)-abeo-1α,25-dihydroxy-22-homo-21-norvitamin D_3 (**2a**) reveals that the side chain introduced at position C18 adopts the same orientation in the ligand binding pocket as the side chain of 1α,25-dihydroxyvitamin D_3.

Introduction

The growth inhibition, prodifferentiating, and immunomodulatory activity of 1α,25-dihydroxyvitamin D_3 (**1a**), the hormonally active form of vitamin D_3 (**1b**), opens new avenues for the use of this compound in cancer, psoriasis, and immune-related disorders such as multiple sclerosis and inflammatory bowel disease. However, the therapeutic applicability of compound **1a** is limited by hypercalcemia, a condition in which serum calcium levels are elevated, which occurs when supraphysiological doses of **1a** are administered.[1] Therefore, it remains a major challenge to design and synthesize analogues of **1a** that have the best possible balance between the advantageous antiproliferative and prodifferentiating effects on the one hand and the adverse calcemic activity on the other hand.



1a: X = Y = OH
1b: X = Y = H

2a: *n* = 4
2b: *n* = 5

One of our research strategies is focused on the synthesis of vitamin D_3 analogues with side chains attached to the angular C18 methyl group. Most of the reported C18 substituted analogues are linked to C18 through an oxygen atom and carry residual groups at C17.[2] We recently reported the synthesis of novel analogues of vitamin D_3 in which side chains homologous to that of the natural hormone are linked to C18 through a C–C bond and that have no substituents on C17.[3] However, the structural analysis and biological activity of these compounds was not yet reported. In this paper we present an improved synthetic route to two of these analogues, 20(17→18)-abeo-1α,25-dihydroxy-22-homo-21-norvitamin D_3 (**2a**) and 20(17→18)-abeo-1α,25-dihydroxy-22,23-dihomo-21-norvitamin

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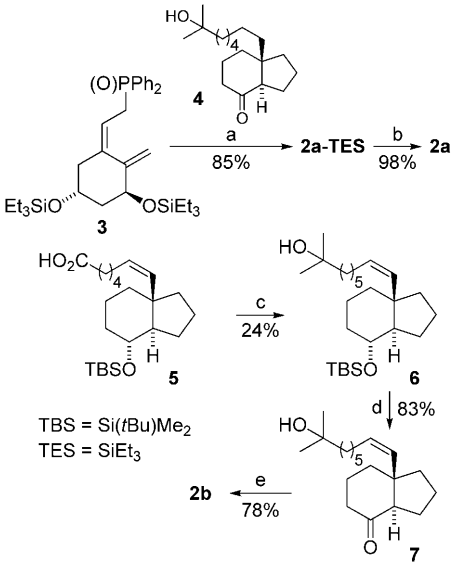
D_3 (**2b**). A structural study of the seven-membered C18 side chain analogue **2a** complexed to hVDR was performed to investigate the binding mode of this newly introduced side chain at C18. Furthermore, the biological activity profile of these analogues was determined in several cancer cell lines.

Results and Discussion

Chemistry

The target compounds **2a** and **2b** were synthesized by modifying our previous strategy as outlined in Scheme 1.[3] The replacement of the *tert*-butyldimethylsilyl protecting group by



Scheme 1. Synthesis of **2a** and **2b**: a) *n*BuLi, THF, −78 °C, then **4**, 3 h; b) HF–Py, CH_2Cl_2, RT, 1 h; c) 1. $LiAlH_4$, Et_2O, Δ, 12 h, 2. *p*-TsCl, DMAP, Py, CH_2Cl_2, 0 °C, 48 h, 3. NaCN, DMSO, 90 °C, 2 h, 4. *i*Bu_2AlH, CH_2Cl_2, −10 °C, 2 h, 5. MeLi, Et_2O, −78 °C, 1 h, 6. PDC, CH_2Cl_2, RT, 24 h, 7. MeLi, Et_2O, −78 °C, 2 h; d) 1. 48% HF–Py, CH_3CN, CH_2Cl_2, Et_3N, RT, 48 h, 2. H_2, 5% Pd/C, EtOAc, RT, 16 h, 3. PDC, CH_2Cl_2, RT, 24 h; e) 1. **3**–Li anion, THF, −78 °C, 6 h, 2. HF–Py, CH_2Cl_2, RT, 2 h (12 steps from **5**: 15.7% yield).

the triethylsilyl group was thought to have benefits at the final deprotection-purification stage. To set the vitamin D triene system corresponding to **2a**, we coupled hydroxyketone **4** with the lithium anion of phosphine oxide **3** in THF at −78 °C to obtain the protected vitamin D_3 analogue **2a-TES** in 85% yield. Desilylation of **2a-TES** took place cleanly using pyridinium fluoride to give the desired vitamin D_3 analogue **2a** in high yield. This result validates the use of phosphine oxide **3** in the syntheses of future vitamin D_3 analogues following Lythgoe's Wittig–Horner approach.[4] The synthesis of **2b** commenced with known carboxylic acid **5**,[3] which was converted into alcohol **6** in 24% yield through a seven-step sequence involving reduction of the carboxylic group, tosylation, displacement of the resulting tosylate with sodium cyanide, reduction of the resulting nitrile to the homologated aldehyde, methylation, oxidation, and methylation of the resulting ketone. Desilylation of **6** followed by hydrogenation of the double bond and oxidation of the secondary hydroxy group provided ketone **7** in 82% yield. Coupling of ketone **7** with the lithium anion of phosphine oxide **3** afforded, after desilylation, the desired eight-membered vitamin D_3 side-chain analogue **2b** in 78% yield over the three steps (12 steps from **5**, 15.7% overall yield).

Structural analysis

A structural study of the hVDR–**2a** complex was performed to gain more insight into the interaction of the **2a** analogue with the VDR. In this complex, the protein adopted the canonical conformation of all previously reported structures of VDR bound to agonist and superagonist ligands with helix H12 folded in the agonistic position.[5] The seven-membered side chain introduced at position C18 adopted the same orientation in the pocket. An adaptation of its conformation was observed to maintain the hydrogen bonds forming the anchoring points. Compared with the structure of the hVDR–**1a** complex, the atomic coordinates of hVDR bound to compound **2a** showed a root-mean-square deviation of 0.25 Å of all Cα atoms. The sizes of the ligands were 396 and 391 $Å^3$ for compound **1a** and **2a**, respectively. The volume of the ligand binding cavity was 673 and 638 $Å^3$ and the ligand occupied 61 and 59% of the pocket for **1a** and **2a**, respectively.

The A and seco B rings of the C18 side chain analogue **2a** presented conformations similar to those of the natural ligand (Figure 1a and b). As a consequence of the modified side chain, the C and D rings were shifted by 0.4 Å. The distance between the 1-hydroxy and the 25-hydroxy groups varied from 13.1 Å for **1a** to 12.8 Å for analogue **2a** complex. All the residues of VDR forming the binding pocket adopted the same conformation as those of the VDR–**1a** complex except for the side chain of Ile271 (H5). The interactions between the ligand and the receptor involved hydrophobic contacts and electrostatic interactions. The hydroxy groups made the same hydrogen bonds as in the VDR–**1a** complex, 1-OH with Ser237 (H3) and Arg274 (H5), 3-OH with Tyr143 (H1) and Ser278 (β0), and the 25-OH with His305 (loop H6–H7) and His397 (H11). As a result of the modification of the ligand, the side chain of compound **2a** took another pathway in the pocket and made additional contacts with the CB atom of Leu230 (H3) at 3.9 Å of C20 and 3.6 Å of C22 atom, respectively. Its side chain, however, lost a contact with the CD2 atom of Leu309 (H7) at 3.8 Å of C21 for **1a** (Figure 1c). The elongated side chain of **2a** induced contacts between C26 and Leu404 (H11), C26 and Leu414 (loop H11–H12), and C27 and Phe422 (H12) at the end of the ligand. A Cα atom of His305 was shifted by 0.4 Å to maintain the hydrogen bonds with the hydroxy group.

To confirm the crystallographic data, several VDR mutants were used to investigate the capacity of compound **2a** to transactivate a vitamin D response element (VDRE)-containing reporter construct. Compound **1a** and the seven-membered C18 side chain analogue **2a** were unable to transactivate the reporter construct when cells were transfected with VDRs car-

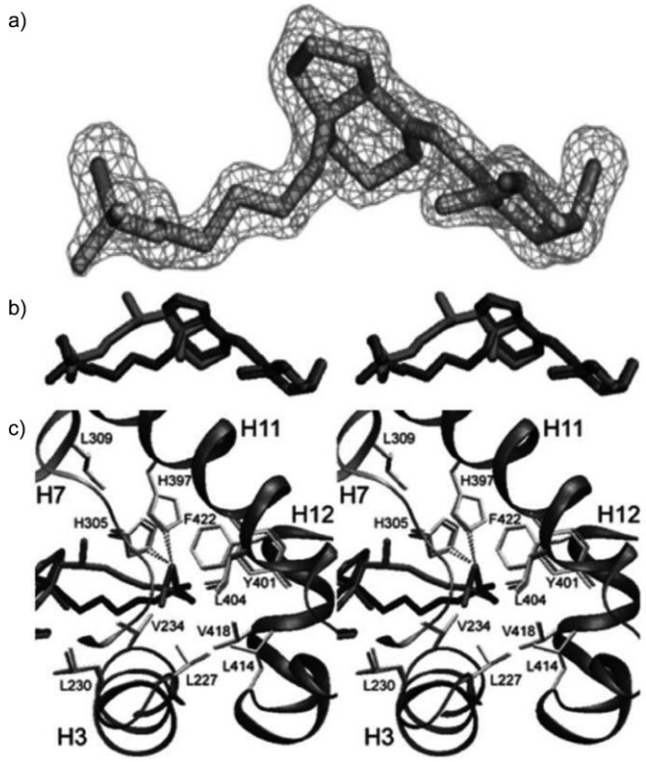


Figure 1. Conformation of the VDR-bound **2a** analogue. a) Compound **2a** is shown in its F_o-F_c electron density omit map contoured at 3.0 σ; the ligand is shown in stick representation. b) A stereo view of the ligand conformations of **1a** (dark gray) and **2a** (light gray) in their ligand binding pockets. c) Superposition of the VDR–**2a** (gray) and VDR–**1a** complexes. The view is restricted to the region of the protein (H3, H7, H11, and H12) that contains the side chain of the ligand. Only residues closer than 4.0 Å are shown. Leu309 and Tyr401 for the **2a** complex and Leu230, Leu404, Leu414, and Phe422 for the **1a** complex are shown for comparison. The ligands are shown in stick representation in light gray for analogue **2a** and dark gray for compound **1a**. Hydrogen bonds formed by the 25-OH groups are shown as dashed lines.

rying either the Leu233Ala, Ile271Ala, Arg274Ala, Trp286Ala, His397Ala, or Tyr401Ala mutation (Figure 2). The transactivation potency of compound **1a** was moderately decreased when cells were transfected with the VDR mutants Val234Ala, Ile268Ala, Val300Ala, and His305Ala (50% of the activity of wild-type VDR), whereas transfection with the mutants Ser275Ala and Ser278Ala had little effect on the transactivation capacity of compound **1a**. Introduction of a seven-membered side chain at C18 rendered analogue **2a** less potent than **1a** in transactivating a VDRE-containing reporter construct in cells transfected with the VDR mutants Ile268Ala, Ser275Ala, Val300Ala, and His305Ala. These findings are in agreement with the crystal structure and suggested that interactions with these amino acids became more critical when a side chain is introduced at C18 instead of at position C17. The transactivating capacity of the VDR mutant Val234Ala became more potent in cells that were incubated with compound **2a**, which confirmed crystallographic data that showed a weaker interaction between this amino acid and the side chain of the **2a** analogue.

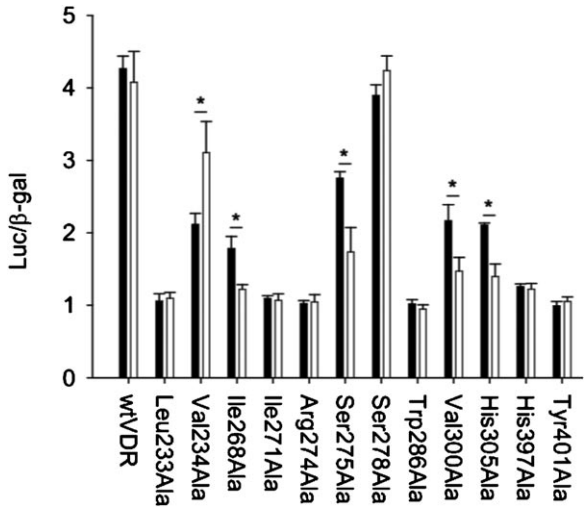


Figure 2. Transactivating potency of various VDR point mutants in COS-1 cells. Cells were stimulated with compound **1a** (black bars) or the analogue **2a** (open bars), each applied at their EC_{50} concentrations (6 nM for compound **1a** and 3 nM for analogue **2a**). Luciferase activity was normalized for β-galactosidase activity (Luc/β-gal) and expressed relative to transfected vehicle-stimulated cells. Bars represent the mean ± SD of three independent experiments (*$p < 0.01$, Student's *t*-test).

Heterodimerization with RXR

To assess whether VDR bound to **2a** was able to heterodimerize with RXR, we monitored its interaction by electrospray ionization mass spectrometry (ESI-MS) under nondenaturing conditions. Addition of fivefold molar excess of **2a** in the heterodimer VDR–RXR LBDs resulted in the appearance of a novel series of mass/charge (*m/z*) ions corresponding to a fully bound VDR–**2a**–RXR (see Supporting Information for details). The different conformation of the VDR-bound **2a** analogue does not affect the VDR–RXR heterodimer stability. We then monitored by ESI-MS the recruitment of the SRC-1 NR2 co-activator peptide to VDR–**2a**–RXR complex (data not shown) and found it to be similar to VDR–**1a**–RXR.

Biological evaluation

Despite the fact that the analogue **2a** fitted well in the VDR binding pocket and that the hydrogen bonds, which form the anchoring points, were maintained, the affinity of compound **2a** for pig VDR was greatly diminished (10% of the affinity of compound **1a**; Table 1). Moreover, binding to the transport protein vitamin D binding protein (hDBP) was significantly decreased (2.5% of the affinity of compound **1a**). Homologation of the side chain in compound **2b** further decreased the binding affinity to DBP and VDR (1.3 and 3.3% of the affinity of **1a**, respectively). The low affinity for hDBP may be explained by attachment of the **2a** and **2b** side chain fragments to C18 that orientates the side chain hydroxy groups toward the β-face of the CD rings. Notwithstanding this low affinity for VDR, but in accordance with the transactivation results, the seven-membered C18 side chain analogue **2a** was as potent as compound **1a** in decreasing the proliferation of human breast adenocarci-

Table 1. In vitro binding affinities, antiproliferative activities, and in vivo calcemic effects of compound **1a** and its 20(17→18)-abeo1α,25-dihydroxy-21-norvitamin D_3 analogues **2a** and **2b**.

Compd	Binding EC_{50} [nM][a]		Growth Inhib. EC_{50} [nM][b]	Calcemic Act.[c]
	DBP	VDR	MCF-7	[μg kg^{-1} day^{-1}]
1a	50	0.2	60	0.1
2a	2000	2	50	>60
2b	4000	6	200	>100

[a] Binding of compounds **1a**, **2a**, and **2b** to human DBP and pig VDR is expressed as the concentration needed to displace 50% of [^{3}H]1α,25(OH)$_2$D$_3$ from DBP or VDR. [b] The antiproliferative effects of 1α,25(OH)$_2$D$_3$ and its analogues on MCF-7 cells are expressed as the concentrations required for half-maximal inhibition of [^{3}H]thymidine incorporation. [c] The calcemic activity of compounds **1a**, **2a**, and **2b** was determined in mice ($n=6$ per group) by intraperitoneal injections for seven consecutive days. This activity is expressed as the maximal dose that can be administered without exceeding the serum calcium concentration observed when mice were treated with compound **1a** at 0.1 μg kg^{-1} day^{-1}. Serum calcium in mice treated with vehicle was 11.5 ± 0.4 mg dL^{-1}, 14.5 ± 0.7 mg dL^{-1} in mice treated with compound **1a** at 0.1 μg kg^{-1} day^{-1}, 11.9 ± 0.7 mg dL^{-1} upon treatment with compound **2a** at 60 μg kg^{-1} day^{-1}, and 11.4 ± 0.4 mg dL^{-1} in mice treated with compound **2b** at 100 μg kg^{-1} day^{-1}. Serum calcium levels in mice treated with compounds **2a** or **2b** were not different from control mice, but were significantly lower than in mice treated with compound **1a** at 0.1 μg kg^{-1} day^{-1} ($p<0.01$, one-way ANOVA followed by Fisher LSD test).

noma MCF-7 cells (Table 1). Addition of an extra carbon atom in the side chain in compound **2b** decreased the growth-inhibitory potential of this analogue because threefold higher concentrations of this compound were required to obtain a 50% decrease in proliferation. The ability to induce cell differentiation was investigated in human colon cancer SW480-ADH cells, which upon incubation with compound **1a** undergo epithelial differentiation with increased adhesiveness as a result of a drastic change in their pattern of gene expression that includes the induction of E-cadherin, occludin, and several other adhesion proteins.[6] Both C18 side chain analogues induced a similar morphological change as compound **1a**, promoting the formation of compact epithelioid cell islands, but again the analogue with the longer side chain (**2b**) was less efficient (Figure 3a). Likewise, compounds **2a** and **1a** were equipotent in increasing the cellular content of E-cadherin, a hallmark of the differentiated phenotype, whereas the **2b** analogue was less potent (Figure 3b). In agreement with these observations, analogue **2a** and the parent molecule **1a**, but not analogue **2b**, increased VDR expression (Figure 3b). The mechanistic basis for the discrepancy between receptor binding and biological activity may be attributed to changes in VDR conformation and a modified interaction of liganded VDR with co-activator molecules, which alters the structural and functional properties of the entire DNA-binding complex.[7]

Interestingly, introduction of a side chain at position C18 led to a decrease in calcemic activity. Indeed, in comparison with compound **1a**, at least 600-fold higher doses of the seven- or eight-membered C18 side chain analogues could be administered on a daily basis in NMRI vitamin D-replete mice (Table 1). The lower calcemic activity may be due, in part, to the low binding affinity of these compounds for DBP, which results in an increased clearance. However, other mechanisms such as increased metabolic degradation, tissue-specific differences in the binding affinities of compound **1a** and its analogues for the VDR, and different signaling pathways have been suggested to contribute.[7,8] The low calcemic activity of compound **2a** together with its potent antiproliferative and prodifferentiating activity make this compound appealing for therapeutic application.

Conclusions

We describe improved syntheses of two des-side chain analogues of vitamin D_3 with substituents at C18. In the VDR crystallographic study we demonstrated that the seven-membered side chain introduced at position C18 in compound **2a** adopts the same orientation in the ligand binding pocket as the side chain of compound **1a** with maintenance of the hydrogen bonds that form the anchoring points of the ligand. Mutational analysis confirmed the interactions of compound **2a** with the amino acids lining the VDR ligand binding pocket as determined in the crystal structure. Despite the fact that analogue **2a** fitted well in the VDR binding pocket, it displayed a weak binding to VDR and homologation of the side chain further decreased the affinity. Nevertheless both des-side chain analogues of vitamin D_3 possessed significant antiproliferative and prodifferentiating properties with greatly decreased calcemic effects. This biological profile makes these analogues, and especially compound **2a**, potential candidates for the treatment of hyperproliferative disorders such as breast or colon cancer.

Experimental Section

Chemistry

In addition to NMR, HPLC analysis was used to determine the purity (>95%) of vitamin D_3 analogues.

20(17→18)-abeo-3-(Triethylsilyl)-1α-[(triethylsilyl)oxy]-25-hydroxy-22-homo-21-norvitamin D_3 (2a-TES): A solution of *n*BuLi (0.45 mL, 1.14 mmol, 2.5 M in hexanes) was added to a solution of phosphine oxide **3** (0.702 g, 1.204 mmol) in dry THF (8 mL) at −78 °C. The deep-red solution was stirred for 1 h. A solution of ketone **4** (0.063 g, 0.277 mmol) in dry THF (8 mL) was added dropwise. The reaction mixture was stirred in the dark at −78 °C for 3 h and at −30 °C for 4 h. H_2O (0.5 mL) was added and the resulting mixture was concentrated to give a residue which was dissolved in Et_2O (100 mL). The combined organic phase was washed with saturated $NaHCO_3$ (20 mL), brine (3×20 mL), H_2O (60 mL), dried, filtered, and concentrated. The residue was purified by flash chromatography (SiO_2, 2×15 cm, 6–12% Et_2O/hexanes) to give the pro-

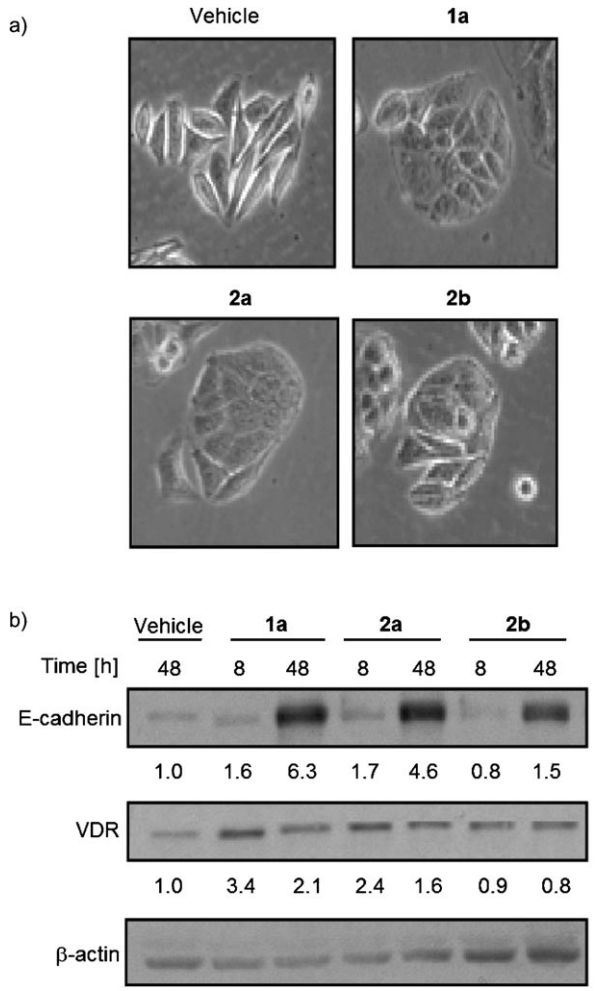


Figure 3. Analogues **2a** and **2b** induce an adhesive epithelial phenotype in SW480-ADH cells. a) Differentiation of SW480-ADH cells demonstrated by phase-contrast micrographs of cells treated with each compound at 100 nM or vehicle for 48 h. A representative experiment is shown. b) Western blot analysis of the expression of E-cadherin, VDR, and β-actin (loading control) at 8 and 48 h treatment with each compound at 100 nM. Numbers show the quantification of E-cadherin induction (ratio of levels in treated versus untreated cells) after normalization to β-actin. A representative experiment is shown.

tected analogue **2a-TES** [0.125 g, 85%, R_f = 0.8 (30% EtOAc/hexanes), colorless oil].

20(17→18)-abeo-1α,25-Dihydroxy-22,23-dihomo-21-norvitamin D_3 (2a): HF–pyridine complex (10 drops) was slowly added to a solution of **2a-TES** (0.125 g, 0.194 mmol) in dry CH_3CN (6 mL), dry CH_2CL_2 (4 mL), and dry Et_3N (3 mL). The reaction mixture was stirred in the dark at RT for 1 h. Saturated $NaHCO_3$ (30 mL) was added slowly and the aqueous layer was extracted with Et_2O (4× 20 mL). The combined organic layer was washed with brine (2× 25 mL), dried, filtered, and concentrated. The residue was purified by flash chromatography (SiO_2, 1.5×15 cm, 10–12% *i*PrOH/hexanes) to give **2a** [0.079 g, 98%, R_f = 0.4 (20% *i*PrOH/hexanes), white solid].

20(17→18)-abeo-3-(Triethylsilyl)-1α-[(triethylsilyl)oxy]-25-hydroxy-22,23-dihomo-21-norvitamin D_3 (2b-TES): A solution of *n*BuLi (0.41 mL, 1.02 mmol, 2.5 M in hexanes) was added to a solution of phosphine oxide **3** (0.627 g, 1.076 mmol) in dry THF (5 mL) at −78 °C. The deep-red solution was stirred for 1 h. A solution of ketone **7** (0.050 g, 0.17 mmol) in dry THF (5 mL) was added dropwise. The reaction mixture was stirred in the dark at −78 °C for 2 h and at −55 °C for 4 h. H_2O (0.5 mL) was added. Concentration of the reaction mixture gave a residue which was dissolved in Et_2O (100 mL). The combined organic phase was washed with saturated $NaHCO_3$ (20 mL), saturated NaCl (3×20 mL), H_2O (60 mL), dried, filtered, and concentrated. The residue was purified by flash chromatography (SiO_2, 2×15 cm, 6–12% Et_2O/hexanes) to give the protected analogue **2b-TES** [0.091 g, 82%, R_f = 0.8 (30% EtOAc/hexanes), colorless oil].

20(17→18)-abeo-1α,25-Dihydroxy-22,23-dihomo-21-norvitamin D_3 (2b): HF–pyridine complex (10 drops) was slowly added to a solution of **2b-TES** (0.085 g, 0.136 mmol) in dry CH_3CN (3 mL), dry CH_2Cl_2 (1 mL) and dry Et_3N (1 mL). The reaction mixture was stirred in the dark at RT for 1.5 h. Saturated $NaHCO_3$ (30 mL) was added slowly and the aqueous layer was extracted with Et_2O (4×20 mL). The combined organic layer was washed with brine (2×25 mL), dried, filtered, and concentrated. The residue was purified by flash chromatography (SiO_2, 1.5×15 cm, 10–12% *i*PrOH/hexanes) to give **2b** [0.05 g, 95%, R_f = 0.5 (30% *i*PrOH/hexanes), white solid].

Details on the syntheses of compounds **3**, **6a**, **6b**, **6c**, **6d**, **6e**, **6f**, **6g**, **6**, **7a**, and **7** are described in the Supporting Information.

Structural analysis of hDVR complexed to compound 2a: Details on expression, purification, crystallization, and subsequent X-ray crystallography, data collection, and processing can be found in the Supporting Information. The accession number for the coordinates of the complex reported herein is PDB ID: 3P8X. The methodology applied for the analysis of heterodimerization with RXR is described in the Supporting Information. The transactivating assay was performed as described by Verlinden et al.[7]

Biological activity

Affinity of the analogues to hDBP and pVDR and their antiproliferative activity were determined as described previously.[8] The protocol for in vivo evaluation of calcemic activity was approved by the ethical committee for animal research at the Katholieke Universiteit Leuven and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The ability of the compounds to induce differentiation of SW480-ADH colon cancer cells was measured as described by Palmer et al.[6]

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Keywords: vitamin D analogues · biological activity · cancer · steroids · vitamins

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