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C-6 functionalized analogs of 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3: Synthesis and binding analysis with vitamin D-binding protein and vitamin D receptor

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Abstract

In this article, we describe the development of a general synthetic strategy to functionalize the C-6 position of vitamin D_3 and its biologically important metabolites, i.e. 25-hydroxyvitamin D_3 (25-OH-D_3) and 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. We employed Mazur's cyclovitamin D method to synthesize vitamin D_3 analogs with several functionalities at the C-6 position. In addition, we synthesized 6-(3-hydroxypropyl) and 6-[(2-bromoacetoxy)propyl] derivatives of 25-OH-D_3 **15** and **16**, respectively, and 6-(3-hydroxypropyl) derivative of 1,25$(OH)_2D_3$ **17**. Competitive binding assays of **15**–**17** with human serum vitamin D-binding protein showed that all these analogs specifically bound to this protein, although with significantly lower affinity than the 25-OH-D_3, the strongest natural binder, but with comparable affinity with 1,25$(OH)_2D_3$, the hormone. On the other hand, 6-[3-hydroxypropyl], 1α,25-dihydroxyvitamin D_3 **17** did not show any specific binding for recombinant nuclear vitamin D receptor. These results indicated that the region containing the C-6 position of the parent seco-steroid [1,25$(OH)_2D_3$] may be an important recognition marker towards vitamin D receptor binding. Information, delineated in this article, will be important for evaluating structure-activity relationship in synthetic analogs of vitamin D and its metabolites.

Keywords: Analogs of vitamin D and its metabolites – synthesis; Vitamin D – binding protein; Vitamin D receptor; Binding analysis of synthetic analogs

Introduction

Recent discovery of the anti-proliferative/pro-differentiative/anti-cancer properties of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$; $R_1 = R_2 = OH$; Fig. 1], the dihydroxylated metabolite of vitamin D_3 ($R_1 = R_2 = H$; Fig. 1), has spurred strong interest in developing a new generation of 1,25$(OH)_2D_3$-based drugs with broad spectrum benefits and reduced toxicity [1]. Such effort has led to the development of numerous analogs of vitamin D_3 and its biologically important metabolites. For example, Calcipotriene (MC-903, Calcipotriol, Dovenex), a side-chain analog of 1,25$(OH)_2D_3$ is currently a Federal Drug Administration-approved drug for psoriasis, a hyperproliferative skin disorder [2,3]. Additionally, several other analogs are presently under scrutiny as potential drugs for cancers of various organs. However, a clear direction in designing a new generation of 1,25$(OH)_2D_3$-based drugs, which would require exhaustive structure-function studies with analogs having modifications at different parts of the parent seco-steroid, is still lacking [1].

We elected to introduce modifications at the C-6 position of vitamin D_3, 25-OH-D_3, and 1,25$(OH)_2D_3$ because currently no knowledge about the effect of this modification on function is available. Although Sheves and Mazur [4] synthesized 6-methyl vitamin D_3 in 1977, they did not extend this synthetic methodology to 25-OH-D_3 or 1,25$(OH)_2D_3$, the biologically relevant metabolites of vitamin D_3. In the present communication we describe the synthesis of novel C-6-modified analogs of vitamin D_3, 25-OH-D_3, and 1,25$(OH)_2D_3$, including an affinity-labeling analog of 25-

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Note: Stereochemistries of the 3- and 1-hydroxyl groups of vitamin D sterols are designated according to cholesterol nomenclature.

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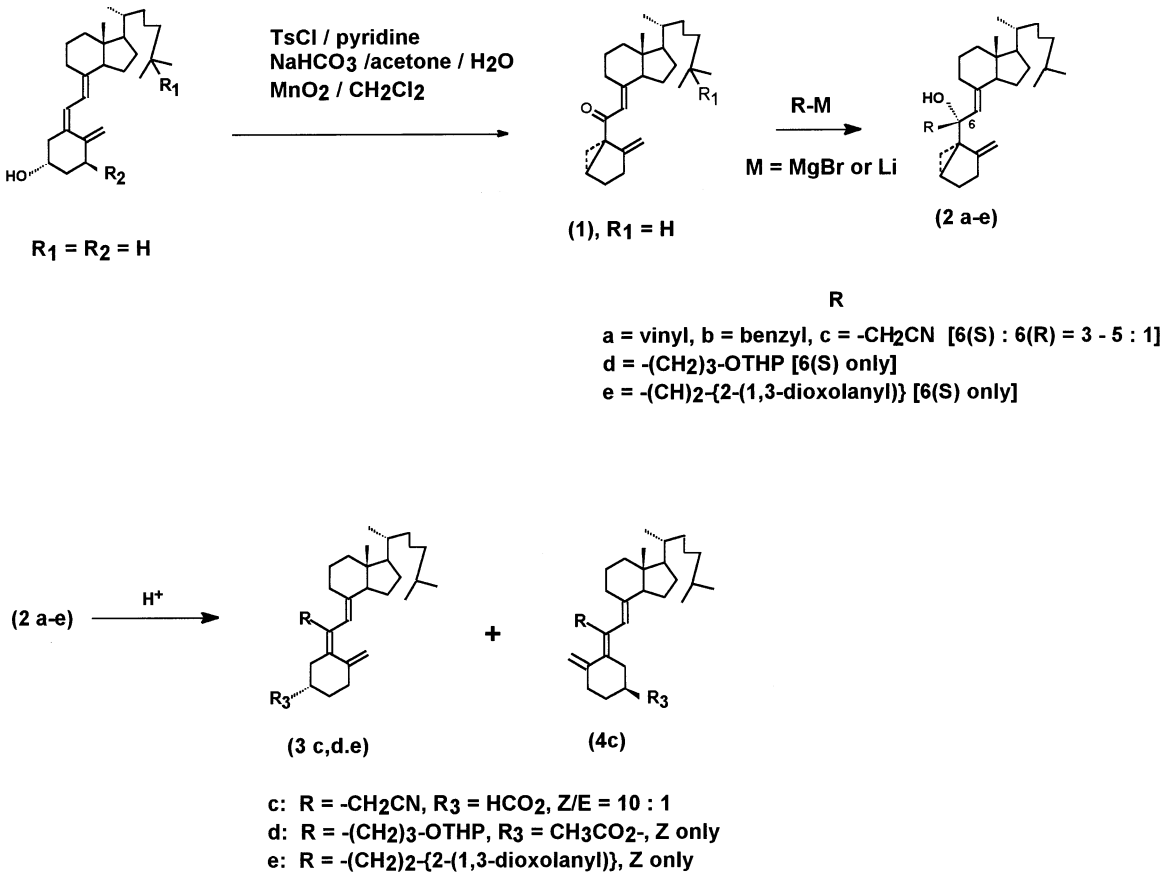


Fig. 1. Synthetic scheme involving nucleophilic addition to 3,5-cyclovitamin D_3 6-ketone and acid-catalyzed cyclo-reversion of the adducts.

OH-D_3. We also describe the results of competitive binding studies of these analogs with vitamin D-binding protein (DBP), a serum protein that specifically binds and transports vitamin D_3 and its metabolites to target organs, and vitamin D receptor (VDR), which specifically binds $1,25(OH)_2D_3$ in the nucleus of the target cells to trigger genomic actions that are responsible for multiple physiological properties of $1,25(OH)_2D_3$ [5,6].

2. Experimental

All the reagents were purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Anhydrous tetrahydrofuran (THF) and ether were obtained by distilling them from Na-benzophenone ketyl in an argon-atmosphere. Anhydrous CH_2Cl_2 was produced by distilling it from CaH_2. Nuclear magnetic resonance (NMR) spectra of the samples were obtained in $CDCl_3$-solution with tetramethylsilane (TMS) as the internal standard (unless mentioned otherwise) on either a 270 MHz (JEOL GSX 270, JEOL USA, Peabody, MA, USA) or a 400 MHz (JEOL GSX 400) spectrometer. Infrared spectra of samples in $CHCl_3$-solution were obtained in a Perkin Elmer 1430 IR Spectrophotometer (Norwalk, CT). Electrospray ionization mass spectrometry analysis of some samples was performed on a VG Quattro triple quadrupole mass spectrometer (Micromass, Inc., Manchester, UK) in positive ion mode. Reconstituted samples (1 μl) were introduced by flow injection in a solvent consisting of acetonitrile and 0.1% aqueous trifluoroacetic acid (60:40). Collision experiments were conducted with a collision energy of 30 V under argon. DBP (Gc-gloulin) and other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, unless otherwise mentioned. 25-Hydroxy[26(27)-^{3}H]vitamin D_3 (specific activity 20 Ci/mM) and 1,25-dihydroxy[26(27)-^{3}H]vitamin D_3 (specific activity 18 Ci/mM) were obtained from New England Nuclear, Boston, MA, USA. $1,25(OH)_2D_3$ and 25-OH-D_3 were generous gifts from Drs Milan R. Uskokovic (Hoffman-La Roche, Inc., Nutley, NJ, USA) and Richard Gray and James Yager (Amoco Research Center, Naperville, IL, USA) respectively.

2.1. 6(S)-[2-(1,3-dioxolanyl)ethyl]-3,5-cyclovitamin D_3 (2e)

To a mixture of ketone 1 (R_1 = H, 167 mg, 0.43 mmol) and excess Mg (20.8 mg, 0.87 mmol) was added anhydrous THF (2 ml) under a N_2 atmosphere, and the mixture was heated at 80°C. 2-(2-Bromoethyl)-1,3-dioxolane (71.8 μl, 0.6 mmol) was added into the reaction flask with a syringe.

After heating the mixture for 15 min, it was cooled to 0°C and diluted with 2 ml of ice-cold H_2O. After extraction with ethyl acetate (EtOAc) (3 × 5 ml), the organic phase was dried with anhydrous $MgSO_4$ and concentrated in vacuo. Preparative thin-layer chromotography (TLC) (15% EtOAc/hexane) gave 170 mg (74%) of the product as an oil. ^{1}H NMR: δ 0.54 (3H, s), 0.88 (6H, d, J = 6.6 Hz), 0.92 (3H, d, J = 6.1 Hz), 3.80–3.980 (4H, m), 4.82 (1H, t, J =), 4.9 (2H, bs), 5.19 (1H, s). ^{13}C NMR: δ 12.0, 14.56, 19.0, 22.29, 23.0, 23.9, 24.0, 25.0, 26.5, 27.5, 27.7, 27.9, 29.4, 31.3, 34.5, 36.4, 39.2, 40.9, 45.9, 56.5, 65.2, 76.1, 105, 106, 124, 142, 151.0. IR: 3480 cm^{-1}.

In a similar fashion, 6(S)-[3-(2-tetrahydro-2H-pyranyl) propoxy-3,5-cyclovitamin D_3 (**2d**) [^{1}H NMR: δ 0.54 (3H, s), 0.87 (6H, d, J = 6.9 Hz), 0.92 (3H, d, J = 6 Hz), 1.05–2.33 (m), 3.25–3.90 (4H, m), 4.60 (1H, t, J = 6.8 Hz), 4.89 (2H, bs), 5.18 (1H, s)], and 6(S)-benzyl-3,5-cyclovitamin D_3 (**2b**) (mixed with ~20% of 6(R)-isomer) [^{1}H NMR: δ 0.524 (3H, s), 0.88, (6H, d, J = 6.7 Hz), 0.93 (3H, d, J = 6.1 Hz), 1.06–2.3 (m), 3.15 (m), 5.01 (2H, bs), 5.29 (1H), [δ 5.45 and 5.52 for the 6(R)-diastereoisomer], and 7.2 (m) were obtained in 78.5% and 94.4% yields, respectively, by reaction of ketone **1** (R_1 = H, 100 mg, 0.26 mmol) with 2-(3-bromopropoxy)tetrahydro-2H-pyran (81.2 mg, 0.4 mmol) and Mg (12.5 mg, 0.5 mmol), and ketone **1** (R_1 = H, 30 mg, 0.08 mmol) with benzylbromide (18.6 mg, 0.1 mmol) and Mg (3.7 mg, 0.15 mmol), respectively.

*2.2. 6(S)-Cyanomethyl-3,5-cyclovitamin D_3 (**2c**)*

n-BuLi (1 M solution in hexane, 195 μl, 0.2 mmol) was added to a solution of acetonitrile, freshly distilled from CaH_2 (13.5 μl, 0.26 mmol) in anhydrous THF (2.7 ml) in a round-bottomed flask equipped with a stir bar at −78°C in a N_2 atmosphere. The solution was stirred at −78°C for 30 min, and the ketone **1** (R_1 = H, 50 mg, 0.13 mmol, in 2.7 ml THF) was added to this lithio-acetonitrile solution via a cannula. The reaction mixture was allowed to warm from −78°C to 25°C in 2 h. Water (5 ml) was added to the reaction mixture, and the organic phase was extracted with EtOAc (3 × 5 ml). The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated in vacuo. Preparative TLC (5% EtOAc/hexane) produced 30.5 mg (55%) of compound 2c. ^{1}H NMR: δ 0.55 (3H, s), 0.87 (6H, d, J = 6.6 Hz), 0.93 (3H, d, J = 6.2 Hz), 2.91–2.93 (2H, d), 4.98 (1H, bs), 5.09 (1H, bs), 5.15 (1H, bs). IR: 2245 and 3400 cm^{-1}. Mass spectrum (MS) calculated for $C_{29}H_{45}NO$: 423.68, observed 424.6 $[M + H]^+$.

*2.3. 6(S)-Vinyl-3,5-cyclovitamin D_3 (**2a**)*

To a mixture of ketone 1 (R_1 = H, 30 mg, 0.08 mmol) and excess Mg (7.5 mg, 0.3 mmol) in 1.0 ml THF at room temperature was added vinyl bromide (0.2 ml of 1 M solution in THF, 0.2 mmol). The reaction mixture was allowed to stir at 25°C for 30 m. After quenching with H_2O (4 ml), the organic phase was extracted with EtOAc and dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. Preparative TLC (15% EtOAc/Hexane) gave 20.6 mg (64%) of compound 2a. ^{1}H NMR: δ 0.55 (3H, s), 0.87 (6H, d, J = 6.5 Hz), 0.93 (3H, d, J = 6.2 Hz), 1.04–2.42 (m), 4.9 (5H, m), 5.05 (1H, broad s). ^{13}C NMR: δ 12.01, 14.00, 18.09, 22.61, 22.90, 23.65, 23.84, 25.55, 25.58, 27.90, 28.01, 30.10, 30.98, 35.95, 39.60, 40.06, 40.97, 45.89, 56.02, 56.56, 75.56, 105.6, 114.20, 124.70, 140.09, 144.29, 152.65.

*2.4. 6-Cyanomethyl-5Z-vitamin D_3-3-formate (**3c**)*

A solution of 2c (10 mg) and 97% HCOOH (250 μl) was heated at 55°C for 15 min, and then diluted by addition of H_2 (5 ml). The reaction mixture was extracted with ether (3 × 5 ml); the combined organic phase was dried over anhydrous $MgSO_4$ and concentrated in vacuo. Chromatography of the crude mixture using 20% EtOAc/hexane gave 4.5 mg (42.2%) of compound 3c. ^{1}H NMR: δ 0.64 (3H, s), 0.85 (6H, d, J = 6.6 Hz), 0.92 (3H, d, J = 6 Hz), 1.12–2.48 (m), 3.11–3.44 (2H, m), 4.89 (1H, s), 5.09 (1H, m), 5.19 (1H, s), 5.33 (1H, s), 7.99 (1H, s). ^{13}C NMR: δ 11.96, 18.81, 22.33, 22.55, 22.60, 22.80, 23.24, 23.28, 23.89, 27.59, 27.99, 29.89, 31.83, 31.91, 36.10, 37.15, 39.49, 40.19, 45.62, 55.55, 56.58, 70.78, 76.86, 77.0, 77.47, 113.53, 116.94, 118.55, 123.22, 136.53, 145.71, 146.62, 160.29.

*2.5. 6-Cyanomethyl-5E-vitamin D_3-formate (**4c**, ~4% yield)*

^{1}H NMR: δ 0.56 (3H, s), 0.89 (6H, d, J = 6.6 Hz), 0.96 (3H, d, J = 6.2 Hz), 1.04–2.31 (m), 2.95–3.15 (2H, m), 4.96 (1H, s), 5.06 (1H, m), 5.11 (1H, s), 5.34 (1H, s), 8.02 (1H, s).

*2.6. 6-[2-(1,3-Dioxolanyl)ethyl]vitamin D_3-acetate (**3e**)*

A solution of 6(S)-[2-(1,3-dioxolanyl)ethyl]-3,5-cyclovitamin D_3 (2e, 10 mg) in 400 μl of glacial HOAc was heated at 60°C for 15 min, and then gently quenched with an ice-cold saturated $NaHCO_3$ solution. The neutralized mixture was extracted with EtOAc (3 × 5 ml). The combined organic extract was dried over anhydrous $MgSO_4$ and concentrated in vacuo. Preparative TLC (5% EtOAc/hexane) produced 5.4 mg (50%) of compound 3e. ^{1}H NMR: δ 0.55 (3H, s), 0.87 (6H, d, J = 6.8 Hz), 0.94 (3H, d, J = 6.1 Hz), 1.15–1.75 (m), 1.98, (3H, s), 2.10–2.57 (m), 3.88 (4H, m), 4.71–4.89 (2H, m), 4.94, (1H, s), 5.24 (1H, s). Correlation spectroscopy (COSY) spectrum of 3e confirmed the assignment of δ 4.71 and 4.94 as the C_{19}-geminal protons. The 5,6-*Z*-stereochemistry of this compound was further confirmed by irradiation at δ 5.24 (C_7-H), which showed nuclear Overhauser effect with one of the C_{19} protons. ^{13}C NMR: δ 12.04, 18.86, 21.32, 22.40, 22.54, 22.81, 23.88, 27.82, 27.98, 28.95, 30.02, 30.20, 32.58, 32.01, 38.82, 39.46, 40.31, 45.32, 55.51, 56.54, 64.06, 72.79, 76.51,

77.10, 77.19 77.45, 111.60, 119.71, 131.78, 133.41, 142.45, 147.06, 170.43. MS [mass/charge (m/e)] calculated for $C_{34}H_{54}O_4$: 526.402, found 526.5. Infrared 1735 cm^{-1}.

*2.7. 6-[3-(2-tetrahydro-2H-pyranyl)propyl]vitamin D_3-acetate (**3d**)*

In a similar fashion, 37% of 3d was obtained. ^{1}H NMR: δ 0.56 (3H, s), 0.86 (6H, d, J = 6.6 Hz), 0.98 (3H, d, J = 6.2 Hz), 1.02–1.98 (m), 1.97 (3H, s), 2.02–2.31 (m), 3.34–3.83 (4H, m), 4.52, (1H, t, J = 2.95 Hz), 4.72, (1H, s), 4.82, (1H, m), 4.92 (1H, s), 5.26, (1H, s). ^{13}C NMR: δ 12.47, 18.83, 19.69, 21.53, 22.40, 22.53, 22.80, 23.88, 28.87, 28.88, 28.95, 29.90, 32.90, 33.01, 36.12, 37.92, 39.56, 40.32, 45.52, 55.61, 56.55, 62.54, 67.34, 72.01, 76.58, 77.10, 77.19, 77.45, 98.95, 111.85, 120.01, 131.79, 133.42, 142.36, 146.07, 170.49.

*2.8. 6-[3-(hydroxypropyl)]vitamin D_3-acetate (**14**)*

6-[3-(2-tetrahydro-2H-pyranyl)propyl]vitamin D_3-acetate (3d) was dissolved in methanol/H_2O (3:1, 1 ml) and *p*-TsOH (0.1 equivalent) was added to the solution. The reaction was stirred at room temperature for 1 h, followed by the addition of water and extraction with EtOAC. Preparative TLC of the reaction mixture (5% EtOAc/hexane) produced the compound 10.

*2.9. Attempted deprotection of the aldehyde group in (**3e**): Formation of the intramolecular Diels-Alder Adduct (**5**)*

A solution of compound 3e (10 mg) and p-TsOH (0.1 equivalent) in acetone (0.5 ml) was stirred at 25°C for 2 h when TLC indicated the complete absence of the starting material. Acetone was evaporated, and the crude reaction mixture was subjected directly to preparative TLC (5% EtOAc/hexane) to produce 4.8 mg (52.4%) of a product identified as 5. ^{1}H NMR: δ 0.59 (3H, s), 0.89 (6H, d, J = 6.5 Hz), 0.92 (3H, d, J = 6.2 Hz), 1.23 (3H, s), 1.31–1.99 (m), 1.6 (3H, s), 2.0 (3H, s), 2.09–2.19 (m), 2.55 (m), 2.88 (dd), 4.59 (1H, m), 4.80 (1H, m), 5.09 (1H, s). ^{13}C NMR: δ 12.06, 14.56, 18.99, 21.90, 22.99, 23.24, 23.53, 23.89, 27.82, 28.98, 29.85, 30.05, 32.32, 32.68, 32.02, 36.29, 38.59, 40.53, 41.45, 45.56, 54.88, 56.55, 71.25, 73.68, 81.99, 119.89, 123.68, 132.66, 146.79, 170.89. MS: calculated for $C_{32}H_{50}O_3$: 482.4, observed: 483.8 $[M + H]^+$. Sub-fragmentation of the 483.8 peak produced peaks at 423.0 (loss of acetate group as acetic acid), 405.4 (this is likely because of the loss of another 18 mass units from 423.0 as water, indicating that, upon fragmentation, the pyran ring opened up), and 247.2 (loss of C, D rings - H).

2.10. 25-Hydroxy, 6-[3-(2-tetrahydro-2H-pyranyl propyl)]-3,5-cyclovitamin D_3

To a mixture of the ketone 1 (R_1 = OH, Fig. 1) (prepared from 25-OH-D_3 by the same procedure as for vitamin D_3) (50 mg, 0.12 mmol) and excess Mg (12 mg, 0.5 mmol) was added anhydrous THF (2 ml) under N_2 atmosphere, and the mixture was heated at 80°C. 6-[3-(2-tetrahydro-2H-pyranyl propyl)] bromide (75 μl, 0.5 mmol), was added into the reaction flask by using a syringe. After heating the mixture for 15 min, it was cooled to 0°C and diluted with 2 ml ice-cold H_2O. After extraction with EtOAc (3 × 5 ml), the organic phase was dried with anhydrous $MgSO_4$ and concentrated in vacuo. Preparative TLC (30% EtOAc/hexane) gave 49.5 mg, (75%) of the product. ^{1}H NMR: δ 0.54 (3H, s), 0.87–0.92 (9H, m,), 1.05–2.31, (m), 3.25–3.51 (m), 3.67–3.90, (m), 4.54 (1H, t, J = 2.96 Hz), 4.90 (2H, m), 5.18 (1H, s).

*2.11. 25-Hydroxy, 6-[3-(2-tetrahydro-2H-pyranyl)propoxy]vitamin D_3-acetate (**13**)*

A solution of 10 mg of 25-hydroxy, 6-[3-(2-tetrahydro-2H-pyranyl propyl)]-3,5-cyclovitamin D_3 in 400 μl of glacial HOAc was stirred at room temperature for 1 h and then gently quenched with an ice-cold, saturated $NaHCO_3$ solution. The neutralized mixture was extracted with EtOAc (3 × 5 ml). The combined organic extract was dried over anhydrous $MgSO_4$ and concentrated in vacuo. Preparative TLC (25% EtOAc/hexane) produced 4.4 mg (40%) of compound 13. ^{1}H NMR: δ 0.56 (3H, s), 0.9 (3H, d, J = 6.7 Hz), 1.2 (6H, s), 1.02–1.98 (m), 1.97 (3H, s), 2.02–2.31 (m), 3.34–3.83 (4H, m), 4.52 (1H, t), 4.72 (1H, s), 4.82 (1H, m), 4.92 (1H, s), 5.26 (1H, s). ^{13}C NMR: δ 12.47, 18.83, 19.69, 21.53, 22.40, 22.53, 22.80, 23.88, 28.87, 28.88, 28.95, 29.90, 32.90, 33.01, 36.12, 37.92, 39.56, 40.32, 45.52, 55.61, 56.55, 62.54, 67.34, 72.01, 76.58, 77.10, 77.19, 77.45, 98.95, 111.85, 120.01, 131.79, 133.42, 142.36, 146.07, 170.49.

2.12. 25-hydroxy-6-[3-(2-tetrahydro-2H-pyranyl propyl)]vitamin D_3

Compound **13** (30 mg) was dissolved in 1.0 ml MeOH and treated with K_2CO_3 (21.3 mg) for 20 min. The reaction was then diluted with H_2O (2 ml) and extracted with EtOAc (2 × 5 ml), dried over anhydrous $MgSO_4$, and concentrated in vacuo to give 25-hydroxy-6-[3-(2-tetrahydro-2H-pyranyl propyl)]vitamin D_3 (14.6 mg, 51.4%). ^{1}H NMR: δ 0.58 (3H, s), 0.92 (3H, d, J = 6.3 Hz), 1.21 (6H, s), 1.07–2.58 (m), 3.28–3.88 (9H, m), 4.53 (1H, bs), 4.70 (1H, s), 4.92 (s), 5.30 (s).

*2.13. 25-Hydroxy, 6-[3-hydroxypropyl]vitamin D_3 (**15**)*

25-Hydroxy, 6-[3-(2-tetrahydro-2H-pyranyl propyl)]vitamin D_3 (5 mg) was dissolved in methanol/H_2O (3:1, 1 ml) and p-TsOH (catalytic amount) was added to the solution. The reaction was stirred at room temperature for 1 h. Water (3 ml) was added into the mixture and extracted with EtOAc. The organic phase was dried with anhydrous $MgSO_4$ and evaporated in vacuo. Preparative TLC (5%

EtOAc/Hexane) gave 2.4 mg (56%) of compound 15. ^{1}H NMR: δ 0.59 (3H, s), 0.92 (3H, d, J = 6Hz), 19 (6H, s), 1.12–2.68 (m), 3.49 (2H, m), 3.84 (1H, s), 4.71 (1H, s), 4.96 (1H, s), 5.38 (1H, s). Ultra violet spectrum of compound 11 (in methanol) had a λ_{max} at 239 nm, which correlated well with the λ_{max} for 6-methylvitamin D_3 [4].

2.14. 25-Hydroxy, 6-[3-(2-tetrahydro-2H-pyranyl propyl)]-3-tert. butyldiphenylsilylvitamin D_3

To a mixture of 25-hydroxy; 6-[3-(2-tetrahydro-2H-pyranyl propyl)]vitamin D_3 (12 mg, 0.02 mmol); imidazole (7.5 mg, 0.15 mmol); and N,N′-dimethylaminopyridine (DMAP) (catalytic), in 1 ml of dimethylformamide, was added tert-butyldiphenylsilylchloride (40 μl, 0.15 mmol). The reaction mixture was stirred for 1 h, from 0°C to 25°C. The mixture was then diluted with H_2O (2 ml) and extracted with EtOAc (3 × 5 ml), dried over $MgSO_4$, and evaporated in vacuo. The residue was purified by preparative TLC using 5% EtOAc/hexane to give (14.9 mg, 87%) of the product 25-hydroxy, 6-[3-(2-tetrahydro-2H-pyranylpropyl)]-3-tert-butyldiphenylsilyloxyvitamin D_3. ^{1}H NMR: δ 0.55 (3H, s), 0.89 (3H, d, 6.1 Hz), 0.94 (9H, s), 1.2 (6H, s), 1.05 (s), 1.14–2.48 (m), 3.27–3.89 (9H, m), 4.52 (1H, bs), 4.68 (1H, s), 4.86 (s), 5.21 (s), 7.26–7.44 (m), 7.58–7.72 (m).

2.15. 25-Hydroxy, 6-(3-hydroxypropyl), 3-tert. butyldiphenylsilylvitamin D_3

A mixture of 25-hydroxy-6-[3-(2-tetrahydro-2H-pyranyl) propyl]-3-tert-butyldiphenylsilylvitamin D_3 (10 mg) in 2 ml of a solvent mixture (AcOH:H_2O:THF; 4:2:1) was refluxed at 50°C for 30 min with stirring. The mixture was cooled to room temperature, and most of the solvent was removed under argon. The mixture was then diluted with H_2O (3 ml) and extracted with EtOAc (3 × 3 ml). The organic phase was dried over anhydrous $MgSO_4$ and concentrated in vacuo. The residue was purified using preparative TLC (25% EtOAc/hexane, as the eluent) to give (11.6 mg, 69%) of the desired product. ^{1}H NMR: δ 0.57 (3H, s), 0.89 (3H, d, J = 6.2 Hz), 0.98 (9H, s), 1.2 (6H, s), 1.17-2.41 (m), 3.49 (2H, t, J = 7.1 Hz), 3.82 (1H, m), 4.62 (1H, s), 4.88 (1H, s), 5.22 (1H, s), 7.29–7.41 (m), 7.48–7.64 (m).

2.16. 25-Hydroxy, 3-tert. butyldiphenylsilylvitamin D_3-6-(3-hydroxypropyl)-2-bromoacetate

A mixture of 25-hydroxy, 6-(3-hydroxypropyl), 3-tert-butyldiphenylsilylvitamin D_3 (5 mg, 0.01 mmol), $BrCH_2CO_2H$ (4.4 mg, 0.03 mmol), bromoacetic acid (DCC) (10 mg, 0.05 mmol) and DMAP (catalytic) was stirred in 1 ml of anhydrous CH_2Cl_2 at 25°C for 15 min. After the addition of 5 ml H_2O to quench the reaction, 5 ml of EtOAC was added. The organic layer was separated from the aqueous phase, dried with anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by preparative TLC (5% EtOAC/hexane) to give (4.4 mg, 75%) of 25-hydroxy, 3-tert-butyldiphenylsilylvitamin D_3-6-(3-hydroxypropyl)-2-bromoacetate.

*2.17. 25-Hydroxyvitamin D_3-6-(3-hydroxypropyl)-2-bromoacetate (**16**)*

A solution of 25-hydroxy, 3-tert-butyldiphenylsilylvitamin D_3-6-(3-hydroxypropyl)-2-bromoacetate (2 mg) and 48% aqueous hydrofluoric acid (8 μl) in 0.5 ml of acetonitrile was stirred in an argon atmosphere overnight followed by neutralization of the solution with saturated sodium bicarbonate solution and extraction of the aqueous solution with ether. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under argon. The reaction mixture was purified by preparative TLC to give 0.8 mg (55.8%) of compound 12. The UV spectrum of 12 in methanol displayed a triene absorption maximum at 239 nm. ^{1}H NMR: δ 0.56 (3H, s), 0.92 (3H, d, J = 6.3 Hz), 1.2 (6H, s), 1.02–2.41 (m), 3.77 (2H, s), 3.80 (1H, m), 4.07 (2H, t, J = 7 Hz), 4.61 (1H, s), 4.82 (1H, s), 5.19 (1H, s). This compound underwent complete isomerization to the previtamin D_3 compound, 25-hydroxy-pre-vitamin D_3-6-(3-hydroxypropyl)-3-bromoacetate, upon heating in methanol at 60°C for 1 h. ^{1}H NMR: δ 0.82–0.83 (overlapping singlets), 0.94 (3H, s), 0.95 (3H, s), 1.24 (bs), 1.02–2.21 (m), 3.81 (2H, s), 3.85 (1H, m), 4.08 (2H, m), 5.14 (1H, broad singlet), 5.82 (2H, s).

2.18. 25-Hydroxy, 6-[3-(2-tetrahydro-2H-pyranyl propyl)], 1α-hydroxyvitamin D_3-acetate and 25-hydroxy, 6-[3-(2-tetrahydro-2H-pyranyl propyl)], 1β-hydroxyvitamin D_3-acetate

To a mixture of 13 (40 mg, 0.07 mmol) and SeO_2 (22.8 mg, 0.21 mmol) in anhydrous CH_2Cl_2 (1 ml) was added tert-butyl hydroperoxide (90% in 1:1 H_2O/tert-butanol, 20 μl, 0.21 mmol) at 0°C. After stirring at 0°C for 30 min, the mixture was allowed to warm to 25°C, and after a total of 3 h the reaction was quenched by the addition of H_2O. The aqueous reaction mixture was extracted with EtOAc (3 × 5 ml), and the organic phase was dried with anhydrous $MgSO_4$ and concentrated in vacuo to give the crude product. Purification by preparative TLC (40% EtOAc/hexane) gave 19.5 mg (47.5%) of an inseparable mixture of hydroxylated products. ^{1}H NMR: δ 0.59 (3H, two overlapping singlets), 0.91 (3H, d, J = 6H), 1.21 (6H, s), 1.15–2.71 (m), 1.99 (3H, two overlapping singlets in approximately 3:1), 3.31–3.87 (4H, m), 3.96 (1H, m), 4.14 (1H, m), 4.38 (1H, m), 4.47 (1H, m), 4.9 (1H, m), 5.18–5.31 (m).

The above mixture (50 mg) was treated with K_2CO_3 (34.4 mg) in MeOH (1 ml) to produce a mixture of 1α- and 1β,25-dihydroxy, 6-[3-(2-tetrahydro-2H-pyranyl propyl)]-vitamin D_3 (26.1 mg, 55% combined). ^{1}H NMR: δ 0.59 (3H, two overlapping singlets), 0.92 (3H, d, J = 6.1 Hz),

1.17 (6H, s), 1.09–2.71 (m), 3.31–3.87 (4H, m), 4.15 (1H, m), 4.38 (1H, m), 4.52 (1H, m), 4.84 (1H, m), 5.15–5.21 (m), 5.32 (m).

The diastereoisomeric compounds, 1α,25-dihydroxy-6-(3-hydroxypropyl)vitamin D_3 (**17**) and 1β,25-dihydroxy-6-(3-hydroxypropyl)vitamin D_3 (**18**) (8.7 mg, 51% combined), separable by preparative TLC (40% EtOAC/hexane) were obtained from 10 mg of a mixture of 1α- and 1β-25-dihydroxy-6-[3-(2-tetrahydro-2H-pyranyl propyl)]vitamin D_3 and a catalytic amount of p-TsOH in MeOH-H_2O (3:1). Compound 17, 1α-isomer (6.5 mg, 38%), ^{1}H NMR: δ 0.59 (3H, s), 0.93 (3H, d, J = 6 Hz), 1.2 (6H, s), 1.12–2.11 (m), 2.31–2.40 (m), 2.61–2.79 (m), 3.50 (2H, m), 4.16 (1H, m), 4.41 (1H, m), 4.86 (1H, s), 5.21 (1H, s), 5.36 (1H, s). Compound 18, 1β-isomer (2.17 mg, 13%), ^{1}H NMR: δ 0.59 (3H, s), 0.91 (3H, s), 0.92 (3H, s), 1.05–2.43 (m), 2.70 (m), 3.05 (m), 3.61 (2H, m), 4.08 (1H, s), 4.40 (1H, s), 4.88 (1H, s), 5.16 (1H, s), 5.42 (1H, s).

*2.19. Competitive radioligand binding assays of 25-OH-D_3 and analogs (**15**), (**16**), and (**17**)*

These assays were according to published procedure [8]. Briefly, solutions, containing human serum DBP (100 ng); ^{3}H-25-OH-D_3 (3000 cpm); and 25-OH-D_3 (2, 4, 9, 18, 40, 80 pmol) or the analogs, 15: (2, 4.1, 8.2, 16.3, 32.7, 65.4 nmol), 16: (2, 4, 8, 15.8, 31.6, 63.2 nmol), and 17: (1.6, 3.2, 6.5, 13, 26, 51.8 nmol), or 1,25$(OH)_2D_3$ (0.15, 0.6, 2.4, 4.8, 9.6, 19.2 nmol) in 10 μl of ethanol; and 490 μl of assay buffer containing 50 mM Tris-HCl, 150 mM sodium chloride, 1.5 mM of ethylenediamine tetraacetic acid, and 0.1% Triton X 100 (Sigma Chemical Co., St. Louis, MO, USA), pH 8.3, were incubated at 4°C for 12 h followed by the addition of 100 μl of Dextran-coated charcoal. The samples were mixed by vortexing and incubated at 4°C for 20 min and centrifuged at 1200 $\times$ *g* for 15 min. The supernatant from each sample was mixed with 5 ml of liquid scintillation cocktail and counted in a liquid scintillation counter for radioactivity.

*2.20. Competitive radioligand binding assays of 1,25$(OH)_2D_3$ and (**17**) with recombinant human vitamin D receptor*

These assays were carried out according to the published procedure [9] with recombinant human VDR (50 ng/tube), ^{3}H-1,25$(OH)_2D_3$ (3000 cpm), rat liver nuclear extract (10 μg protein/tube), and ethanolic solutions (5 μl each) of various doses of either 1,25$(OH)_2D_3$ (0.02, 0.04, 0.08, 0.15, 0.30, 1.2, and 4.8 pmol) or the analog 17 (0.05, 0.16, 0.47, 1.4, 4.2, and 12.6 nmol) in buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 300 mM KCl, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium molybdate, and 5 mM dithiothreitol (DTT)]. Briefly, samples were incubated at 4°C for 14 h followed by the addition of 100 μl of Dextran-coated charcoal. The samples were mixed by vortexing and incubated at 4°C for 20 min and centrifuged at 1200 $\times$ *g* for 15 min. The supernatant from each sample was mixed with 5 ml of scintillation cocktail and counted in a liquid scintillation counter for radioactivity.

3. Results and discussion

3.1. Synthetic studies

Conversion of vitamin D_3 to the i-steroid, 3,5-cyclovitamin D_3, and the solvolysis of the latter back to vitamin D_3, is a well established procedure [10]. Earlier, Sheves and Mazur [4] treated 6-oxo-3,5-cyclovitamin D_3 (**1**, R_1 = H) with CH_3Li to obtain a 3:1 mixture of C-6 epimeric alcohols. They further noted that both the alcohols, upon acidic hydrolysis, produced a 1:1 mixture of 6-methyl-*Z*-vitamin D_3 (*cis*-triene geometry, as in the natural compound) and 6-methyl-E-vitamin D_3 (*trans*-triene geometry).

We chose to exploit Mazur's [4,7] procedure further for synthesizing C-6-modified analogs of vitamin D_3 and its metabolites. To this end, we selected nucleophiles with varying steric bulk and latent functionalities to study the stereochemistry of 1,2-addition to **1** in some detail and to elaborate these functional groups at a later stage, to potentially synthesize affinity/photoaffinity probes, which could be used to map the vitamin D sterol-binding pockets of DBP and VDR [8–23].

We observed that the addition of various nucleophiles to **1** predominantly produced one C-6 stereoisomer **2a**–**3**; and the extent of stereoselectivity depended on the steric bulk of the nucleophiles, as judged by the C-18 methyl-absorption at $\delta \sim 0.5$ in the ^{1}H NMR of the adducts. For example, high stereoselectivities were observed with reagents containing bulky dioxalanyl and pyranyl groups, which produced single stereoisomers **2d**, **e**. However, for groups with less steric demand, i.e. vinyl, benzyl, and cyanomethyl, the addition was less stereoselective (3–5:1 of the epimers, **2a**–**c**) as judged from two C-18 methyl signals in the NMR.

Treatment of the major isomer of 6-cyanomethyl 3,5-cyclovitamin D_3 **2c** with *p*-TsOH and AcOH [4,23] produced intractable mixture whereas, formic acid, under similar reaction conditions, produced 6-cyanomethyl 5,6-*Z*-vitamin D_3-formate 3c and 6-cyanomethyl 5,6-E-vitamin D_3-formate **4c** at 10:1. In contrast, adducts 2d and 2e were converted exclusively to the 5,6-*Z*-vitamin D_3 derivatives (**3d**,**e**, Fig. 1) by treatment with acetic acid. Earlier, Sheves and Mazur [4] reported that acidic solvolysis (p-TsOH/aqueous dioxane) of 6(R)-or 6(S)-methyl 3,5-cyclovitamin D_3 produced a 1:1 mixture of desirable 6-methyl-5,6-*Z*-vitamin D_3 and undesirable 6-methyl-5,6-E-vitamin D_3. However, in our case, the acid-catalyzed cycloreversion produced predominantly [as in the case of **2c**] or exclusively (as in the case of **2d** and **2e**) the desired 5,6-*Z*-vitamin D_3 derivatives **3c**,**d**,**e**.

Stereochemical outcome of the nucleophilic addition to

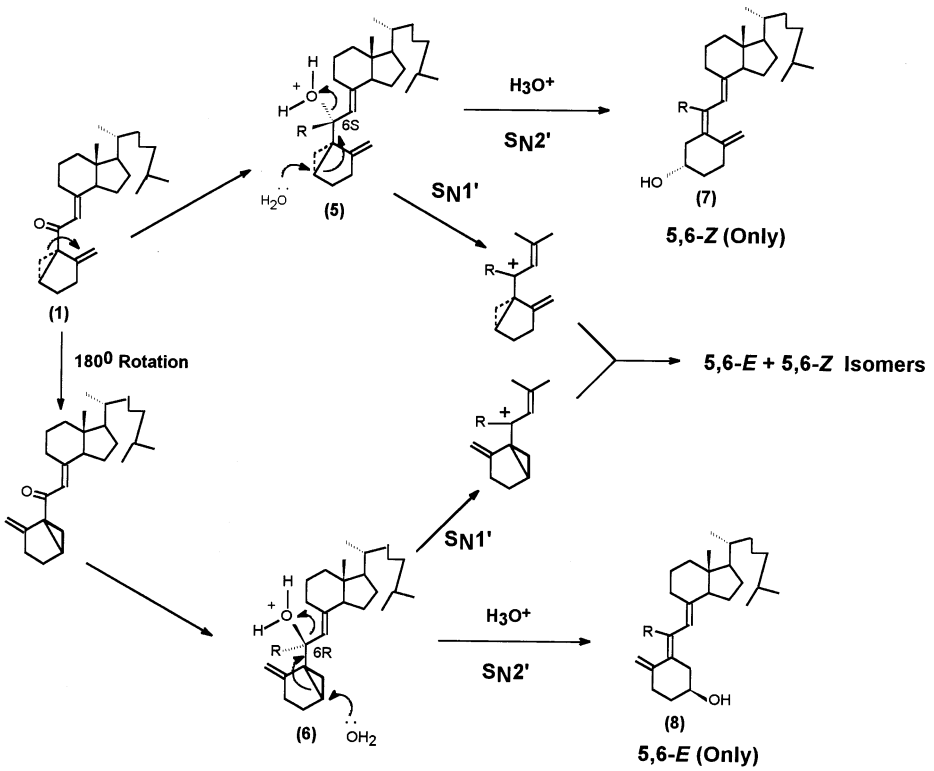


Fig. 2. Proposed mechanism for the acid-catalyzed solvolysis of the 3,5-cyclovitamin D_3 and its C-6 derivatives.

ketone 1 and acid-catalyzed solvolysis of the adducts **2a–e** deserved a close scrutiny. It is appropriate to assume that the nucleophilic attack at C-6 of ketone **1** would take place from the side opposite to that of the cyclopropyl envelope flap, particularly in the cases of bulky nucleophiles (e.g. **2d** and **2e**), producing, predominantly the 6(S)-*i*-steroid (**5**, Fig. 2). However, a 180° rotation along the 5,6-bond would bring the cyclopropyl group into the *β*-side, and the resultant nucleophilic attack would predominantly produce the 6(R)-*i*-steroid **6**.

It has been demonstrated that the cycloreversion step has a combination of S_N1' and S_N2' characters [4,7,24]. As shown in Fig. 2, a completely S_N2' mechanism should exclusively produce a 5,6-*Z*-vitamin **7** from a 6(S)-*i*-steroid **5**, and a 5,6-E-vitamin **8** from a 6(R)-*i*-steroid **6**. On the other hand, a predominant S_N1' character of the solvolysis step will produce a cyclopropylcarbonium ion intermediate, which in turn would produce an equal amount of 5,6-*Z* and 5,6-E-vitamins in the mixture. In our case, acidic solvolysis predominantly produced the 5,6-*Z*-isomers (Fig. 1), strongly suggesting that the solvolysis step might have an exclusively S_N2' character. Furthermore, a predominance of 5,6-*Z*-vitamin in the cycloreversion step dictated an S stereochemistry for C-6, and largely excluded rotation around 5,6-bond of the ketone **1**, which would produce 6(R)-adducts, and subsequently 5,6(E)-vitamins.

6-[2-(1,3-Dioxolanyl)ethyl]-vitamin D_3-acetate **3e**, containing a protected aldehyde group, was of particular interest to us. Aldehyde analogs of biological molecules are well known affinity-labeling agents because aldehydes can form Schiff's bases with basic amino acids of a protein under suitable reaction conditions [25]. We observed that acid-treatment (TsOH, HCl, formic acid/25°C) of **3e** quantitatively converted it into a single product 9 (Fig. 3). ^{1}H-NMR of **9** contained no aldehyde peak. Moreover, ^{13}C NMR spectrum contained two ^{13}C-O peaks at δ 73.68 and 81.99, in addition to the $^{13}C_3$-O peak at δ 71.25 (CH_3CO), which indicated the formation of a product by intramolecular rearrangement, which was supported by the mass spectrum of **9** ($MW_{calc.}$ 482.8; observed: 483.8 [M + H^+]). The presence of a 3H singlet at δ1.6 (allylic methyl absorption in the ^{1}H NMR spectrum of this compound) was unusual because it was not consistent with the parent seco-steroid structure. As a result, a structure such as **11**, formed by the direct cycloaddition between the aldehyde group (in **10**) and the 5(6), 10(19)-*cis*-diene part of the vitamin D_3 derivative, was ruled out.

Sheves and Mazur [4] reported that C-6-methylvitamin D_3 isomerized to 5,6-E-6-methylvitamin D_3 and 6-methyl tachysterol in the presence of visible light or iodine. This raised the possibility that either compound **3e** or the intermediate aldehyde **10** might have been converted to the corresponding 5,6-E-vitamin and the tachysterol derivative followed by an intramolecular cyclization. However, similar acidic treatments of compounds **3** and **13** exclusively produced the desired 5,6-*Z*-vitamins (Fig. 4), ruling out the possibility of acid-catalyzed isomerization of the 5,6-*Z*-triene structure.

It was reported earlier that C-6 methylvitamin D_3 isomerized to the pre-vitamin D_3 isomer upon brief heating at 90°C

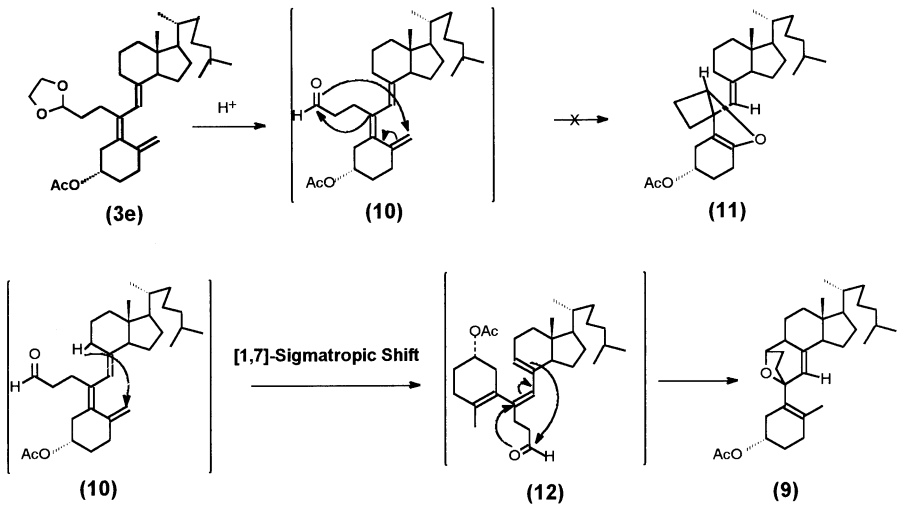
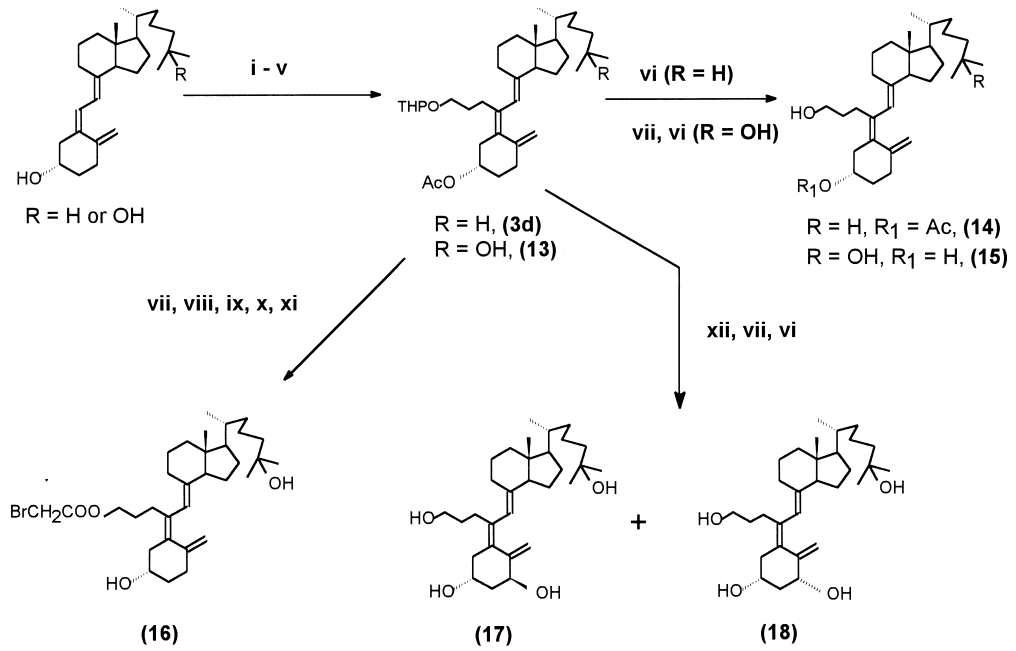


Fig. 3. Proposed mechanism for the acid-catalyzed intramolecular cycloaddition reaction of 6-[2-(1,3-dioxolanyl)ethyl]-vitamin D_3-acetate 3e.

[4]. This involved a 1,7-sigmatropic shift in a Woodward-Hoffman-allowed mode [26]. Recently, Garcia et al. [27] also observed that the equilibrium between C-6 methylvitamin D_3 and C-6 methyl pre-vitamin D_3 lies completely in the favor of the pre-vitamin D_3 form ($t_{1/2}$ = 15.3 h at 37°C). We also found that heating compounds **15**–**17** at 60°C in methanol for 1 h converted them completely to the corresponding pre-vitamin D_3 forms. Although compounds **10** was largely not expected to form the pre-vitamin form **12** under the mild reaction conditions (TsOH or formic acid, 25°C), the equilibrium mixture would certainly be contaminated with a small amount of **12**. We projected that the intermediate **12** might undergo an intramolecular hetero-Diels-Alder reaction to produce **9** [28]. Such a process would drive the **6** ⇄ **8** to the right, leading to the accumulation of the product **9**. Observed spectral data of the prod-



i = TsCl / pyridine / 4^{0}C, **ii** = $NaHCO_3$ / Acetone-H_2O / 60^{0}C, **iii** = MnO_2 / CH_2Cl_2, **iv** = THPO-$(CH_2)_3$-MgBr / THF
v = AcOH / 25^{0}C, **vi** = MeOH-H_2O / TsOH, **vii** = K_2CO_3 / MeOH, **viii** = TBDPSiCl / Imidadole / DMF,
ix = AcOH / THF-H_2O / 50^{0}C, **x** = $BrCH_2CO_2H$ / DCC / DMAP / CH_2Cl_2, **xi** = 0.5% aqueous HF /CH_3CN,
xii = SeO_2 / t-BuOOH / CH_2Cl_2

Fig. 4. Scheme for the synthesis of C-6-modified analogs of 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3.

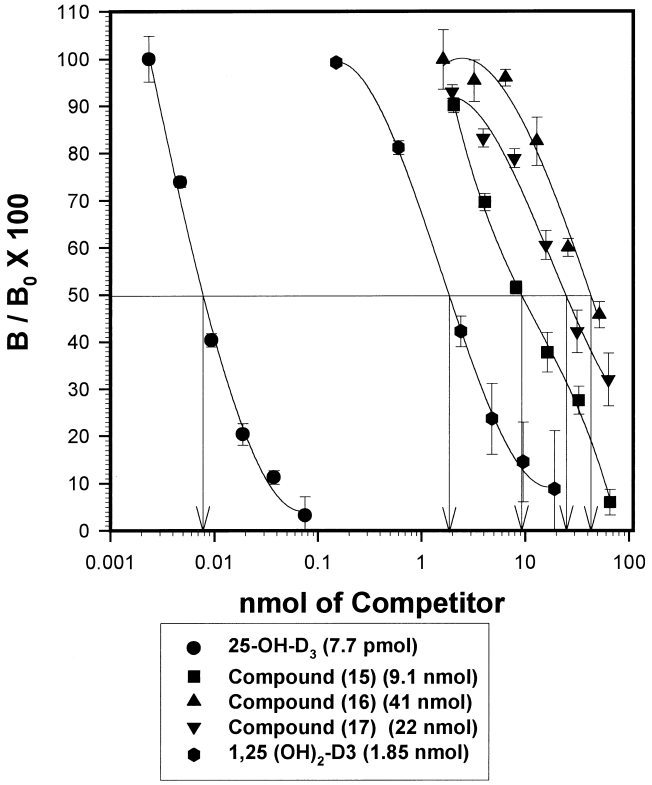


Fig. 5. Competitive radioligand binding assays of 25-OH-D_3, 1,25$(OH)_2D_3$ and the analogs **15**, **16**, and **7**. Briefly, solutions, containing human serum DBP (100 ng), ^{3}H-25-OH-D_3 (3000 cpm) and ethanolic solutions of 25-OH-D_3 (2, 4, 9, 18, 40, 80 pmol) or the analogs, **15**: (2, 4.1, 8.2, 16.3, 32.7, 65.4 nmol), 16: (2, 4, 8, 15.8, 31.6, 63.2 nmol), and **17**: (1.6, 3.2, 6.5, 13, 26, 51.8 nmol), or 1,25$(OH)_2D_3$ (0.15, 0.6, 2.4, 4.8, 9.6, 19.2 nmol) in the assay buffer were incubated at 4°C for 12 h followed by the addition of 100 μl of Dextran-coated charcoal. The samples were mixed by vortexing and incubated at 4°C for 20 min and centrifuged at 1200 × *g* for 15 min. Supernatant from each sample was mixed with 5 ml of liquid scintillation cocktail and counted in a liquid scintillation counter for radioactivity.

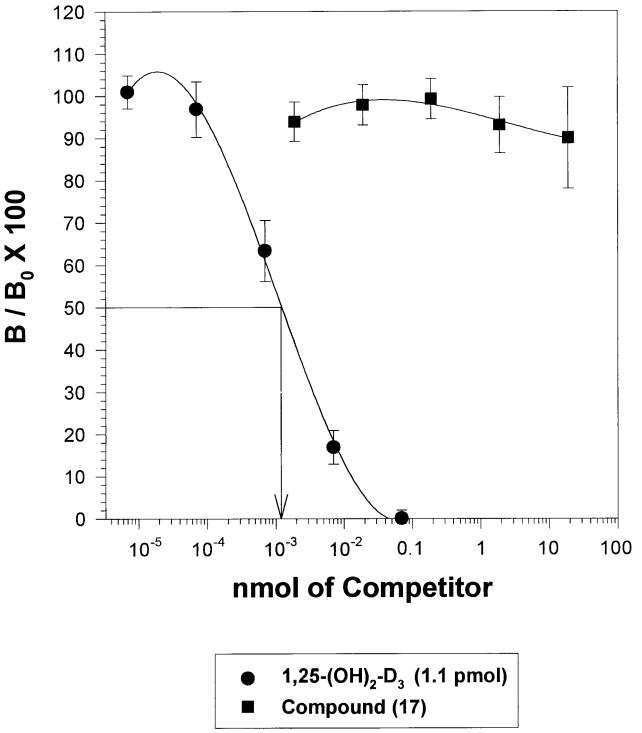


Fig. 6. Competitive radioligand binding assays of 1,25$(OH)_2D_3$ and **17** with recombinant human vitamin D receptor (VDR). Recombinant human VDR (50 ng/tube), ^{3}H-1,25$(OH)_2D_3$ (3000 cpm), rat liver nuclear extract (10 μg protein/tube), and ethanolic solutions of various doses of either 1,25$(OH)_2D_3$ (0.02, 0.04, 0.08, 0.15, 0.30, 1.2, and 4.8 pmol) or the analog **17** (0.05, 0.16, 0.47, 1.4, 4.2, and 12.6 nmol) in KTED buffer. The samples were incubated at 4°C for 14 h followed by the addition of 100 μl of Dextran-coated charcoal, mixed by vortexing and incubated at 4°C for 20 min, followed by centrifugation at 1200 × *g* for 15 min. Supernatant from each sample was mixed with 5 ml of scintillation cocktail and counted in a liquid scintillation counter for radioactivity.

uct **5** matched with the assigned structure. However, the true identity of **9**, including stereochemical assignments, would require further chemical and spectroscopic analysis, which is beyond the scope of this study.

Failure to deprotect the aldehyde in **3e** turned our attention to **3d**, which upon removal of the THP-protecting group, produced the alcohol **14**, which was suitable for further modification (attachment of an affinity probe) (Fig. 4). Having established the viability of our general synthetic scheme with commercially available vitamin D_3, we used 25-OH-D_3 as the starting material to synthesize C6-modified derivatives of 25-OH-D_3 and 1,25$(OH)_2D_3$ by standard synthetic manipulations (Fig. 4). The bromoacetate affinity analog of 25-OH-D_3 **16** was synthesized from **13** (Fig. 4).

3.2. Biochemical studies

These studies included competitive binding assays of various analogs with DBP and VDR to determine the binding affinities of these analogs for these proteins. In the DBP assays, binding of a fixed amount of radiolabeled natural ligand, i.e. ^{3}H-25-OH-D_3, to human serum, DBP was competed out by increasing concentrations of either 25-OH-D_3 or analogs **15**–**17**. As shown in Fig. 5, there was an ~1000–5000-fold decrease in the binding-affinity of DBP for the C6-modified analogs **15**, **16**, and **17** compared to 25-OH-D_3. Interestingly, the relative binding-affinity of **13**, containing a 1α-OH group, was found to be similar to those without it **15** and **16**, although the binding affinity of DBP for 1,25$(OH)_2D_3$ was 240 times less than that for 25-OH-D_3 (Fig. 5).

It is well established that VDR-binding affinity of 1,25$(OH)_2D_3$ is ~1000 times higher than that of 25-OH-D_3. Furthermore, 1β,25-dihydroxyvitamin D_3, the synthetic isomer of 1,25$(OH)_2D_3$ containing an unnatural 1β-hydroxyl group, does not display any binding towards VDR [1]. Hence, we tested the VDR-binding affinity of **17** only because only this analog contained the 1α-hydroxyl group as in the natural hormone. Results of these assays (Fig. 6) demonstrated that, although 1,25$(OH)_2D_3$ produced a dose-dependent displacement of ^{3}H-1,25$(OH)_2D_3$ from VDR, compound **17** failed to do so even at a very high concentration, indicating a lack of specific binding of **17** for VDR.

It is noteworthy that the UV-maxima of the C-6-modified

analogs shifted hypsochromically to 239 nm from a typical UV-maximum of 265 nm of the unmodified seco-steroids. This indicated that the co-planarity of the triene part of these molecules was significantly compromised, probably resulting in subtle structural changes in the three-dimensional geometry of these molecules. Importantly, these structural changes were enough to seriously compromise DBP- and VDR-binding.

In conclusion, we have developed synthetic procedures to obtain C6-functionalized analogs of vitamin D_3 and its biologically relevant metabolites. DBP-binding studies of these analogs demonstrated that modification of the C6-position significantly decreased DBP-binding, in sharp contrast to our recent observation that C-19-modification of 25-OH-D_3 is not significantly detrimental towards DBP-binding [29]. On the other hand, the C-6-modified 1,25$(OH)_2D_3$-analog **17** did not show any specific binding affinity for VDR, demonstrating, for the first time, that the triene part of 1,25$(OH)_2D_3$ is an important recognition marker for this process. Because VDR binding is a prerequisite for the majority of the biological functions of the vitamin D hormone, this information will be an important consideration in developing biologically relevant vitamin D analogs.

Acknowledgements

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