Synthesis, Structure, and Biological Activity of des-Side Chain Analogues
of 1α,25-Dihydroxyvitamin D₃ with Substituents at C-18

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● Antonio Mouriño dedicates this paper to Argentinean Professors Rita H.
Rossi, Julio C. Podestá, Manuel González Sierra and Oscar S. Giordano,
for their dedication and contribution to organic chemistry.
ABSTRACT An improved synthetic route to des-side chain analogues of 1α,25-dihydroxyvitamin D₃ with substituents at C-18 and their biological activity is reported. These analogues displayed significant antiproliferative and prodifferentiating effects with a greatly reduced 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 D₃ (2a), revealed that the side chain introduced at position C-18 adopts the same orientation in the ligand binding pocket as the side chain of 1α,25-dihydroxyvitamin D₃.

Introduction

The growth-inhibitory, prodifferentiating, and immunomodulatory activity of 1α,25-dihydroxyvitamin D₃ (1a, Fig. 1), the hormonally active form of vitamin D₃ (1b), opens perspectives 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 1α,25-dihydroxyvitamin D₃ is limited due to hypercalcemia. Therefore, it remains a major challenge to design and synthesize analogues of 1α,25-dihydroxyvitamin D₃ that have the best possible balance between the advantageous antiproliferative and prodifferentiating effects and the adverse calcemic activity.

One of our research strategies is focused on the synthesis of 1α,25-
dihydroxyvitamin D₃-analogues with side chains attached to the angular C-18 methyl group. Most of the reported C-18 substituted analogues are linked to C-18 through an oxygen atom and carry residual groups at C-17.² We recently reported the synthesis of novel 1α,25-dihydroxyvitamin D₃-analogues in which side chains homologous to that of the natural hormone are linked to C-18 through a C-C bond and that have no substituents on C-17.³ 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 D₃ (2a, Fig. 1) and 20(17→18)-abeo-1α,25-dihydroxy-22,23-dihomo-21-norvitamin D₃ (2b). A structural study of the 7-membered C-18 side chain analogue 2a complexed to hVDR was performed in order to investigate the binding mode of this newly introduced side chain at C-18. 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 modified procedures of our previous strategy³ as outlined in Scheme 1. The replacement of the tert-butyldimethylsilyl protecting group by 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 protected vitamin D₃ analogue 2a-TES in 85% yield. Desilylation of 2a-TES took place cleanly using pyridinium fluoride to give the desired vitamin D₃ analogue 2a in high yield. This result validates the use of phosphine oxide 3 in the syntheses of
future vitamin D₃ analogues following Lythgoe’s Wittig-Horner approach.⁴ The synthesis of 2b commenced with known carboxylic acid 5,³ which was converted to alcohol 6 in 24% yield by 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 hydroxyl 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 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 in 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 7-membered side chain introduced at position C-18 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 hVDR-1α,25(OH)₂D₃ complex, the atomic coordinates of hVDR bound to compound 2a showed root-mean-square deviation of 0.25 Å of all Cα atoms. The sizes of the ligands were 391 and 396 Å³ for analogue 2a and 1α,25(OH)₂D₃, respectively. The volume of the ligand binding cavity was 638 and 673 Å³ and the ligand occupied 59% and 61% of the pocket for 2a and 1α,25(OH)₂D₃,
respectively. The A and secoB rings of the C-18 side chain analogue 2a presented conformations similar to those of the natural ligand (Fig. 2A-B). As a consequence of the modified side chain, the C and D rings were shifted by around 0.4 Å. The distance between the 1-hydroxy and the 25-hydroxy groups varied from 13.1 Å for 1α,25(OH)₂D₃ 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/1α,25(OH)₂D₃ except for the side chain of Ile271 (H5). The interactions between the ligand and the receptor involved hydrophobic contacts and electrostatic interactions. The hydroxyl groups made the same hydrogen bonds as VDR/1α,25(OH)₂D₃ 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). Because 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 C-20 and 3.6 Å of C-22 atom, respectively. Its side chain, however, lost a contact with the CD2 atom of Leu309 (H7) at 3.8 Å of C-21 for 1α,25(OH)₂D₃ (Fig. 2C). The elongated side chain of 2a induced contacts between the C-26 and Leu404 (H11), the C-26 and Leu414 (loop H11-H12), C-27 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 hydroxyl 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. 1α,25(OH)₂D₃ as well as the 7-membered C-18 side chain analogue 2a were unable to transactivate the
reporter construct when cells were transfected with VDRs carrying either the Leu233Ala, Ile271Ala, Arg274Ala, Trp286Ala, His397Ala, or Tyr401Ala mutation (Fig. 3). The transactivation potency of 1α,25(OH)2D3 was moderately reduced 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 1α,25(OH)2D3. Introduction of a 7-membered side chain at C-18 rendered analogue 2a less potent than 1α,25(OH)2D3 in transactivating a VDRE-containing reporter construct in cells transfected with the VDR mutants Ile268Ala, Ser275Ala, Val300Ala, and His305Ala. These findings were in agreement with the crystal structure and suggested that interactions with these amino acids became more critical when a side chain was introduced at C-18 instead of at position C-17. 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 this 2a analogue.

**Heterodimerization with RXR.** To assess whether VDR bound to DSA was able to heterodimerize with RXR, we monitored its interaction with by ESI-MS under non-denaturating 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.6 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 coactivator peptide to VDR-2a/RXR complex (data not shown) that was similar to VDR-1α,25(OH)₂D₃/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 reduced (2.5% of the affinity of 1α,25(OH)₂D₃) (Table 1). Also the binding to the transport protein vitamin D binding protein (hDBP) was significantly reduced (10% of the affinity of 1α,25(OH)₂D₃). Homologation of the side chain in compound 2b further decreased the binding affinity to DBP and VDR (1.3% and 4% of the affinity of 1α,25(OH)₂D₃, respectively). The low affinity for the DBP can be explained by attachment of the 2a and 2b side-chain fragments to the C18 that orientates the side-chain hydroxyl groups towards the β-face of the CD rings. The lower affinity of 2a and 2b for DBP may also explain their reduced calcemic activity. Notwithstanding this low affinity for VDR, but in accordance with the transactivation results, the 7-membered C-18 side chain analogue 2a was as potent as 1α,25(OH)₂D₃ in reducing the proliferation of human breast adenocarcinoma MCF-7 cells (Table 1). Addition of an extra C-atom in the side chain in compound 2b decreased the growth-inhibitory potential of this analogue because 3-fold 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 1α,25(OH)₂D₃ undergoes 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. Both C-18 side chain analogues induced a similar morphological change as 1α,25(OH)₂D₃, promoting the formation of compact epithelioid cell islands, but again the analogue with the longer side chain (2b) was less efficient (Fig. 4A). Likewise, compound 2a and 1α,25(OH)₂D₃ were equipotent in increasing the cellular content of E-cadherin, a hallmark of the differentiated phenotype, while the 2b analogue was less potent (Fig. 4B). In agreement with this, analogue 2a as well as 1α,25(OH)₂D₃, but not analogue 2b, increased VDR expression (Fig. 4B).

Interestingly, introduction of a side chain at position C-18 led to an interesting decrease of calcemic activity. Indeed, when compared to 1α,25(OH)₂D₃, at least 600-fold higher doses of the 7- or 8-membered C-18 side chain analogues could be administered on a daily basis in NMRI vitamin D-replete mice (Table 1). This low calcemic activity makes these compounds, especially compound 2a, appealing for therapeutic application.

Conclusions

We describe improved syntheses of two des-side chain analogues of 1α,25-dihydroxyvitamin D₃ with substituents at C-18. In the VDR structural study we demonstrated that the 7-membered side chain introduced at position C-18 in compound 2a adopted the same orientation in the ligand binding pocket as the side chain of 1α,25-dihydroxyvitamin D₃ 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 crystallization study. 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 1α,25-dihydroxyvitamin D₃ possessed significant antiproliferative and prodifferentiating properties with greatly reduced 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 analogues.

20(17→18)-abeo-3-(Triethylsilyl)-1α-[(triethylsilyl)oxy]-25-hydroxy-22-homo-21-norvitamin D₃ (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. H₂O (0.5 mL) was added and the resulting mixture was concentrated to give a residue which was dissolved in Et₂O (100 mL). The combined organic phase was washed with saturated NaHCO₃ (20 mL), brine (3x20 mL), H₂O (60 mL), dried, filtered, and concentrated. The residue was purified by flash chromatography (SiO₂, 2x15 cm, 6-12% EtOAc-hexanes) to give the protected analogue 2a-TES [0.125 g, 85%, Rf = 0.8 (30 % EtOAc-hexanes), colorless oil].
HF-pyridine complex (10 drops) was slowly added to a solution of 2a-TES (0.125 g, 0.194 mmol) in dry CH₃CN (6 mL), dry CH₂Cl₂ (4 mL) and dry Et₃N (3 mL). The reaction mixture was stirred in the dark at rt for 1 h. Saturated NaHCO₃ (30 mL) was added slowly and the aqueous layer was extracted with Et₂O (4x20 mL). The combined organic layer was washed with brine (2x25 mL), dried, filtered and concentrated. The residue was purified by flash chromatography (SiO₂, 1.5x15 cm, 10-12% i-PrOH-hexanes) to give 2a [0.079 g, 98%, Rf = 0.4 (20% i-PrOH-hexanes), white solid].

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. H₂O (0.5 mL) was added. Concentration gave a residue which was dissolved in Et₂O (100 mL). The combined organic phase was washed with saturated NaHCO₃ (20 mL), saturated NaCl (3x20 mL), H₂O (60 mL), dried, filtered, and concentrated. The residue was purified by flash chromatography (SiO₂, 2x15 cm, 6-12% Et₂O-hexanes) to give protected analogue 2b-TES [0.091 g, 82%, Rf = 0.8 (30 % EtOAc-hexanes), colorless oil].
(0.085 g, 0.136 mmol) in dry CH$_3$CN (3 mL), dry CH$_2$Cl$_2$ (1 mL) and dry Et$_3$N (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$_2$O (4x20 mL). The combined organic layer was washed with brine (2x25 mL), dried, filtered and concentrated. The residue was purified by flash chromatography (SiO$_2$, 1.5x15 cm, 10-12% $t$-PrOH-hexanes) to give 2b [0.05 g, 95%, $R_f=0.5$ (30% $t$-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 Supplementary Material.

**Structural analysis of hDVR complexed to compound 2a, biological activity and electrospray ionization mass spectrometry** are shown in the supplementary material.

**Figure legends**

**Figure 1.** Chemical structure of 1$\alpha$,25(OH)$_2$D$_3$ and 20(17→18)-abeo-analogues.

**Figure 2.** 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 $\sigma$. The ligand is shown in stick representation with carbon and oxygen atoms in green and red, respectively. (B) A stereo view of the ligand conformations of 1$\alpha$,25(OH)$_2$D$_3$ (red) and compound 2a (blue) in their ligand binding pockets. (C) Superposition of the VDR·2a (gray) and VDR·1$\alpha$,25(OH)$_2$D$_3$ (yellow) complexes. The view is restricted to the region of the protein (H3, H7, H11, and H12), which contains the side chain of the ligand. Only residues closer
than 4.0 Å are shown. Leu309 and Tyr401 for the compound 2a complex and Leu230, Leu404, Leu414, and Phe422 for the 1α,25(OH)2D3 complex are shown for comparisons. The ligands are shown in stick representation in blue for analogue 2a and red for 1α,25(OH)2D3, respectively. The hydrogen bonds formed by the 25-OH groups are shown in blue (compound 2a) and red (1α,25(OH)2D3) dashed lines.

**Figure 3.** Transactivating potency of different VDR point mutants in COS-1 cells. Cells were stimulated with 1α,25(OH)2D3 or the analogue 2a, each applied at their EC50-concentrations (6 x 10^{-9} M for 1α,25(OH)2D3 and 3 x 10^{-9} M for analogue 2a). Open bars represent results for 1α,25(OH)2D3 and black bars for compound 2a. Bars represent means and S.D. of three independent experiments.

**Figure 4.** 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 10^{-7} M of each compound or vehicle for 48 h. A representative experiment is shown. (B) Western blot analysis of the expression of the expression of E-cadherin, VDR and β-actin (loading control) at 8 h and 48 h of treatment with 10^{-7} M of each compound. Numbers show the quantification of the E-cadherin induction (ratio of levels in treated versus untreated cells) after normalization to β-actin. A representative experiment is shown.

*Protein Data Bank Accession Number* - The accession number for the coordinates of the complex reported in this article is PDB ID (3P8X)
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Supporting Information Available: Experimental procedures and crystallographic information files. This material is available free of charge via the Internet at http://pubs.acs.org.

Figure 1. Chemical structure of 1α,25(OH)₂D₃ and 20(17→18)-abeo-analogues.
**Figure 2.** Conformation of the VDR-bound 2a analogue.
**Figure 3.** Transactivating potency of different VDR point mutants in COS-1 cells.

**Figure 4.** Analogues 2a and 2b induce an adhesive epithelial phenotype in SW480-ADH cells.

A

![Cell images](image)

B

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Scheme 1. Synthesis of 2a and 2b

$^a$Key: (a) $n$-BuLi, THF, -78 °C; 4. (b) HF-pyridine, CH$_2$Cl$_2$, (c) LiAlH$_4$, Et$_2$O, Δ; $p$-TsCl, DMAP, py, CH$_2$Cl$_2$, 0 °C; NaCN, DMSO, 90 °C; $i$-Bu$_2$AlH, CH$_2$Cl$_2$, 0 °C; MeLi, Et$_2$O; PDC, CH$_2$Cl$_2$; MeLi, Et$_2$O. (d) 48% HF-Py, CH$_3$CN, CH$_2$Cl$_2$, Et$_3$N; H$_2$, 5% Pd-C, EtOAc; PDC, CH$_2$Cl$_2$. (e) 3-Li-anion; HF-pyridine, CH$_2$Cl$_2$ (12 steps from 5, 15.7% yield).

Table 1. In vitro binding affinities and antiproliferative activities and in vivo
calcemic effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and its $20(17\rightarrow18)$-abeo-$1\alpha,25$-dihydroxy-21-norvitamin D$_3$ analogues.

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<td>Growth-inhibitory activity (EC50)</td>
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<td>$5 \times 10^{-8} \text{ M}$</td>
<td>$2 \times 10^{-7} \text{ M}$</td>
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<td>Calcemic activity (max applicable dose)</td>
<td>&gt; $60$ µg/kg/day</td>
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The binding of $1\alpha,25(\text{OH})_2\text{D}_3$ and the compounds $2a$ and $2b$ to human DBP and pig VDR was expressed by their dissociation constants. The antiproliferative effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and its analogues on MCF-7 cells were expressed as the concentrations required for the half-maximal inhibition of $[^3\text{H}]$thymidine incorporation. The calcemic activity of $1\alpha,25(\text{OH})_2\text{D}_3$ and analogues $2a$ and $2b$ was determined in mice by intraperitoneal injections during 7 consecutive days. This activity was expressed as the maximal dose that could be administered without exceeding a serum calcium concentration observed when mice were treated with $0.1$ µg/kg/day $1\alpha,25(\text{OH})_2\text{D}_3$.

**References**


(6) For details, see supplementary material.


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