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Synthesis and biological activity of previtamin D_3 analogues with A-ring modifications

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ABSTRACT

Synthesis of two novel 6-*s*-*cis* analogues of 1α,25-dihydroxyvitamin D_3 are described using shikimic acid and its 4-*epi* isomer as versatile chiral starting materials. These derivatives contain a 2β-(3′-hydroxypropoxy) moiety or a 2β,3β-epoxy group into 1α,25-$(OH)_2$-19-*nor*-pre-D_3. The synthesized analogues were found to be not suitable for binding to the vitamin D receptor and showed weak binding affinity toward the vitamin D-binding protein. The new derivatives failed to inhibit cell proliferation.

1. Introduction

In the last decade, conformational, biological, physiological and chemical studies have enhanced our understanding of vitamin D mechanism of action.[1] The discovered biological activities of 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3 (**1**), Fig. 1], the hormonally active form, allow for application of vitamin D_3 (**2**) for the treatment of diseases such as cancer, psoriasis, osteoporosis, rheumatoid arthritis, and multiple sclerosis.[2] The biological responses of 1α,25-$(OH)_2$-D_3 are mediated via binding to the nuclear vitamin D receptor (VDR),[3] which belongs to the nuclear receptor superfamily and acts as a ligand-dependent transcription factor. In addition to these relatively slow (hours to days) genomic effects,[4] 1α,25-$(OH)_2$-D_3 generates a variety of non-genomic, rapid responses (seconds to minutes); some examples include the stimulation of intestinal Ca^{2+} transport (transcaltachia),[5] secretion of insulin by pancreatic β-cells,[6] opening of voltage–gated Ca^{2+} and Cl^- channels,[7] and the rapid migration of endothelial cells.[8] There is controversy as to the nature of the receptor that initiates non-genomic actions. In 1994 it was postulated a plasma membrane-associated receptor (VDR_{mem}).[9] However, new evidence indicates that these rapid actions are mediated by the classical VDR located near or associated with the plasma membrane or its caveolae components.[10]

The conformational flexibility of vitamin D_3 and its metabolites is unique among the steroid hormones.[11] This *seco* steroids can undergo a rotation around the 6,7 carbon-carbon single bond

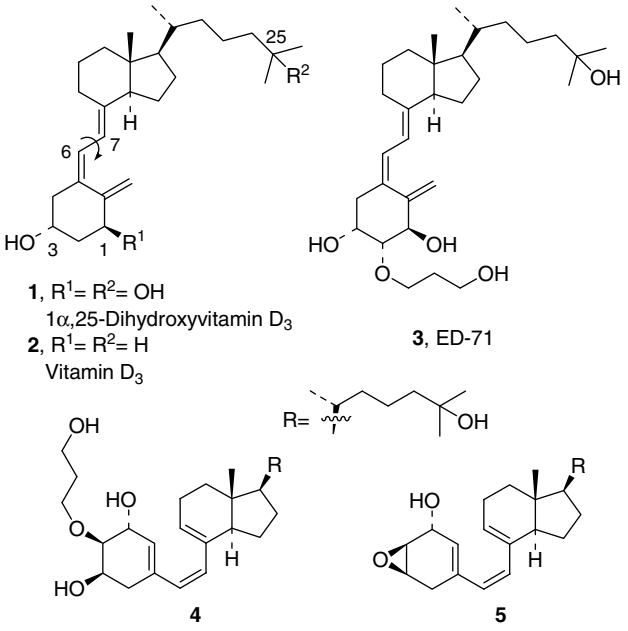

Figure 1. Structure of 1α,25-dihydroxyvitamin D_3 and its derivatives.

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which generates a wide array of molecular shapes extending from the 6-*s*-*cis* (steroid-like conformation) to the more stable extended 6-*s*-*trans* conformation. It is well documented that a 6-*s*-*trans* conformation is required for efficient gene transcription, while 6-*s*-*cis* locked metabolites activate a variety of non-genomic responses.[12] Scarce examples of 6-*s*-*cis* locked derivatives of vitamin D are described in the literature. This include the synthesis of several provitamin D diastereoisomers,[12c] stable previtamin D derivatives which are unable to undergo rearrangement to the respective vitamin D form by virtue of the absence of the C-19 methyl group,[13] or a 9,19-methano-bridged analogue of 1α,25-$(OH)_2$-D_3.[14] Surprisingly, the first previtamin D analogue, characterized by the presence of a *trans*-fused decalin CD-ring system, with genomic activities equivalent to 1α,25-$(OH)_2$-D_3 has been described.[15] This analogue interacted as efficiently as the natural hormone with the VDR and uses the same contact points within the receptor as did 1α,25-$(OH)_2$-D_3.

There are multiple vitamin D analogues currently used as treatment for a variety of diseases as well as several others in clinical trials. Of note is the ED-71 (**3**, Fig. 1), an analogue developed by Chugai Pharmaceuticals Co., which is currently under phase III clinical studies for the treatment of osteoporosis and bone fracture prevention.[16] This derivative has a 2β-(3′-hydroxypropoxy) group attached to the C-2 position of 1α,25-$(OH)_2$-D_3. As a consequence of their interesting biological profile, synthesis of ED-71 analogues has received considerable attention. Thus, synthesis of 19-*nor*,[17] 3-*epi*,[18] 2-hydroxyalkyl,[19] 2-fluoroalkyl,[20] and 2-hydroxyalkoxy[21] derivatives, or analogues of ED-71 having side chain modifications[22] have been described.

To elucidate further the structure–activity relationships of the natural hormone and its analogues we focused our attention on examining the introduction of a 2β-(3′-hydroxypropoxy) group at C-2 in 1α,25-$(OH)_2$-19-*nor*-pre-D_3, a 6-*s*-*cis* locked previtamin D_3 analogue (**4**). This derivative possess the same configuration in the A-ring that the previously synthesized analogue 1α,2β,25-$(OH)_3$-19-*nor*-pre-D_3, the most potent diastereomer in inhibiting proliferation on MCF-7 cells of a series of 2-hydroxy substituted 1α,25-$(OH)_2$-pre-D_3 derivatives.[13a] On the other hand, limited examples are available for vitamin D analogues possessing an epoxide in its skeleton. Introduction of an epoxy group in the side chain created some interesting vitamin D analogues whose cell differentiating activity exceeded their calcemic effects more than 100-fold.[23] Furthermore, some derivatives with the epoxy group at the triene system have been reported.[24] In addition, 3β-(1,2-epoxypropyl)ether-25-hidroxyvitamin D_3 has been described as an affinity labeling reagent of human DBP.[25] Herein, we also synthesized a previtamin D analogue containing an epoxy group in the A-ring (**5**). Results of preliminary evaluation of their biological properties are included.

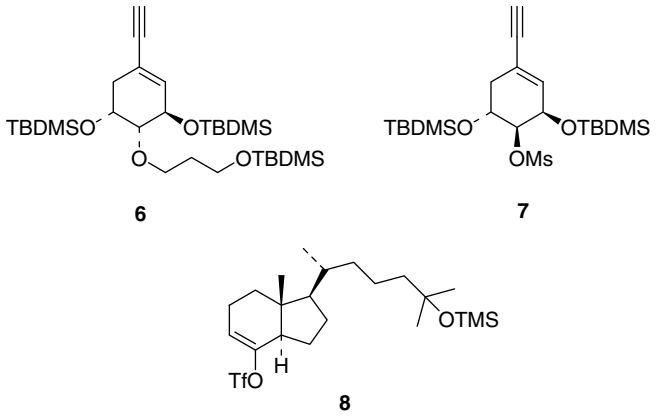


Figure 2. A-ring and CD-ring/side chain precursors.

2. Results and discussion

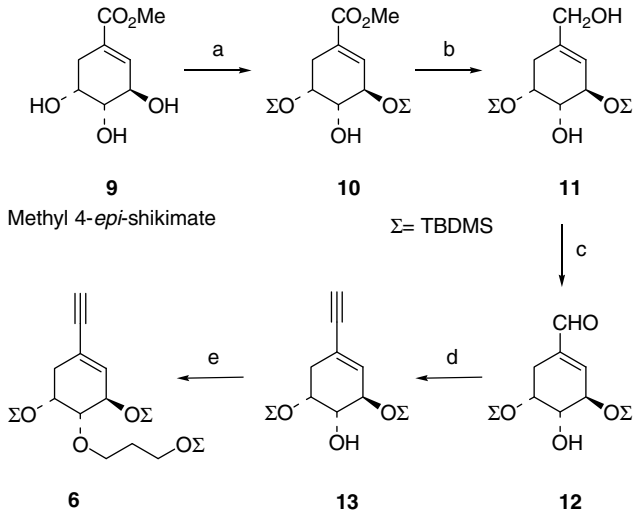
For the synthesis of both analogues **4** and **5**, standard Sonogashira coupling[26] of A-ring precursors **6** and **7** with an enol triflate of the CD-ring/side chain fragment (**8**)[14,27] was employed (Fig. 2).

The A-ring precursor **6** was synthesized from methyl 4-*epi*-shikimate (**9**), a versatile chiral building block with the correct hydroxy-stereochemistry. This compound was previously reported by us from shikimic acid through an efficient approach.[28] The preparation of **6** started with the selective protection of hydroxyl groups at C-3 and C-5 position of **9** (Scheme 1). Treatment of the latter with *tert*-butyldimethylsilyl chloride afforded compound **10**. Transformation of the ester into the aldehyde was best carried out via a two-step sequence. Thus, reduction of **10** with DIBALH gave the alcohol **11**, which upon oxidation of the allylic alcohol with MnO_2 yielded the aldehyde **12**.

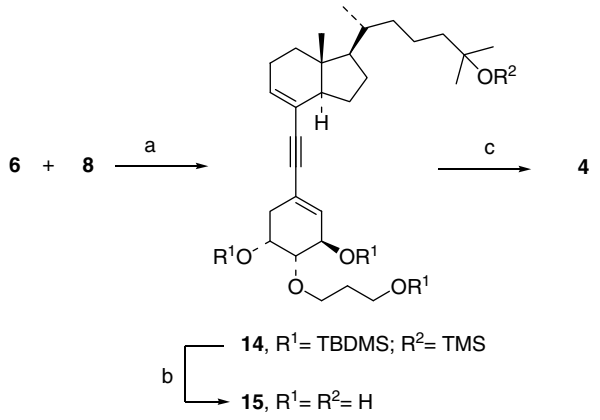
The reaction of the aldehyde with lithium trimethylsilyldiazomethane generates the alkyne **13**. Alkylation of the hydroxyl group at C-2 position was carried out by treatment with 1-bromo-3-[(*tert*-butyldimethylsilyl)oxy]propane to afford the key precursor **6** in high yield.

The 2β-(3′-hydroxypropoxy)-1α,25-$(OH)_2$-19-*nor*-pre-D_3 analogue (**4**) was successfully synthesized according to the reaction sequence shown in Scheme 2. The vinyl triflate **8** was treated with the A-ring synthon **6** in the presence of bis(triphenylphosphine)palladium (II) acetate-copper (I) iodide catalyst and Et_2NH in DMF affording the dienyne **14**, which after silyl ether deprotection with camphor sulfonic acid (CSA) in MeOH gave tetraol **15** in 70% yield. Careful catalytic hydrogenation of **15** in the presence of Lindlar catalyst and quinoline provided the desired previtamin D analogue **4**.

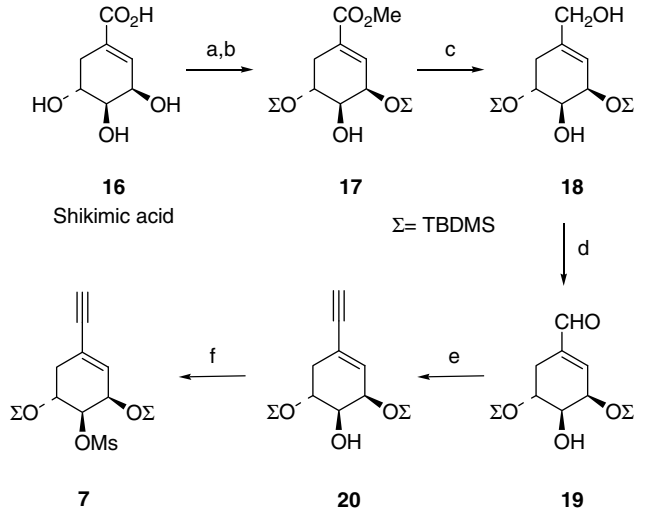
Synthesis of analogue **5** begins with A-ring precursor **7**, which was prepared starting from shikimic acid (Scheme 3). Transformation of **16** into **20** was performed in a similar manner as above described for **13**. Commercial shikimic acid was esterified and selectively protected to afford **17**. Reduction of the ester to aldehyde **19** was followed by formation of the enyne **20** by reaction



Scheme 1. Reagents and conditions: (a) TBDMSCl, Et_3N, DMAP, DMF, 0 °C → rt, 2 h (65%); (b) DIBALH, Et_2O, −78 °C, 2 h (81%); (c) MnO_2, CH_2Cl_2, 18 h (88%); (d) $TMSCHN_2$, ^nBuLi, THF, −78 °C → rt, 8 h (56%); (e) $Br(CH_2)_3OTBDMS$, NaH, DMF, −10 °C, 24 h (80%).



Scheme 2. Reagents and conditions: (a) $(PPh_3)_2Pd(OAc)_2$, CuI, Et_2NH, DMF, 4 h; (b) (−)-CSA, MeOH, overnight (70%, two steps); (c) H_2, Lindlar catalyst, quinoline, MeOH, 20 min (82% yield).



Scheme 3. Reagents and conditions: (a) HCl, MeOH, 60 °C, 6 h (quantitative); (b) TBDMSCl, Et_3N, DMAP, DMF, 0 °C → rt, 4 h (75%); (c) DIBALH, Et_2O, −78 °C, 3 h (75%); (d) MnO_2, CH_2Cl_2, 16 h (91%); (e) $TMSCHN_2$, ^nBuLi, THF, −78 °C → rt, 7 h (56%); (f) MsCl, pyridine, 2 h (89%).

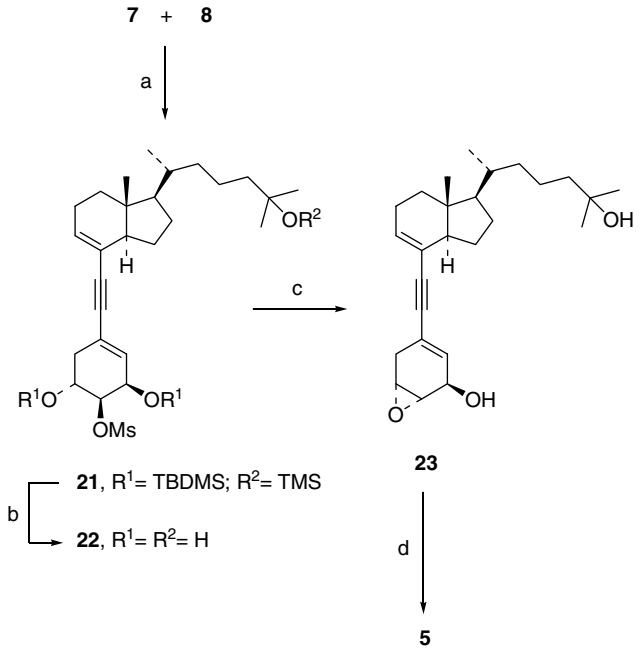
with $TMSCHN_2$. The resulting product **20** was treated with mesyl chloride to give mesylate **7**.

Sonogashira coupling of **7** with the CD-ring/side chain fragment **8** followed by direct desilylation afforded the corresponding dienyne **22** (Scheme 4). The latter was converted to epoxide **23** by treatment with DBU in THF at room temperature in 58% overall yield for the three steps. Compound **23** was next subjected to hydrogenation under standard conditions (Lindlar catalyst) to give the 2β,3β-epoxy-1α,25-$(OH)_2$-19-*nor*-pre-D_3 analogue **5**.

3. Biological evaluation

The synthesized 6-*s*-*cis* locked analogues **4** and **5** with the structural modification at the A-ring were examined for the binding affinity to the vitamin D receptor (VDR) and vitamin D-binding protein (DBP). In addition, the capacity to inhibit breast cancer MCF-7 cell proliferation was evaluated. The results are summarized in Table 1, the activities being shown as percentages of that of 1α,25-$(OH)_2$-D_3.

The 2β-(3′-hydroxypropoxy)-1α,25-$(OH)_2$-19-*nor*-pre-D_3 analogue **4** possessed no affinity for the VDR (Fig. 3). Introduction of a



Scheme 4. Reagents and conditions: (a) $(PPh_3)_2Pd(OAc)_2$, CuI, Et_2NH, DMF, 4 h; (b) (−)-CSA, MeOH, overnight; (c) DBU, THF, 24 h (58%, three steps); (d) H_2, Lindlar catalyst, quinoline, MeOH, 20 min (85% yield).

Table 1
Biological activity of 19-*nor*-pre-D_3 analogues

Compound	VDR (%)	hDBP (%)	MCF-7 (%)
1α,25-$(OH)_2$-D_3	100	100	100
1α,2β,25-$(OH)_3$-19-*nor*-pre-D_3[13a]	2	8	8
4	0	0.5	0
5	0	0.9	0

Summary of the in vitro effects of A-ring modified 19-*nor*-pre-D_3 analogues on receptor binding (VDR), interaction with human vitamin D-binding protein (hDBP), and inhibition of breast cancer MCF-7 cell proliferation. The in vitro effect is expressed as percentage activity at EC_{50} in comparison with 1α,25-$(OH)_2$-D_3 (=100% activity).

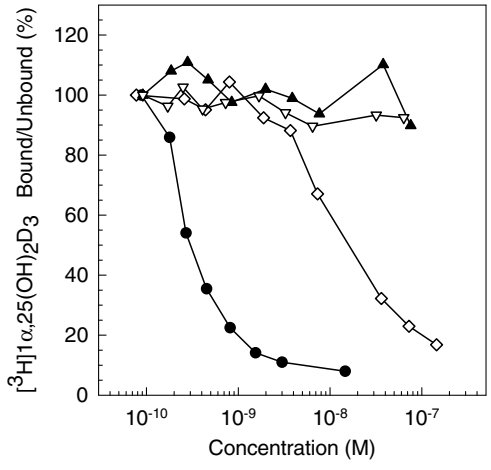


Figure 3. Affinity of 1α,25-$(OH)_2$-D_3 and 19-*nor*-pre-D_3 analogues for pig vitamin D receptor. 1α,25-$(OH)_2$-D_3 (●); 1α,2β,25-$(OH)_3$-19-*nor*-pre-D_3 (◊); **4** (▽); **5** (▲).

3′-hydroxypropyl group into the 1α,2β,25-$(OH)_3$-19-*nor*-pre-D_3[13a] decreased drastically its binding affinity. Similarly, 2β,3β-epoxy-1α,25-$(OH)_2$-19-*nor*-pre-D_3 analogue **5** did not bind to the VDR.

Both analogues displayed also markedly decreased affinity to the human DBP compared with the binding affinity of the analogue carrying a 2β-OH substituent. The epoxy derivative **5** probed to be 8-fold and 100-fold less potent than 1α,2β,25-$(OH)_3$-19-*nor*-pre-D_3[13a] and 1α,25-$(OH)_2$-D_3,[15] respectively, to bind to DBP.

Since the 19-*nor*-pre-D_3 analogues **4** and **5** have no affinity at all for the VDR, these analogues were unable to inhibit MCF-7 breast cancer cell proliferation (Fig. 4).

4. Conclusions

We have described the synthesis and biological evaluation of 6-*s-cis* locked vitamin D analogues with structural modifications at the A-ring. These novel target compounds have been prepared in order to investigate important structure–activity features. We have demonstrated the versatility of shikimic acid and its 4-*epi* isomer for the synthesis of vitamin D analogues. Data from biological assays indicate that 2β-(3′-hydroxypropoxy)-1α,25-$(OH)_2$-19-*nor*-pre-D_3 and 2β,3β-epoxy-1α,25-$(OH)_2$-19-*nor*-pre-D_3 possessed no affinity for the vitamin D receptor and bound very poorly to the vitamin D-binding protein. Unfortunately these analogues showed no antiproliferative activity.

5. Experimental

5.1. General

Synthesis of **8**[14,27] and **9**[28] was previously reported. HPLC semi-preparative was performed using a Zorbax Sil PrepHT column, 7 μm, 250 × 21.2 mm. Column chromatography was performed over silica 60 Å (230–400 mesh) or silica 60 Å (32–63 μm) pH 7.

5.2. 1α,25-Dihydroxy-2β-(3′-hydroxypropoxy)-19-*nor*-previtamin D_3 (4)

A flask containing Lindlar catalyst (45 mg) was exposed to a positive pressure of hydrogen gas (balloon). A solution of **15** (17 mg, 0.036 mmol) in MeOH (1.8 mL) and quinoline (130 μL of 0.17 M in hexane, 0.022 mmol) were added. The reaction was stirred vigorously during 20 min. The mixture was filtered on Celite, concentrated, and the crude subjected to flash chromatography using silica 60 Å (32–63 μm) pH 7 (gradient elution with 10–30% acetone/CH_2Cl_2). Further purification by HPLC (Zorbax sil PrepHT, 10 mL/min, hexane/IPA, 70:30) afford **4** in 82% yield. ^{1}H NMR (400.13 MHz, $CDCl_3$): δ 0.69 (s, 3H, Me_{18}), 0.95 (s, 3H, Me_{21}, $^3J_{HH}$ 6.4 Hz), 1.04 (m, 1H), 1.21 (s, 6H, Me_{26} + Me_{27}), 1.2–1.5 (m, 12H), 1.7–2.0 (5H, m), 2.1–2.3 (m, 4H), 2.66 (d, 1H, H_{4e}, $|^2J_{HH}|$ 17.6, $^3J_{HH}$ 5.9 Hz), 3.28 (dd, 1H, H_2, $^3J_{HH}$ 9.8, 4.4 Hz), 3.73 (m, 1H), 3.8–3.9 (m, 3H), 4.04 (ddd, 1H, H_3, $^3J_{HH}$ 9.8, 9.8, 4.4 Hz), 4.46 (dd, 1H, H_1, $^3J_{HH}$ 4.4, 4.4 Hz), 5.99 (s, 1H, H_{10}) and 6.01 (s, 1H, H_9) ppm; (ESI^+, *m*/*z*): 476 [$(M)^+$, 45%], 499 [$(M+Na)^+$, 20%].

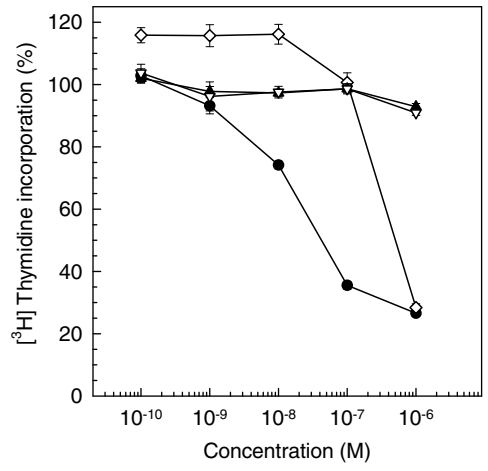


Figure 4. In vitro antiproliferative effects of 1α,25-$(OH)_2$-19-*nor*-pre-D_3 analogues on breast cancer MCF-7 cells. 1α,25-$(OH)_2$-D_3 (●); 1α,2β,25-$(OH)_3$-19-*nor*-pre-D_3 (◊); **4** (▽); **5** (▲).

5.3. 2β,3β-Epoxy-1α,25-dihydroxy-3-deoxy-19-*nor*-previtamin D_3 (5)

A similar procedure as that described for **4** afforded **5** in 85% yield. Flash chromatography (EtOAc) was performed using silica 60 Å (32–63 μm) pH 7. Further purification by HPLC (Zorbax sil PrepHT, 10 mL/min, hexane/IPA, 90:10). ^{1}H NMR (600.13 MHz, $CDCl_3$): δ 0.74 (s, 3H, Me_{18}), 0.99 (s, 3H, Me_{21}, $^3J_{HH}$ 6.1 Hz), 1.10 (m, 1H), 1.24 (s, 6H, Me_{26} + Me_{27}), 1.3–1.6 (m, 11H), 1.9–2.0 (m, 2H), 2.0–2.1 (m, 2H), 2.3–2.4 (m, 3H). 2.72 (d, 1H, H_4, $|^2J_{HH}|$ 19.7), 2.81 (d, 1H, H_4, $|^2J_{HH}|$ 19.6), 3.30 (s, 1H, H_2), 3.38 (s, 1H, H_3), 4.59 (s, 1H, H_1), 5.44 (s, 1H, H_9), 5.69 (s, 1H, H_{10}) and 5.81 (s, 2H, H_6 + H_7) ppm; ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 11.3 (C_{18}), 18.7 (C_{21}), 20.8 (CH_2), 23.2 (CH_2), 24.7 (CH_2), 27.5 (C_4), 28.3 (CH_2), 29.2 (CH), 29.3 (CH), 36.1 (CH_2), 36.2 (C_{26} and C_{27}), 36.4 (CH_2), 42.1 (C), 44.4 (CH_2), 50.8 (C_3), 51.1 (CH) 54.3 (C_2), 63.9 (C_1), 71.1 (C),, 119.6 (C_3), 124.2 (C_{10}), 125.6 (C_9), 129.6 (C_6), 131.9 (C_7), 134.0 (C_5) and 136.4 (C_8) ppm; (ESI^+, *m*/*z*): 423 [$(M+Na)^+$, 100%].

5.4. (3*R*,4*R*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-4-[((3′-*tert*-butyldimethylsilyl)oxy)propoxy]-1-ethynylcyclohex-1-ene (6)

NaH (246 mg, 60% in mineral oil, 5.89 mmol) was added to a solution of **13** (75 mg, 0.20 mmol) in anhydrous DMF at −10 °C. After 10 min, it was added dropwise 1-bromo-3-[(*tert*-butyldimethylsilyl)oxy]propane (220 μg, 1 mmol). The mixture was stirred at this temperature during 24 h. The reaction was quenched with water and allowed to reach room temperature. The aqueous layer was extracted with Et_2O. The combined organic fractions were dried (Na_2SO_4) and concentrated, and the residue purified by flash chromatography using silica 60 Å (32–63 μm) pH 7 (gradient elution with hexane–2% Et_2O/hexane) affording **6** as a colorless oil in 80% yield. IR (NaCl): υ 3455, 3316, 2955, 2885 and 2858 cm^{-1}; ^{1}H NMR (400.13 MHz, $CDCl_3$): δ 0.06 (s, 6H, 2Si*Me*), 0.08 (s, 3H, Si*Me*), 0.09 (s, 6H, 2Si*Me*), 0.10 (s, 3H, Si*Me*), 0.88 (s, 9H, SiC*Me*$_3$), 0.91 (s, 9H, SiC*Me*$_3$), 1.78 (q, 2H, $2H_2'$, $^3J_{HH}$ 6.4 Hz), 2.21 (dd, 1H, H_{6e}, $|^2J_{HH}|$ 16.4, $^3J_{HH}$ 4.4 Hz), 2.39 (dd, 1H, H_{6a}, $|^2J_{HH}|$ 16.4, $^3J_{HH}$ 8 Hz), 2.86 (s, 1H, H_8), 3.63 (t, 2H, $H_{3'}$, $^3J_{HH}$ 6.4 Hz), 3.69 (t, 2H, $2H_{1'}$), 3.74 (br s, 1H, H_4), 3.79 (br s, 1H, H_3), 3.94 (ddd, 1H, H_5, $^3J_{HH}$ 5.2, 5.2, 1.6 Hz) and 6.08 (br s, 1H, H_2) ppm; ^{13}C NMR (100.13 MHz, $CDCl_3$): δ −5.3 (Si*Me*), −4.8 (Si*Me*), −4.7 (Si*Me*), −4.5 (Si*Me*), −4.4 (Si*Me*), 18.1 (Si*C*), 18.2 (Si*C*), 18.3 (Si*C*), 25.8 (SiC*Me*$_3$), 26.0 (SiC*Me*$_3$), 31.0 ($C_{2'}$), 33.4 (C_6), 59.8 ($C_{3'}$), 66.7 ($C_{1'}$), 68.5 (C_5), 72.3 (C_3), 76.1 (C_8), 78.3 (C_4), 84.2 (C_7), 123.7 (C_1) and 132.0 (C_2) ppm; (ESI^+, *m*/*z*): 577 [$(M+Na)^+$, 100%].

5.5. (3*R*,4*S*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-1-ethynyl-4-(methanesulfonyloxy)cyclohex-1-ene (7)

To a stirred solution of compound **20** (65 mg, 0.17 mmol) in anhydrous pyridine (1 mL), was added methanesulfonyl chloride dropwise (21 μL, 0.26 mmol). Then, the solution was stirred for 2 h at room temperature. The reaction was quenched by adding an aqueous saturated solution of $NaHCO_3$ and extracting with Et_2O. The combined organic fractions were dried (Na_2SO_4) and concentrated, and the residue purified by flash chromatography using silica 60 Å (32–63 μm) pH 7 (gradient eluent, 5–20% Et_2O/hexane) yielding **7** as a colorless oil in 89%. IR (NaCl): υ 3277, 2950, 2929,

2881 and 2851 cm^{-1}; ^1H NMR (300.13 MHz, $CDCl_3$): δ 0.12 (s, 3H, Si*Me*), 0.13 (s, 3H, Si*Me*), 0.15 (s, 3H, Si*Me*), 0.15 (s, 3H, Si*Me*), 0.91 (s, 9H, SiC*Me*$_3$), 0.92 (s, 9H, SiC*Me*$_3$), 2.40 (m, 2H, $2H_6$), 2.92 (s, 1H, H_8), 3.10 (s, 3H, *Me*S), 4.30 (ddd, 1H, H_5, $^3J_{HH}$ 5.4, 5.4, 1.3 Hz), 4.47 (dd, 1H, H_3, $^3J_{HH}$ 4.2, 4.2 Hz), 4.54 (dd, 1H, H_3, $^3J_{HH}$ 1.5, 4.8 Hz) and 5.98 (s, 1H, H_2) ppm; ^{13}C NMR (100.13 MHz, $CDCl_3$): δ −4.9 (Si*Me*), −4.8 (Si*Me*), −4.7 (Si*Me*), 18.0 (Si*C*), 18.1 (Si*C*), 25.7 (SiC*Me*$_3$), 25.8 (SiC*Me*$_3$), 35.4 (C_6), 38.3 (*Me*S) 66.1 (C_5), 68.2 (C_3), 83.2 (C_7), 83.5 (C_4), 120.2 (C_1) and 133.4 (C_2) ppm; (ESI$^+$, *m*/*z*): 483 [(M+Na)$^+$, 100%].

5.6. Methyl (3*R*,4*R*,5*R*)-3,5-di[(*tert*-butyldimethylsilyl)oxy]-4-hydroxycyclohex-1-enecarboxylate (10)

Anhydrous Et_3N (1.2 mL, 8.92 mmol), DMAP (155 mg, 1.27 mmol) and TBDMSCl (1.1 g, 7.65 mmol) were added to a solution of **9** (480 mg, 2.55 mmol) in anhydrous DMF (5 mL) at 0 °C. The mixture was stirred at room temperature during 2 h. Next, the reaction was quenched with water and the aqueous layer was extracted with Et_2O. The combined organic fractions were dried (Na_2SO_4) and concentrated, and the residue purified by flash chromatography using silica 60 Å (230–400 mesh) (gradient elution with 3–5% EtOAc/hexane) affording **10** as a white solid in 65% yield. Mp: 70–72 °C; IR(NaCl): υ 3508, 2958, 2927, 2895, 2854 and 1711 cm^{-1}; ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.09 (s, 3H, 3Si*Me*), 0.10 (s, 3H, Si*Me*), 0.13 (s, 3H, 3Si*Me*), 0.13 (s, 3H, Si*Me*), 0.90 (s, 9H, SiC*Me*$_3$), 0.91 (s, 9H, SiC*Me*$_3$), 2.36 (dd, 1H, H_{6a}, $|^2J_{HH}|$ 17.8, $^3J_{HH}$ 6.7 Hz), 2.54 (dd, 1H, H_{6e}, $|^2J_{HH}|$ 17.8, $^3J_{HH}$ 4.9 Hz), 3.65 (dd, 1H, H_4, $^3J_{HH}$ 4.4, 2.2 Hz), 3.75 (s, 3H, H_8), 4.11 (ddd, 1H, H_5, $^3J_{HH}$ 7.0, 4.8, 2.2 Hz), 4.35 (dd, 1H, H_3, $^3J_{HH}$ 3.7, 3.7 Hz) and 6.71 (br s, 1H, H_2) ppm; ^{13}C NMR (100.13 MHz, $CDCl_3$): δ −4.9 (Si*Me*), −4.8 (Si*Me*), −4.7 (Si*Me*), −4.6 (Si*Me*), 18.1 (Si*C*), 25.8 (SiC*Me*$_3$), 30.3 (C_6), 51.9 (C_8), 67.9 (C_5), 69.7 (C_3), 74.3 (C_4), 128.3 (C_1), 137.1 (C_2) and 167.1 (C_7) ppm; (ESI$^+$, *m*/*z*): 417 [(M+H)$^+$, 100%].

5.7. (3*R*,4*R*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-4-hydroxy-1-hydroxymethylcyclohex-1-ene (11)

DIBALH (2 mL of 1.0 M in toluene, 2 mmol) was added dropwise to a solution of **10** (210 mg, 0.50 mmol) in anhydrous Et_2O (3 mL) at −78 °C, and the reaction was stirred for 2 h at the same temperature. An aqueous solution of potassium and sodium tartrate (1.0 M) was added and the mixture was warmed to room temperature, diluted with Et_2O, and filtered through a short column of silica gel, using additional Et_2O to elute the column. The filtrate was concentrated and the crude was purified by flash chromatography with silica 60 Å (230–400 mesh) (gradient elution with 20–30% EtOAc/hexane) to afford **11** as a white solid in 81% yield. Mp: 97–99 °C; IR(KBr): υ 3505, 2947, 2928, 2895, 2856 and 1666 cm^{-1}; ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.10 (s, 3H, Si*Me*), 0.11 (s, 3H, Si*Me*), 0.11 (s, 3H, Si*Me*), 0.12 (s, 3H, Si*Me*), 0.91 (s, 18H, 2SiC*Me*$_3$), 2.2 (m, 3H, $2H_6$+OH), 3.68 (br s, 1H, H_4), 3.64 (br s, 1H, H_4), 4.04 (m, 2H, H_7), 4.14 (ddd, 1H, H_5, $^3J_{HH}$ 7.2, 7.2, 2.4 Hz), 4.26 (br s, 1H, H_3) and 5.59 (s, 1H, H_2) ppm; ^{13}C NMR (100.13 MHz, $CDCl_3$): δ −4.8 (Si*Me*), −4.7 (Si*Me*), −4.6 (Si*Me*), −4.5 (Si*Me*), 18.1 (Si*C*), 18.2 (Si*C*), 25.8 (SiC*Me*$_3$), 25.8(SiC*Me*$_3$), 31.1 (C_6), 66.2 (C_7), 67.9 (C_5), 69.8 (C_3), 74.9 (C_4), 121.6 (C_2) and 137.8 (C_1) ppm; (ESI$^+$, *m*/*z*): 411 [(M+Na)$^+$, 50%] and 799 [(2M+Na)$^+$, 50%].

5.8. (3*R*,4*R*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-4-hydroxycyclohex-1-enecarbaldehyde (12)

MnO_2 (224 mg, 2.60 mmol) was added to a solution of **11** (100 mg, 0.26 mmol) in anhydrous CH_2Cl_2 (2.5 mL). The reaction mixture was stirred at room temperature for 18 h. The mixture was filtered through a short column of Celite and washed with CH_2Cl_2. The filtrate was concentrated to afford **12** as a white solid (88% yield), which was sufficiently pure for direct use in the next step. This aldehyde is instable and should be kept in the refrigerator. Mp: 52–54 °C; IR(KBr): υ 3506, 2957, 2928, 2894, 2853 and 1665 cm^{-1}; ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.09 (s, 3H, Si*Me*), 0.11 (s, 3H, Si*Me*), 0.16 (s, 6H, Si*Me*), 0.89 (s, 9H, SiC*Me*$_3$), 0.93 (s, 9H, SiC*Me*$_3$), 2.30 (dd, 1H, H_{6a}, $|^2J_{HH}|$ 18, $^3J_{HH}$ 6.4 Hz), 2.48 (dd, 1H, H_{6e}, $|^2J_{HH}|$ 18, $^3J_{HH}$ 4.8 Hz), 3.70 (br s, 1H, H_4), 4.14 (ddd, 1H, H_5, $^3J_{HH}$ 4.8, 4.8, 2 Hz), 4.49 (dd, 1H, H_3, $^3J_{HH}$ 3.6, 3.6 Hz), 6.51 (s, 1H, H_2) and 9.52 (s, 1H, H_7) ppm; ^{13}C NMR (100.13 MHz, $CDCl_3$): δ −4.9 (Si*Me*), −4.8 (Si*Me*), −4.7 (Si*Me*), −4.6 (Si*Me*), 18.1 (Si*C*), 18.0 (Si*C*), 25.7 (SiC*Me*$_3$), 25.8 (SiC*Me*$_3$), 27.6 (C_6), 67.8 (C_5), 70.0 (C_3), 74.9 (C_4), 138.7 (C_1), 147.0 (C_2) and 193.6 (C_7) ppm; (ESI$^+$, *m*/*z*): 409 [(M+Na)$^+$, 30%] and 795 [(2M+Na)$^+$, 20%].

5.9. (3*R*,4*R*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-1-ethynyl-4-hydroxycyclohex-1-ene (13)

^{n}BuLi (0.38 mL of 1.6 M in hexane, 0.60 mmol) was added to a solution of $TMSCHN_2$ (0.29 mL of 2.0 M in hexane, 0.57 mmol) at −78 °C. To this solution was added **12** (255 mg, 0.51 mmol) in anhydrous THF (2 mL). The mixture was stirred and allowed to reach room temperature during 8 h. The reaction was poured into H_2O/Et_2O and the aqueous layer extracted with Et_2O. The combined organic fractions were dried (Na_2SO_4) and concentrated, and the residue purified by flash chromatography using silica 60 Å (32–63 μm) pH 7 (gradient elution with hexane–20% Et_2O/hexane) to afford **13** as a colorless oil in 56% yield. IR(NaCl): υ 3466, 3316, 2955, 2930, 2895 and 2857 cm^{-1}; ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.10 (s, 3H, 2Si*Me*), 0.10 (s, 3H, Si*Me*), 0.11 (s, 3H, Si*Me*), 0.12 (s, 3H, Si*Me*), 0.90 (s, 9H, SiC*Me*$_3$), 0.91 (s, 9H, SiC*Me*$_3$), 2.32 (m, 2H, $2H_6$), 2.86 (s, 1H, H_8), 3.64 (br s, 1H, H_4), 4.10 (ddd, 1H, H_5, $^3J_{HH}$ 5.6, 5.6, 2 Hz), 4.28 (dd, 1H, H_3, $^3J_{HH}$ 4, 4 Hz) and 5.99 (br s, 1H, H_2) ppm; ^{13}C NMR (100.13 MHz, $CDCl_3$): δ −4.8 (Si*Me*), −4.7 (Si*Me*), −4.6 (Si*Me*), 18.1 (Si*C*), 25.8 (SiC*Me*$_3$), 34.8 (C_6), 67.5 (C_5), 69.7 (C_3), 74.2 (C_4), 76.3 (C_8), 83.8 (C_7), 119.3 (C_1) and 134.5 (C_2) ppm; (ESI$^+$, *m*/*z*): 405 [(M+Na)$^+$, 100%].

5.10. 1α,25-dihydroxy-2β-(3′-hydroxypropoxy)-6,7-dehydro-19-*nor*-previtamin D_3 (15)

CuI (1 mg, 0.005 mmol), $(PPh_3)_2Pd(OAc)_2$ (1 mg, 0.001 mmol), and anhydrous Et_2NH (350 μL) were added to a solution of **6** (25 mg, 0.045 mmol) and **8** (24 mg, 0.049 mmol) in anhydrous DMF (350 μL). The reaction was monitored by TLC (hexane). After 4 h, the mixture was poured into H_2O/Et_2O and the aqueous layer extracted with Et_2O. The combined organic fractions were dried (Na_2SO_4) and concentrated. (−)-CSA (63 mg, 0.27 mmol) was added to a solution of this crude in MeOH (1.4 mL) at 0 °C and the reaction was stirred overnight a room temperature. The mixture was poured into $NaHCO_3$ (aqueous)/EtOAc and the aqueous layer extracted with EtOAc. The combined organic fractions were dried (Na_2SO_4) and concentrated, and the residue was subjected to flash chromatography using silica 60 Å (32–63 μm) pH 7 (gradient elution with 90% EtOAc/hexane–EtOAc) to afford **15** as a colorless oil in 70% yield. ^1H NMR (400.13 MHz, $CDCl_3$): δ 0.70 (s, 3H, *Me*$_{18}$), 0.96 (s, 3H, *Me*$_{21}$, $^3J_{HH}$ 6.5 Hz, 1.04 (m, 1H), 1.23 (s, 6H, *Me*$_{26}$ + *Me*$_{27}$), 1.2–1.5 (m, 9H), 1.7–2.1 (5H, m), 2.1–2.3 (m, 4H), 2.40 (d, 1H, $|^2J_{HH}|$ 17.4 Hz), 2.52 (d, 1H, $|^2J_{HH}|$ 17.4 Hz), 3.73 (m, 2H), 3.8–3.9 (m, 3H), 4.08 (br s, 1H), 4.16 (br s, 1H) and 5.99 (br s, 2H, H_9 + H_{10}) ppm; ^{13}C NMR (100.13 MHz, $CDCl_3$): δ 11.0 (C_{18}), 18.7 (C_{21}), 20.8 (CH_2), 23.9 (CH_2), 25.2 (CH_2), 28.0 (CH_2), 29.2 y 29.4 (C_{26}+C_{27}), 30.9 (CH), 32.0 (CH_2), 35.9 (CH), 36.1 (CH_2), 36.4 (CH), 41.8 (C), 44.4 (CH_2), 49.9 (CH), 54.7 (CH), 61.6 (CH_2), 68.0 (CH), 68.4 (CH_2), 71.1 (C), 72.4 (CH), 87.6 (C), 89.6 (C), 120.6 (C),

122.3 (C), 129.2 (CH) y 134.3 (CH) ppm; (ESI$^+$, *m*/*z*): 497 [(M+Na)$^+$, 100%], 971 [(2M+Na)$^+$, 50%].

5.11. Methyl (3*R*,4*S*,5*R*)-3,5-di[(*tert*-butyldimethylsilyl)oxy]-4-hydroxycyclohex-1-enecarboxylate (17)

A similar procedure as that described for **10** afforded **17**. This compound was previously described.[28]

5.12. (3*R*,4*S*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-4-hydroxy-1-hydroxymethylcyclohex-1-ene (18)

A similar procedure as that described for **11** afforded **18** as a white solid in 75% yield. Mp: 116–117 °C; IR(KBr): υ 3323, 2952, 2927, 2892 and 2858 cm^{-1}; ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 0.09 (s, 3H, Si*Me*), 0.10 (s, 3H, Si*Me*), 0.14 (s, 6H, 2Si*Me*), 0.89 (s, 9H, SiC*Me*$_3$), 0.93 (s, 9H, SiC*Me*$_3$), 1.94 (dd, 1H, H_{6a}, $|^2J_{HH}|$ 17.7, $^3J_{HH}$ 4.5 Hz), 2.42 (ddd, 1H, H_{6e}, $|^2J_{HH}|$ 17.7, $^3J_{HH}$ 3.8, $|^4J_{HH}|$ 2.1 Hz), 3.64 (br s, 1H, H_4), 4.04 (br s, 2H, H_7), 4.13 (ddd, 1H, H_5, $^3J_{HH}$ 11.1, 4.8, 4.8 Hz), 4.43 (s, 1H, H_3) and 5.53 (s, 1H, H_2) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ −4.8 (Si*Me*), −4.4 (Si*Me*), 18.2 (Si*C*), 18.0 (Si*C*), 25.9 (SiC*Me*$_3$), 27.8(SiC*Me*$_3$), 31.91 (C_6), 66.2 (C_7), 67.7 (C_3), 68.6 (C_5), 71.9 (C_4), 121.6 (C_2) and 137.4 (C_1) ppm; (ESI$^+$, *m*/*z*): 411 [(M+Na)$^+$, 30%] and 799 [(2M+Na)$^+$, 40%].

5.13. (3*R*,4*S*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-4-hydroxycyclohex-1-enecarbaldehyde (19)

A similar procedure as that described for **12** afforded **19** as a white solid in 91% yield. Mp: 72–73 °C; IR(KBr): υ 3947, 2952, 2925, 2883, 2849 and 1668 cm^{-1}; ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 0.08 (s, 3H, Si*Me*), 0.09 (s, 3H, Si*Me*), 0.18 (s, 3H, Si*Me*), 0.19 (s, 3H, Si*Me*), 0.86 (s, 9H, SiC*Me*$_3$), 0.96 (s, 9H, SiC*Me*$_3$), 2.24 (d, 1H, H_{6a}, $|^2J_{HH}|$ 13.5 Hz), 2.51 (ddd, 1H, H_{6e}, $|^2J_{HH}|$ 13.5, $^3J_{HH}$ 0.9, $|^4J_{HH}|$ 0.9 Hz), 2.58 (s, 1H, OH), 3.80 (dd, 1H, H_4, $^3J_{HH}$ 3, 3 Hz), 4.25 (ddd, 1H, H_5, $^3J_{HH}$ 5.7, 3, 3 Hz), 4.68 (br s, 1H, H_3), 6.43 (s, 1H, H_2) and 9.50 (s, 1H, H_7) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ −4.8 (Si*Me*), −4.7 (Si*Me*), −5.0 (Si*Me*), 17.9 (Si*C*), 18.2 (Si*C*), 25.6 (SiC*Me*$_3$), 25.8 (SiC*Me*$_3$), 26.5 (C_6), 67.8 (C_3), 68.0 (C_5), 71.2 (C_4), 138.8 (C_1), 147.4 (C_2) and 194.0 (C_7) ppm; (ESI$^+$, *m*/*z*): 409 [(M+Na)$^+$, 35%] and 795.0 [(2M+Na)$^+$, 15%].

5.14. (3*R*,4*S*,5*R*)-3,5-Di[(*tert*-butyldimethylsilyl)oxy]-1-ethynyl-4-hydroxycyclohex-1-ene (20)

A similar procedure as that described for **13** afforded **20** as a colorless oil in 56% yield. IR(NaCl): υ 3452, 3316, 2954, 2937, 2888 and 2858 cm^{-1}; ^{1}H NMR (400.13 MHz, $CDCl_3$): δ 0.09 (s, 6H, 2Si*Me*), 0.12 (s, 3H, Si*Me*), 0.13 (s, 3H, Si*Me*), 0.89 (s, 9H, SiC*Me*$_3$), 0.93 (s, 9H, SiC*Me*$_3$), 2.06 (d, 1H, H_{6a}, $|^2J_{HH}|$ 18 Hz), 2.58 (d, 2H, H_{6e} + OH), 2.86 (s, 1H, H_8), 3.64 (br s, 1H, H_4), 4.11 (ddd, 1H, H_5, $^3J_{HH}$ 5.6, 5.6, 5.6 Hz), 4.44 (s, 1H, H_3) and 5.93 (s, 1H, H_2) ppm; ^{13}C NMR (100.61 MHz, $CDCl_3$): δ −4.9 (Si*Me*), −4.8 (Si*Me*), −4.6 (Si*Me*), 18.1 (Si*C*), 18.0 (Si*C*), 25.8 (SiC*Me*$_3$), 25.7 (SiC*Me*$_3$), 34.7 (C_6), 67.36 (C_3), 68.1 (C_5), 70.9 (C_4), 76.2 (C_8), 84.07 (C_7), 119.20 (C_1) and 134.74 (C_2) ppm; (ESI$^+$, *m*/*z*): 405 [(M+Na)$^+$, 100%].

5.15. 2β,3β-Epoxy-1α,25-dihydroxy-6,7-dehydro-3-deoxy-19-*nor*-previtamin D_3 (23)

CuI (2.5 mg, 0.013 mmol), $(PPh_3)_2Pd(OAc)_2$ (3 mg, 0.004 mmol), and anhydrous Et_2NH (1 mL) were added to a solution of **7** (70 mg, 0.130 mmol) and **8** (70 mg, 0.143 mmol) in anhydrous DMF (1 mL). The reaction was monitored by TLC (hexane). After 4 h, the mixture was poured into H_2O/Et_2O and the aqueous layer extracted with Et_2O. The combined organic fractions were dried (Na_2SO_4) and concentrated. (−)-CSA (182 mg, 0.78 mmol) was added to a solution of this crude in MeOH (1.4 mL) at 0 °C and the reaction was stirred overnight a room temperature. The mixture was poured into $NaHCO_3$ (aqueous)/EtOAc and the aqueous layer extracted with EtOAc. The combined organic fractions were dried (Na_2SO_4) and concentrated. To a solution of this crude in anhydrous THF (1.3 mL) was added dropwise DBU (40 μL, 0.26 mmol). After stirring the mixture at room temperature for 24 h, the solvent was concentrated in vacuo, and the residue purified by flash chromatography using silica 60 Å (32–63 μm) pH 7 (gradient elution with 50% EtOAc/hexane–EtOAc) to afford **23** as a colorless oil in 58% yield. ^{1}H NMR (600.15 MHz, $CDCl_3$): δ 0.70 (s, 3H, *Me*$_{18}$), 0.96 (s, 3H, *Me*$_{21}$, $^3J_{HH}$ 6.4 Hz), 1.09 (m, 1H), 1.23 (s, 6H, *Me*$_{26}$+*Me*$_{27}$), 1.3–1.8 (m, 13H), 1.9–2.1 (2H, m), 2.1–2.2 (m, 3H), 2.64 (d, 1H, H_4, $|^2J_{HH}|$ 19.2 Hz), 2.72 (d, 1H, H_4, $|^2J_{HH}|$ 20.4 Hz), 3.27 (m, 1H, H_2), 3.35 (s, 1H, H_3), 4.57 (s, 1H, $H_{1,}$), 5.92 (br s, 1H, H_{10}) and 6.0 (br s, 1H, H_9) ppm; ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 11.0 (C_{18}), 18.7 (C_{21}), 20.8 (CH_2), 23.9 (CH_2), 25.2 (CH_2), 27.9 (CH_2), 29.2 (C_{26} + C_{27}), 29.3 (C_4), 29.4 (CH), 35.8 (CH_2), 36.2 (CH), 36.3 (CH_2), 41.8 (C), 44.4 (CH_2), 49.9 (CH), 50.2 (C_3), 52.6 (C_2), 63.5 (C_1), 71.1 (C), 87.8 (C_6), 90.2 (C_7), 119.6 (C_5), 122.0 (C_8), 128.4 (C_{10}) and 134.8 (C_9) ppm; (ESI$^+$, *m*/*z*): 421 [(M+Na)$^+$, 100%].

6. In vitro biological evaluation

6.1. Cell proliferation assay

As a measure of cell proliferation, [^{3}H]thymidine incorporation of breast cancer MCF-7 (ATCC, Rockville, MD) was determined after a 72 h incubation period with various concentrations of 1α,25-$(OH)_2$-D_3, analogues or vehicle as described previously.[29]

6.2. Binding studies

The affinity of 1α,25-$(OH)_2$-D_3 and its analogues to the vitamin D receptor was evaluated by their ability to compete with [^{3}H]1α,25-$(OH)_2$-D_3 for binding to high speed supernatant from intestinal mucosa homogenates obtained from normal pigs as described previously.[29] The relative affinity of the analogues was calculated from their concentration needed to displace 50% of [^{3}H]1α,25-$(OH)_2$-D_3 from its receptor compared with the activity of 1α,25-$(OH)_2$-D_3 (assigned a 100% value).

Binding of vitamin D analogues to the human vitamin D-binding protein (hDBP) was performed at 4 °C as described previously.[30]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.10.053.

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