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Versatile synthesis and biological evaluation of 1,3-diamino-substituted 1α,25-dihydroxyvitamin D_3 analogues

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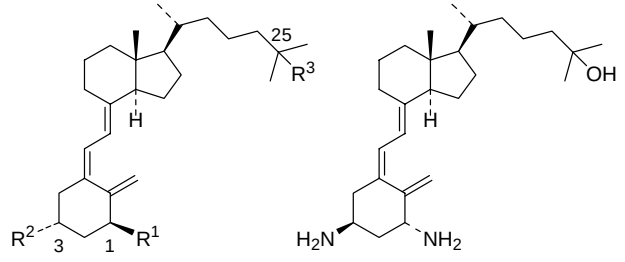
Abstract—A concise route to 1α,3β-diamino-25-hydroxy-3-deoxyvitamin D_3 (**5**) and 1β,3α-diamino-25-hydroxy-3-deoxyvitamin D_3 (**6**) has been developed starting from (*R*)- or (*S*)-carvone for the construction of the modified A-ring fragments. The conversion of the hydroxyl group to amine function with complete inversion of the configuration was efficiently accomplished by Mitsunobu reaction using phthalimide as nucleophile or activation of the hydroxyl group as mesylate followed by reaction with NaN_3. Diamino **5** and **6** as well as monoamino **3**, **4**, **30**, and **31** vitamin D_3 derivatives have shown poor binding to VDR compared with 1α,25-dihydroxyvitamin D_3. The most active compound in the inhibition of MCF-7 cell proliferation and HL 60 cell differentiation was 1α-amino analogue **3**. Also, very low in vivo calcemic effects of derivatives **3** and **4** were found.

1. Introduction

1α,25-Dihydroxyvitamin D_3 [**1**, 1α,25-$(OH)_2$-D_3, Fig. 1] has been recognized as the hormonally active metabolite of vitamin D_3 (**2**) involved in intestinal calcium absorption, and bone resorption and mineralization. In addition, **1** regulates cell differentiation, cell proliferation, and immune effects.[1] Because of the biological profile of 1α,25-$(OH)_2$-D_3, structurally modified compounds have been prepared as sensitive molecular biology probes and as potential new drugs of high efficacy and low toxicity.[2] Eight vitamin D analogues are currently in use as drugs for chemotherapy of various human diseases and four new derivatives in human clinical trials.[2a] Of considerable interest is the development by Leo Pharmaceutical of a compound called seocalcitol[3] (Leo EB 1089), characterized by an altered side-chain structure featuring 26,27-dimethyl groups and two double bonds. Most of the analogues are altered in the side chain of the CD-ring moiety, although modifications in the A-ring, less accessible synthetically, provide vitamin D derivatives with an unique biological profile.[4]



1, R^1= R^2= R^3= OH
2, R^1= R^3= H; R^2= OH
3, R^1= NH_2; R^2= R^3= OH
4, R^1= R^3= OH; R^2= NH_2
5, R^1= R^2= NH_2; R^3= OH

Figure 1.

1α-Fluoro-16,23-diene-20-*epi* derivative Ro 26-9228 (Hoffmann-La Roche)[5] and 2β-(3-hydroxypropoxy) analogue ED-71 (Chugai)[6] are both used in human clinical trials as promising candidates for the treatment of osteoporosis. Another analogue with a substitution in

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the A-ring on C-2 (2-methylene, 2MD) induces bone formation in vitro and in vivo.[7]

To investigate the structure–function relationships of this hormone are of interest the synthesis and biological evaluation of novel A-ring modified vitamin D analogues. Recently, we reported the synthesis of 1α- and 3β-amino derivatives of 1α,25-$(OH)_2$-D_3 **3** and **4**, respectively.[8] Although hydroxyl groups at C-1 and C-25 positions are considered essential for binding to nVDR and DBP proteins, various vitamin D derivatives with structural changes at the 1-position have showed significant biological activities. Among them, the Ro 26-9228 derivative, above mentioned, and 1-hydroxyalkyl-25-hydroxyvitamin D_3 analogues,[9] which are known to retain calcitriol's antiproliferative activity in murine keratinocytes even though these synthetic homologues are significantly less effective than calcitriol in binding to the 1α,25-$(OH)_2$-D_3 receptor. In addition, orientation of the two hydroxyl groups on the A-ring is important in the biological profile of such derivatives. Thus, the C-1 epimer of 1α,25-$(OH)_2$-D_3 was shown to be an antagonist of nongenomic, but not genomic, actions by Norman and co-workers.[10] Also, the C-3 epimer was shown to be produced in the vitamin D metabolic pathway in certain cell lines.[11] Herein, we wish to describe the synthesis of 1α,3β- and 1β,3α-diamino derivatives of vitamin D_3 **5** and **6**, respectively, and also the biological significance of both the amino substitution and stereochemistry at C-1 and/or C-3 position.

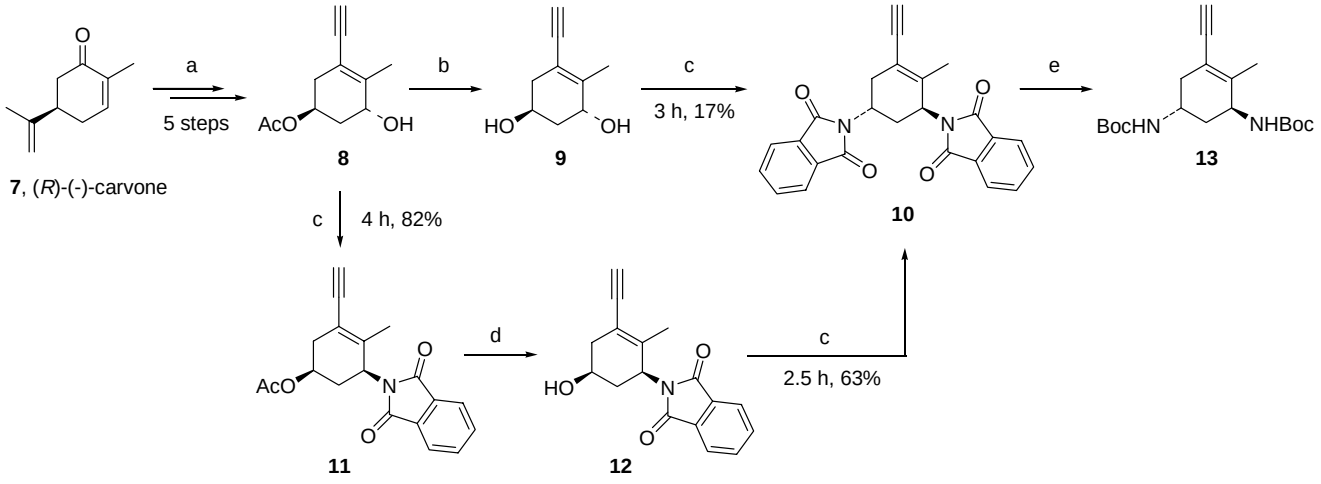
2. Results and discussion

To synthesize the diamino analogues, we used a convergent route[12] based on the palladium-catalyzed coupling reaction of the A-ring synthon with the CD-ring portion, which are separately prepared. In this approach, a dienyne is semihydrogenated to a previtamin structure that undergoes rearrangement to the corresponding vitamin D analogue.

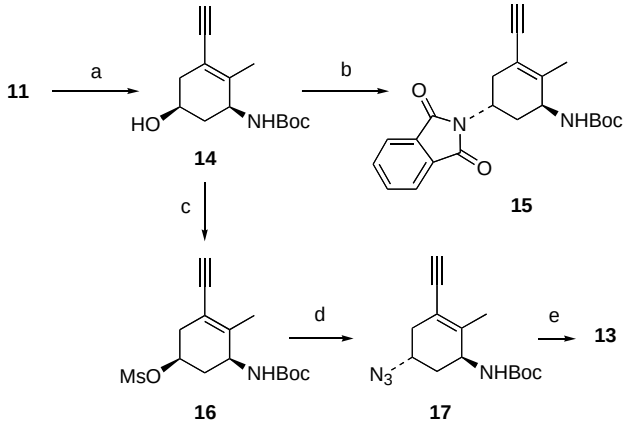
For the synthesis of 1α,3β-A-ring precursor **13** (Scheme 1), we chose (*R*)-carvone as chiral template, which was converted in a highly efficient synthesis to the monoacetate **8** by Okamura et al.[13] We envisaged that the synthesis of **13** could be achieved by Mitsunobu reaction[14] using phthalimide as nucleophilic on the corresponding diol **9**. When **9** was allowed to react with phthalimide in the presence of DEAD and Ph_3P, diimide derivative **10** was obtained with complete inversion of the configuration at both centers. However, simultaneous inversion of both OH groups gives place to low yield (17%). ^1H NMR spectrum of the reaction crude showed the absence of starting material and traces of possible elimination products. The loss of crude weight can be explained by the formation of 1-ethynyl-2-methylbenzene, which was vaporized in the work-up. For this reason, sequential modification of C-3 and C-5 positions was tried. Thus, allylic alcohol **8** was subjected to above-mentioned conditions being isolated compound **11** in 82% yield. Saponification of the acetate ester and subsequent treatment with phthalimide under Mitsunobu conditions gives place to diimide **10**. The reaction of **10** with 8 M $MeNH_2$ in EtOH afforded the corresponding diamino, which is *N*-protected by adding di-*tert*-butyldicarbonate to the reaction mixture. However, the moderate yield of this step prompted us to search for a more efficient route.

Treatment of **11** with $MeNH_2$ in EtOH results in complete deprotection of both the hydroxy and the amino groups to give the corresponding aminoalcohol, which is *N*-protected. The resulting derivative **14** (Scheme 2) was treated with phthalimide to provide the required stereoisomer **15** in a low yield of 30% due to the presence of elimination products. The use of toluene instead of THF, or replacement of Ph_3P by Me_3P did not have significant impact on the yield.

The conversion of alcohol **14** to inverted amine was best achieved by activation of the hydroxyl group as mesylate followed by reaction with NaN_3. This approach provides the azido-substituted compound **17**



Scheme 1. (a) Ref. 13; (b) NaOMe, MeOH, 0 °C, 2.5 h (96%); (c) phthalimide, Ph_3P, DEAD, THF, rt; (d) K_2CO_3, MeOH, 0 °C, 1.5 h (71%); (e) i, $MeNH_2$, EtOH, rt, 24 h, and then 65 °C, 12 h. ii, $(Boc)_2O$, $NaHCO_3$ (aq), $CHCl_3$, rt, 24 h (45%).

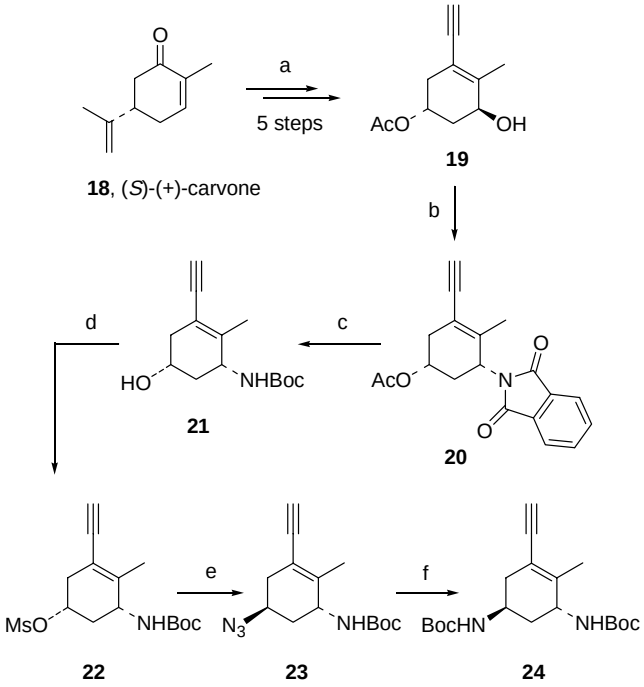


Scheme 2. (a) i, $MeNH_2$, EtOH, rt, 24 h, and then 65 °C, 12 h. ii, $(Boc)_2O$, $NaHCO_3$ (aq), $CHCl_3$, rt, 24 h (88%); (b) phthalimide, Ph_3P, DEAD, THF, rt, 3 h (30%); (c) MsCl, Et_3N, CH_2Cl_2, 0 °C, 3 h (94%); (d) NaN_3, DMF, 65 °C, 6 h (79%); (e) Me_3P, $(Boc)_2O$, 1 M NaOH, THF, 24 h (82%).

with inversion of the configuration at C-3 in good yield. Transformation of azide to amine was accomplished by the Staudinger reaction.[15] Thus, treatment of **17** with Me_3P in the presence of H_2O and Boc_2O afforded the *N*-Boc derivative **13** in 53% yield after flash chromatography. Recently, Vilarrasa et al.[16] have described that the presence of a basic medium, to avoid the protonation of $^tBuOCOO^-$ to $^tBuOCOOH$ and/or to remove CO_2 as HCO_3^- or CO_3^{2-}, is essential to the success of the reaction. Thus, when **17** was treated in degassed THF with Me_3P and Boc_2O in the presence of degassed aqueous 1 M NaOH the yield of **13** was increased up to 82%.

Having established a route for the synthesis of 1α,3β-A-ring precursor, we embarked on the preparation of corresponding stereoisomer 1β,3α-A-ring synthon. The synthesis started from key intermediate **19** (Scheme 3), which has been previously reported by Okamura et al., using (*S*)-(+)-carvone.[17] Substitution of hydroxyl group in **19** by phthalimide and simultaneous inversion of the configuration was carried out by the Mitsunobu method. Subsequent reaction of **20** with $MeNH_2$ gives place to deprotection of both the phthaloyl and acetyl groups yielding the appropriate aminoalcohol which was *N*-protected to give **21**. The latter was transformed to the mesylate **22**, which was then converted to the azide **23** by the treatment with NaN_3. Finally, direct conversion of azido derivative to *N*-Boc compound afforded the 1β,3α-A-ring precursor **24** in high yield. We confirmed unambiguously the stereochemistry of **13** and **24** by NOESY experiments on compounds **14** and **21**. In addition, comparison of the optical rotation of derivatives obtained in Schemes 2 and 3 revealed the formation of corresponding enantiomers.

The B-*seco* steroidal structure was constructed[12a,18] by standard Sonogashira coupling of the A-ring synthons **13** and **24** with the CD-ring portion **25** (Scheme 4), prepared according to the published procedure.[19] The reaction crude was subjected to desily-



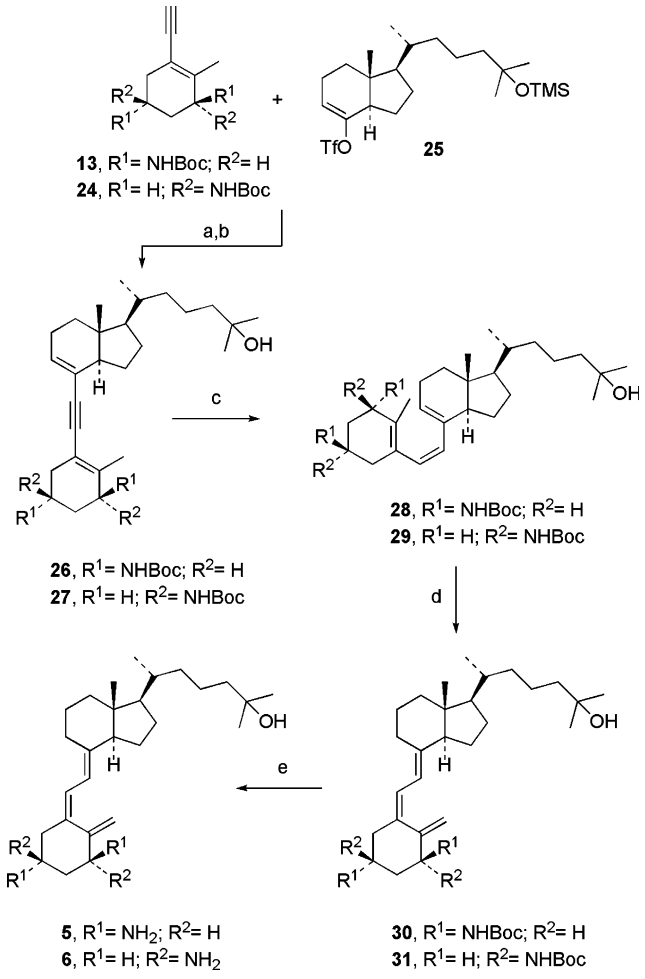
Scheme 3. (a) Ref. 17; (b) phthalimide, Ph_3P, DEAD, THF, rt, 4 h (84%); (c) i, $MeNH_2$, EtOH, rt, 24 h, and 65 °C, 12 h. ii, $(Boc)_2O$, $NaHCO_3$ (aq), $CHCl_3$, rt, 24 h (77%); (d) MsCl, Et_3N, CH_2Cl_2, 0 °C, 3 h (81%); (e) NaN_3, DMF, 65 °C, 6 h (75%); (f) Me_3P, $(Boc)_2O$, 1 M NaOH, THF, 24 h (79%).

lation with tetrabutylammonium fluoride to afford the most stable dienyne derivatives **26** and **27** in 82 and 89% yield, respectively (for coupling and desilylation steps). Subsequent catalytic hydrogenation in the presence of Lindlar catalyst and quinoline poison generated previtamins **28** and **29**. One note concerns the conditions used to reach high conversions in the hydrogenation process, which was conducted in deoxygenated methanol using a vibromatic at 800 cycles/min and with the catalyst pre-dried at 60 °C in vacuum. The thermal isomerization of **28** and **29** (acetone, 80 °C, 4 h) reveals an interequilibrium between the vitamin and the previtamin forms. ^1H NMR analysis of **30** and **31** indicated an equilibrium proportion of the previtamin forms of 28 and 26%, respectively. These minor constituents were readily separable by HPLC being isolated derivatives **30** and **31** in 40–43% yield after purification (hydrogenation and isomerization steps). Finally, deprotection of the amino group with HCl and purification by semi-preparative TLC [5% NH_3(aq)/MeOH] yielded **5** and **6**.

A-ring chair conformations of analogues **5** and **6** are shown in Chart 1, as determined by ^1H NMR analysis. In both cases, the 1-amino group occupies the equatorial position and the 3-amino group the axial position.

3. Biological evaluation

We evaluated the potencies of the amino vitamin D_3 derivatives **3**, **4**, **5**, **6**, **30**, and **31** in terms of their ability



Scheme 4. (a) $Pd(Ph_3P)_2(OAc)_2$, CuI, Et_2NH, DMF, rt, 1 h; (b) Bu_4NF, THF, rt, 12 h (82% for **26** and 89% for **27**, 2 steps); (c) H_2, Lindlar cat., quinoline, MeOH, rt, 30 min; (d) acetone, 80 °C, 4 h (43% for **30** and 40% for **31**, 2 steps); (e) HCl(g), EtOH, rt, 30 min (60% for **5** and 55% for **6**.

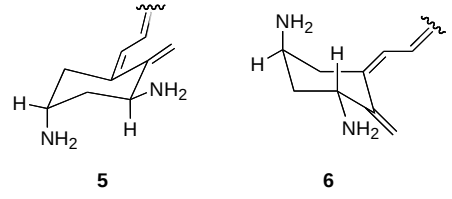


Chart 1.

to bind to the pig intestinal vitamin D receptor in comparison to the natural hormone. In addition, inhibition of MCF-7 cell proliferation and the induction of HL 60 cell differentiation by 1α,25-$(OH)_2$-D_3 and its analogues as well as their calcemic effects were measured.

The relative affinity of the analogues for the VDR was calculated from their concentration required for 50% displacement of [^{3}H]1α,25-$(OH)_2$-D_3 from the receptor protein compared with the activity of 1α,25-$(OH)_2$-D_3 (assigned a value of 100 by definition). These analogues

Table 1. Biological activity of amino derivatives of 1α,25-$(OH)_2$-D_3

Compound	VDR (%)	MCF-7 (%)	HL-60 (%)
1α,25-$(OH)_2$-D_3	100	100	100
3	5	30	20
4	0.4	2	2
5	0	0	0
6	0	0	0
30	0	0	0
31	0	0	0

Summary of the in vitro effects of amino analogues of 1α,25-$(OH)_2$-D_3 on receptor binding (VDR), MCF-7 proliferation, and HL 60 differentiation. The in vitro effect is expressed as percentage activity at EC_{50} in comparison with 1α,25-$(OH)_2$-D_3 (= 100% activity).

bound very poorly to VDR when compared with 1α,25-$(OH)_2$-D_3. As shown in Table 1, the 1α-amino derivative **3** exhibited 5% of affinity compared with the natural hormone; meanwhile, the other analogues show no affinity at all.

As a measure of cell proliferation, [^{3}H]thymidine incorporation of MCF-7 was determined after a 72 h incubation period with various concentrations of 1α, 25-$(OH)_2$-D_3, analogues or ethanol. The most active compound is the 1α-amino derivative **3**, which can inhibit the cell proliferation for 80% at a concentration of 10^{-6} M comparable with 1α,25-$(OH)_2$-D_3 but this compound was three times less potent than 1α,25-$(OH)_2$-D_3 at the EC_{50} concentration (Fig. 2). Also compound **3** was the most potent analogue to stimulate the differentiation of
HL 60 cells (Fig. 3).

Besides the in vitro screening, the in vivo calcemic effects of the compound **3** and **4** were evaluated. Both analogues were much less calcemic than 1α,25-$(OH)_2$-D_3 even at 100-fold higher doses than 1α,25-$(OH)_2$-D_3 (0.1 μg/kg/d) (Fig. 4).

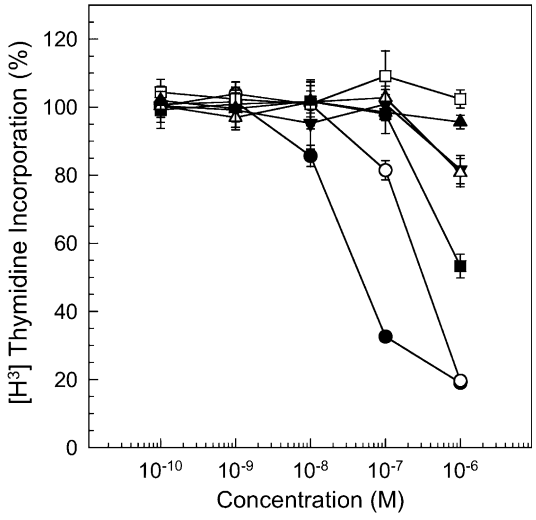


Figure 2. Antiproliferating effects of mono- and diamino 1α,25-$(OH)_2$-D_3 analogues on breast cancer MCF-7 cells. 1α,25-$(OH)_2$-D_3 (●); **3** (○); **4** (■); **5** (□); **6** (▲); **30** (△); **31** (▼).

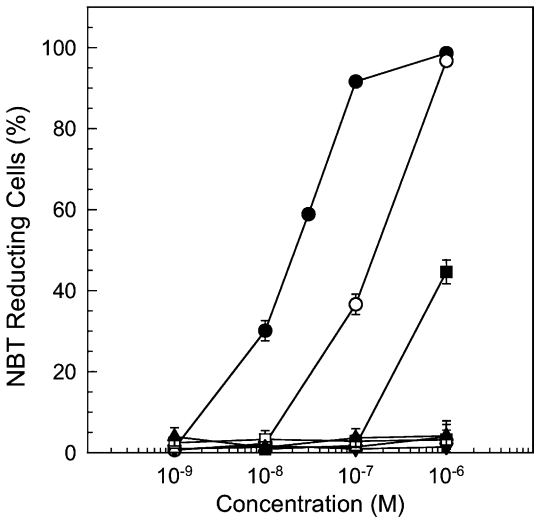


Figure 3. Prodifferentiating effects of mono- and diamino 1α,25-$(OH)_2$-D_3 analogues on promyelocytic HL 60 leukemia cells. 1α,25-$(OH)_2$-D_3 (●); **3** (○); **4** (■); **5** (□); **6** (▲); **30** (△); **31** (▼).

4. Conclusions

We have described the synthesis and biological evaluation of novel 1-, 3-, and 1,3-diamino-substituted vitamin D_3 analogues. Key feature of these approaches is the excellent yield and stereoselectivity of the Mitsunobu reaction, which provide direct introduction of the amino group at C-1 with total inversion of the configuration. The conversion of alcohol at C-3 to inverted amine was best achieved by activation of the hydroxyl group as mesylate followed by reaction with NaN_3. Biological assays on diamino **5** and **6** as well as monoamino **3**, **4**, **30**, and **31** vitamin D_3 derivatives have shown poor binding to VDR. The most active compound in the inhibition of MCF-7 cell proliferation and HL 60 cell differentiation was 1α-amino analogue **3**. In vivo calcemic effects of derivatives **3** and **4** were evaluated showing very low calcemic effect.

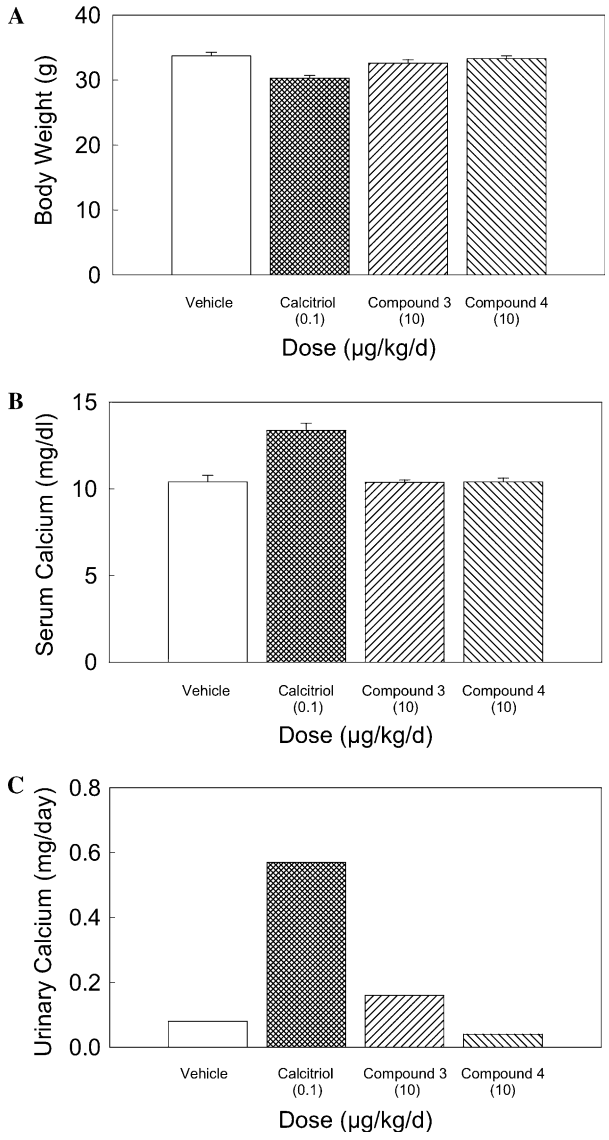


Figure 4. In vivo biological activity of 1- and 3-monoamino 1α,25-$(OH)_2$-D_3 analogues determined by body weight (A) as a marker of toxicity and measuring serum (B) and urine (C) calcium levels in mice after intraperitoneally injections after seven consecutive days. Mice were injected with vehicle (arachis oil), 1α,25-$(OH)_2$-D_3 (0.1 μg/kg/d) or analogues **3** and **4** (10 μg/kg/d).

5. Experimental section

5.1. General spectroscopic and experimental data

Melting points were taken on samples in open capillary tubes and are uncorrected. IR spectra were recorded on an Infrared Fourier Transform spectrophotometer using KBr pellets. Flash chromatography was performed using silica gel 60 (230–400 mesh). ^1H, ^{13}C NMR, and DEPT were obtained using AC-300 (^1H, 300.13 MHz and ^{13}C, 75.5 MHz) or DPX 300 (^1H, 300.13 MHz and ^{13}C, 75.5 MHz) spectrometers for routine experiments. AMX-400 spectrometer operating at 400.13 and 100.61 MHz for ^1H and ^{13}C, respectively, was used for the acquisition of ^1H–^1H homonuclear and ^1H–^{13}C heteronuclear correlation experiments. The chemical shifts are given in delta (δ) values and the coupling constants (J) in Hertz (Hz). ES^+ was used to record mass spectra (MS). Microanalyses were performed on a Perkin-Elmer model 2400 instrument. HPLC was performed using UV detector and a Spherisorb W, 5 μm silica gel column, 250 × 10 mm.

5.2. Synthesis of 1α,3β-diamino-25-hydroxy-3-deoxyvitamin D_3 (5)

A solution of **30** (30.7 mg, 0.05 mmol) in 1.5 mL of ethanol was added to a solution of HCl/EtOH [15 mL, generated by bubbled HCl (g) in EtOH]. The solution was stirred for 0.5 h, and then solvent was removed under reduced pressure. The residue was purified by semipreparative TLC [5% NH_3(aq)/MeOH] to afford a white solid (60% yield). ^1H NMR (MeOH-d_4, 300.13 MHz): δ 0.75 (s, 3H, H_{18}), 1.15 (d, 3H, H_{21}, $^3J_{HH}$ 6.2 Hz), 1.36 (s, 6H, $H_{26} + H_{27}$), 1.2–2.4 (m, 21H, $2H_2 + 2H_9 + 2H_{11} + 2H_{12} + H_{14} + 2H_{15} + 2H_{16} + H_{17} + H_{20} + 2H_{22} + 2H_{23} +$

$2H_{24}$), 2.73 (d, 1H, H_4, $^2J_{HH}$ 13.3 Hz), 3.07 (d, 1H, H_4, $^2J_{HH}$ 13.3 Hz), 3.40 (m, 1H, H_3), 3.76 (m, 1H, H_1), 5.41 (m, 1H, H_{19}), 6.25 (d, 1H, H_7, $^3J_{HH}$ 10.5 Hz) and 6.59 (d, 1H, H_6, $^3J_{HH}$ 10.5 Hz); MS (ES^+, *m/z*): 415 [$(M+H)^+$, 100%].

5.3. Synthesis of 1β,3α-diamino-25-hydroxy-3-deoxyvitamin D_3 (6)

A similar procedure as that described for **5** afforded **6** as a white solid (55% yield). ^1H NMR (MeOH-d_4, 300.13 MHz): δ 0.76 (s, 3H, H_{18}), 1.15 (d, 3H, H_{21}, $^3J_{HH}$ 6.0 Hz), 1.36 (s, 6H, $H_{26} + H_{27}$), 1.2–2.4 (m, 21H,$2H_2 + 2H_9 + 2H_{11} + 2H_{12} + H_{14} + 2H_{15} + 2H_{16} + H_{17} + H_{20} + 2H_{22} + 2H_{23} + 2H_{24}$), 2.71 (dd, 1H, H_4, $^2J_{HH}$ 13.1 Hz, $^3J_{HH}$ 4.3 Hz), 3.07 (dd, 1H, H_4, $^2J_{HH}$ 13.1 Hz, $^3J_{HH}$ 4.3 Hz), 3.36 (m, 1H, H_3), 3.76 (m, 1H, H_1), 5.40 (m, 1H, H_{19}), 6.25 (d, 1H, H_7, $^3J_{HH}$ 11.4 Hz) and 6.58 (d, 1H, H_6, $^3J_{HH}$ 11.4 Hz); MS (ES^+, *m/z*): 415 [$(M+H)^+$, 100%].

5.4. Synthesis of (3*R*,5*S*)-3,5-dihydroxy-1-ethynyl-2-methylcyclohex-1-ene (9)

Acetate **8** (194 mg, 1 mmol) was treated with 0.2 M sodium methoxide in methanol (10 mL). After stirring for 2.5 h at 0 °C, the solution was acidified with Dowex 50X4-400 resin (200–400 mesh). After the removal of the resin by filtration, the solution was evaporated, and the residue was purified by flash chromatography (EtOAc) to give the deprotected diol (96% yield). This compound was previously reported.[17a]

5.5. Synthesis of (3*S*,5*R*)-1-ethynyl-2-methyl-3,5-bis(phthalimido)cyclohex-1-ene (10)

5.5.1. From imide 9. Phthalimide (108 mg, 0.74 mmol), Ph_3P (193 mg, 0.74 mmol) and diethylazodicarboxilate (0.115 mL, 0.74 mmol) were added to a stirred solution of **9** (45 mg, 0.30 mmol) in THF (10 mL). The mixture was stirred for 3 h at room temperature and then evaporated under reduced pressure to leave a residue which was purified by flash chromatography (30% EtOAc/hexane) to afford a white solid (17% yield).

5.5.2. From imide 12. A similar procedure as described in Section 5.5.1 afforded **10** as a white solid (63% yield). Mp: 199–201 °C (decomp.); IR (KBr): υ 3269, 3064, 2916, 2088, 1744, 1711, 1612, and 1468 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 1.88 (s, 3H, H_9), 1.96 (m, 1H, H_4), 2.58 (dd, 1H, H_4, $^2J_{HH}$ 17.0, $^3J_{HH}$ 5.7 Hz), 3.0 (ddd, 1H, H_6, $^2J_{HH}$ 13.5, $^3J_{HH}$ 13.5, $^4J_{HH}$ 6.9 Hz), 3.12 (m, 1H, H_6), 3.18 (s, 1H, H_8), 4.97 (ad, 1H, H_3, $^3J_{HH}$ 6.3 Hz), 5.13 (m, 1H, H_5) and 7.76 (m, 8H, $H_{12} + H_{13} + H_{16} + H_{17}$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 18.7 (C_9), 32.2, 32.5 ($C_4 + C_6$), 43.5 (C_5), 49.5 (C_3), 81.3 (C_8), 82.5 (C_7), 117.9 (C_1), 123.1, 123.4 ($C_{12} + C_{16}$), 131.5, 131.7 ($C_{11} + C_{15}$), 133.9, 134.1 ($C_{13} + C_{17}$), 136.5 (C_2) and 168.1 ($C_{10} + C_{14}$); MS (ES^+, *m/z*): 433 [$(M+Na)^+$, 45%] and 449 [$(M+K)^+$, 100%]; Anal. Calcd (%) for $C_{25}H_{18}N_2O_4$: C, 73.16; H, 4.42; N, 6.83. Found: C, 73.3; H, 4.6; N, 6.7.

5.6. Synthesis of (3*S*,5*S*)-5-acetoxy-1-ethynyl-2-methyl-3-phthalimidocyclohex-1-ene (11)

A similar procedure as that described for **10** afforded **11** as a white solid (82% yield). Mp: 105–107 °C; IR (KBr): υ 3256, 2932, 2104, 1774, 1710, 1606, and 1468 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 1.74 (s, 3H, H_9), 2.04 (s, 3H, H_{11}), 2.18 (m, 1H, H_4), 2.42 (m, 2H, $H_4 + H_6$), 2.63 (d, 1H, H_6, $^2J_{HH}$ 15.1 Hz), 3.14 (s, 1H, H_8), 5.0 (m, 2H, $H_3 + H_5$) and 7.80 (m, 4H, $H_{14} + H_{15}$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 17.2 (C_9), 21.0 (C_{11}), 31.8, 34.8 ($C_4 + C_6$), 49.9 (C_3), 67.6 (C_5), 81.0 (C_8), 82.4 (C_7), 114.7 (C_1), 123.3 (C_{14}), 131.5 (C_{13}), 134.1 (C_{15}), 139.4 (C_2), 167.4 (C_{12}) y 170.1 (C_{10}); MS (ES^+, *m/z*): 346 [$(M+Na)^+$, 100%] and 362 [$(M+K)^+$, 20%]; $[\alpha]_D^{20} = -102.3$ (*c* 1.0, $CHCl_3$); Anal. Calcd (%) for $C_{19}H_{17}NO_4$: C, 70.58; H, 5.30; N, 4.33. Found: C, 70.3; H, 5.5; N, 4.3.

5.7. Synthesis of (3*S*,5*S*)-1-ethynyl-5-hydroxy-2-methyl-3-phthalimidocyclohex-1-ene (12)

To a solution of **11** (200 mg, 0.619 mmol) in MeOH (12 mL) K_2CO_3 (85 mg, 0.619 mmol) was added. The reaction was stirred at 0 °C for 1.5 h and then treated with Dowex 50WX4-400 (200–400 mesh). Solution was filtered, solvents were evaporated under reduced pressure, and the residue was purified by flash chromatography (30% EtOAc/hexane). White solid (71% yield). Mp: 178–180 °C (decomp.). IR (KBr): υ3490, 3268, 2922, 2094, 1768, 1700, and 1460 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 1.77 (s, 3H, H_9), 2.1–2.65 (m, 4H, $H_4 + H_6$), 3.17 (s, 1H, H_8), 4.03 (m, 1H, H_5), 4.96 (m, 1H, H_3) and 7.8 (m, 4H, $H_{12} + H_{13}$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 17.6 (C_9), 35.8, 38.4 ($C_4 + C_6$), 49.4 (C_3), 64.9 (C_5), 80.9 (C_8), 82.9 (C_7), 115.4 (C_1), 123.4 (C_{12}), 131.5 (C_{11}), 134.2 (C_{13}), 138.8 (C_2) y 167.9 (C_{10}); MS (ES^+, *m/z*): 304 [$(M+Na)^+$, 100%] and 320 [$(M+K)^+$, 25%]; $[\alpha]_D^{20} = -117.5$ (*c* 0.9, $CHCl_3$); Anal. Calcd (%) for $C_{17}H_{15}NO_3$: C, 72.58; H, 5.37; N, 4.98. Found: C, 72.7; H, 5.5; N, 5.2.

5.8. Synthesis of (3*S*,5*R*)-3,5-bis[(*tert*-butoxycarbonyl)amino]-1-ethynyl-2-methylcyclohex-1-ene (13)

5.8.1. From diimide 10. Compound **10** (205 mg, 0.5 mmol) was dissolved in 5 mL of a 8 M solution of $MeNH_2$ in ethanol under nitrogen. The mixture was stirred for 24 h at room temperature and then 12 h at 65 °C. Solvent was removed under reduced pressure to leave a residue which was dissolved in $CHCl_3$ (10 mL). Di-*tert*-butyl-dicarbonate (350 mg, 1.6 mmol) and aqueous saturated solution of $NaHCO_3$ (0.5 mL) were added and the mixture was stirred for 24 h at room temperature. The mixture was extracted with $CHCl_3$ (3× 10 mL) and the combined organic layers were dried and evaporated under reduced pressure. The crude was purified by flash chromatography (25% EtOAc/hexane) to afford a white solid (45% yield).

5.8.2. From azide 17. A solution of NaOH (aq) (0.4 mL, 1 M, previously degassed), Me_3P (0.543 mL, 1 M in THF) and di-*tert*-butyl-dicarbonate (237 mg,

1.086 mmol) was added to a stirred solution of azide **17** (100 mg, 0.362 mmol) in THF (4 mL). The mixture was stirred for 24 h at room temperature and then brine was added. The mixture was extracted with $CHCl_3$ and the organic layers were dried and evaporated under reduced pressure lo leave a residue which was purified by flash chromatography (25% EtOAc/hexane) to afford a white solid (82% yield). Mp: 208–210 °C (decomp.). IR (KBr): υ3352, 3284, 2970, 2087, 1680, 1509, 1445, and 1391 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 1.45 (s, 18H, $H_{12} + H_{15}$), 1.73 (m, 1H, H_4), 1.94 (m, 5H, $H_4 + H_6 + H_9$), 2.62 (d, 1H, H_6, $^2J_{HH}$ 16.4 Hz), 3.09 (s, 1H, H_8), 3.72 (s, 1H, H_5), 4.28 (m, 1H, H_3), 4.49 (m, 1H, NH) and 4.65 (d, 1H, NH, $^3J_{HH}$ 8.9 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 18.8 (C_9), 28.3 ($C_{12} + C_{15}$), 35.7 (C_4), 36.4 (C_6), 42.7 (C_5), 49.2 (C_3), 79.5, 79.6 ($C_{11} + C_{14}$), 80.6 (C_8), 82.6 (C_7), 115.5 (C_1), 141.3 (C_2) y 155.1, 155.3 ($C_{10} + C_{13}$); MS (ES^+, *m/z*): 373 [$(M+Na)^+$, 100%]; $[\alpha]_D^{20} = -97.6$ (*c* 0.85, $CHCl_3$); Anal. Calcd (%) for $C_{19}H_{30}N_2O_4$: C, 65.12; H, 8.63; N, 7.99. Found: C, 65.3; H, 8.9; N, 7.7.

5.9. Synthesis of (3*S*,5*S*)-3-[(*tert*-butoxycarbonyl)amino]-1-ethynyl-5-hydroxy-2-methylcyclohex-1-ene (14)

A similar procedure as that described in Section 5.5.1 afforded **14** as a white solid (88% yield). Mp: 188–190 °C (decomp.); IR (KBr): υ3346, 2985, 2937, 2087, 1682, 1519, 1458, and 1369 cm^{-1}; ^1H NMR (MeOH-d_4, 300.13 MHz): δ 1.64 (s, 9H, H_{12}), 1.77 (m, 1H, H_4), 2.05 (s, 3H, H_9), 2.25 (m, 2H, $H_4 + H_6$), 2.57 (d, 1H, H_6, $^2J_{HH}$ 16.5 Hz), 3.63 (s, 1H, H_8), 4.07 (m, 1H, H_5) and 4.37 (m, 1H, H_3); ^{13}C NMR (MeOH-d_4, 75.5 MHz): δ 14.3 (C_9), 28.7 (C_{12}), 38.8, 39.5 ($C_4 + C_6$), 50.9 (C_3), 65.8 (C_5), 80.1 (C_{11}), 81.5 (C_8), 84.2 (C_7), 115.0 (C_1), 143.4 (C_2) and 157.9 (C_{10}); MS (ES^+, *m/z*): 274 [$(M+Na)^+$, 100%] and 290 [$(M+K)^+$, 22%]; $[\alpha]_D^{20} = -175.4$ (*c* 0.73, $CHCl_3$); Anal. Calcd (%) for $C_{14}H_{21}NO_3$: C, 66.91; H, 8.42; N, 5.57. Found: C, 67.1; H, 8.7; N, 5.6.

5.10. Synthesis of (3*S*,5*R*)-3-[(*tert*-butoxycarbonyl)amino]-1-ethynyl-2-methyl-5-phthalimidocyclohex-1-ene (15)

A similar procedure as that described in Section 5.5.1 afforded **15** as a white solid (30% yield). ^1H NMR ($CDCl_3$, 300.13 MHz): δ 1.41 (s, 9H, C *Me*$_3$), 1.9 (m, 1H, H_4), 1.97 (s, 3H, H_9), 2.33 (m, 1H, H_4), 2.72 (m, 1H, H_6), 2.85 (m, 1H, H_6), 3.08 (s, 1H, H_8), 4.4 (m, 2H, $H_3 + H_5$), 4.78 (m, 1H, NH) and 7.7 (m, 4H, $H_{12} + H_{13}$).

5.11. Synthesis of (3*S*,5*S*)-3-[(*tert*-butoxycarbonyl)amino]-1-ethynyl-5-metanesulfonyloxy-2- methylcyclohex-1-ene (16)

Et_3N (0.420 mL, 3 mmol) and MsCl (0.230 mL, 3 mmol) were added to a stirred solution of **14** (375 mg, 1.5 mmol) in anhydrous CH_2Cl_2 (20 mL). The solution was stirred for 3 h at 0 °C. Then, the solvent was removed under reduced pressure to leave a residue, which was purified by flash chromatography (25% EtOAc/hexane) to afford a white solid (94% yield). Mp: 135–137 °C; IR (KBr): υ3367, 3282, 2986, 2944, 2098, 1682, 1527, and 1420 cm^{-1}; ^1H NMR (MeOH-d_4, 300.13 MHz): δ 1.64 (s, 9H, H_{13}), 2.09 (s, 3H, H_9), 2.15 (m, 1H, H_4), 2.43 (m, 1H, H_4), 2.65 (m, 1H, H_6), 2.8 (m, 1H, H_6), 3.3 (s, 3H, H_{10}), 3.73 (s, 1H, H_8), 4.47 (m, 1H, H_3) and 5.12 (m, 1H, H_5); ^{13}C NMR (MeOH-d_4, 75.5 MHz): δ 18.2 (C_9), 28.6 (C_{13}), 36.1, 36.9 ($C_4 + C_6$), 38.3 (C_{10}), 50.1 (C_3), 76.3 (C_5), 80.3 (C_{12}), 82.4 (C_8), 83.3 (C_7), 114.0 (C_1), 143.4 (C_2) and 157.9 (C_{11}); MS (ES^+, *m/z*): 352 [$(M+Na)^+$, 100%] and 368 [$(M+K)^+$, 55%]; $[\alpha]_D^{20} = -35.8$ (*c* 1.06, EtOH); Anal. Calcd (%) for $C_{15}H_{23}NO_5S$: C, 54.69; H, 7.04; N, 4.25. Found: C, 54.8; H, 7.3; N, 4.1.

5.12. Synthesis of (3*S*,5*R*)-5-azido-3-[(*tert*-butoxycarbonyl)amino]-1-ethynyl-2-methylcyclohex-1-ene (17)

NaN_3 (228 mg, 3.5 mmol) was added to a stirred solution of **16** (385 mg, 1.17 mmol) in anhydrous DMF (10 mL). The solution was stirred for 6 h at 65 °C, and then, the solvent was removed under reduced pressure to leave a residue, which was purified by flash chromatography (10% EtOAc/hexane). White solid (79% yield). Mp: 89–91 °C; IR (KBr): υ3310, 2976, 2931, 2094, 1673, 1529, and 1453 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 1.43 (s, 9H, H_{12}), 1.75 (m, 1H, H_4), 1.92 (s, 3H, H_9), 2.0 (m, 1H, H_4), 2.15 (dd, 1H, H_6, $^2J_{HH}$ 16.8, $^3J_{HH}$ 8.9 Hz), 2.49 (dd, 1H, H_6, $^2J_{HH}$ 16.8, $^3J_{HH}$ 4.0 Hz), 3.11 (s, 1H, H_8), 3.6 (m, 1H, H_5), 4.28 (m, 1H, H_3) and 4.61 (d, 1H, NH, $^3J_{HH}$ 8.9 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 18.7 (C_9), 28.2 (C_{12}), 34.5, 34.7 ($C_4 + C_6$), 49.0 (C_3), 53.1 (C_5), 79.7 (C_{11}), 81.0 (C_8), 82.2 (C_7), 114.8 (C_1), 141.3 (C_2) and 155.1 (C_{10}); MS (ES^+, *m/z*): 299 [$(M+Na)^+$, 100%]; $[\alpha]_D^{20} = -127.5$ (*c* 0.83, EtOH); Anal. Calcd (%) for $C_{14}H_{20}N_4O_2$: C, 60.85; H, 7.30; N, 20.28. Found: C, 60.6; H, 7.5; N, 20.5.

5.13. Synthesis of (3*R*,5*R*)-5-acetoxy-1-ethynyl-2-methyl-3-phthalimidocyclohex-1-ene (20)

A similar procedure as that described for **11** afforded **20** as a white solid (84% yield). $[\alpha]_D^{20} = +98.0$ (*c* 0.8, $CHCl_3$).

5.14. Synthesis of (3*R*,5*R*)-3-[(*tert*-butoxycarbonyl)amino]-1-ethynyl-5-hydroxy-2-methylcyclohex-1-ene (21)

A similar procedure as that described for **14** afforded **21** as a white solid (77% yield). $[\alpha]_D^{20} = +177.7$ (c 0.93, $CHCl_3$).

5.15. Synthesis of (3*R*,5*R*)-3-[(*tert*-butoxycarbonyl)amino]-1-ethynyl-5-metanesulfonyloxy-2-methylcyclohex-1-ene (22)

A similar procedure as that described for **16** afforded **22** as a white solid (81% yield). $[\alpha]_D^{20} = +39.5$ (*c* 0.97, EtOH).

5.16. Synthesis of (3*R*,5*S*)-5-azido-3-[(*tert*-butoxycarbonyl)amino]-1-ethynyl-2-methylcyclohex-1-ene (23)

A similar procedure as that described for **17** afforded **23** as a white solid (75% yield). $[\alpha]_D^{20} = +139.7$ (*c* 1.09, EtOH).

5.17. Synthesis of (3*R*,5*S*)-3,5-bis[(*tert*-butoxycarbonyl)amino]-1-ethynyl-2-methylcyclohex-1-ene (24)

A similar procedure as that described for **13** afforded **24** as a white solid (79% yield). $[\alpha]_D^{20} = +92.2$ (*c* 0.5, $CHCl_3$).

5.18. Synthesis of 1α,3β-bis[(*tert*-butoxycarbonyl)amino]-25-hydroxy-6,7-didehydro-3-deoxyprevitamin D_3 (26)

CuI (2.8 mg, 0.015 mmol), $Pd(PPh_3)_2(OAc)_2$ (3.4 mg, 0.067 mmol), and Et_2NH (1.2 mL) were added to a stirred solution of **25** (73 mg, 0.149 mmol) and **13** (57.4 mg, 0.164 mmol) in DMF (1.2 mL). The reaction mixture was stirred at room temperature for 1 h under nitrogen and then poured into water and extracted with diethyl ether (3× 10 mL). The combined ether layers were dried and concentrated to give a crude, which was sufficiently pure for the next step. Although it was possible to purify this compound by flash chromatography, it decomposed in a few hours. TBAF (0.3 mL, 1 M in THF) was added to a solution of the crude in THF (3 mL) at 0 °C, and the reaction was stirred for 12 h at room temperature. THF was evaporated and the crude residue was poured into water/EtOAc. The aqueous layer was extracted with EtOAc (3× 10 mL) and the combined organic layers were dried and evaporated under reduced pressure. The crude was purified by flash chromatography to afford a white solid (82% yield). Mp: 104–106 °C; IR (KBr): υ3451, 3366, 2971, 1700, 1513, 1359, and 1239 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 0.67 (s, 3H, H_{18}), 0.93 (d, 3H, H_{21}, $^3J_{HH}$ 6.3 Hz), 1.20 (s, 6H, $H_{26} + H_{27}$), 1.42 (s, 18H, 2 Me_3CO), 1.89 (s, 3H, H_{19}), 1.0–2.3 (m, 21H, $2H_2 + H_4 + 2H_{11} + 2H_{12} + H_{14} + 2H_{15} + 2H_{16} + H_{17} + H_{20} + 2H_{22} + 2H_{23} + 2H_{24}$ + OH), 2.56 (d, 1H, H_4, $^2J_{HH}$ 14.4 Hz), 3.73 (m, 1H, H_3), 4.26 (m, 1H, H_1), 4.61 (d, 1H, NH, $^3J_{HH}$ 7.5 Hz), 4.78 (d, 1H, NH, $^3J_{HH}$ 9.1 Hz) and 5.94 (d, 1H, H_9, $^3J_{HH}$ 2.6 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 10.9, 18.5, 18.8, 20.7, 24.0, 25.0, 27.8, 28.2, 28.3, 29.0, 29.2, 35.7, 36.0, 36.2, 36.7, 41.7, 42.7, 44.2, 49.2, 49.9, 54.5, 70.8, 79.3, 87.0, 93.2, 116.7, 122.2, 133.6, 138.1, 155.1 and 155.3; MS (ES^+, *m*/*z*): 635 [$(M+Na)^+$, 100%]; Anal. Calcd (%) for $C_{37}H_{60}N_2O_5$: C, 72.51; H, 9.87; N, 4.57. Found: C, 72.6; H, 10.0; N, 4.7.

5.19. Synthesis of 1β,3α-bis[(*tert*-butoxycarbonyl)amino]-25-hydroxy-6,7-didehydro-3-deoxyprevitamin D_3 (27)

A similar procedure as that described for **26** afforded **27** as a white solid (89% yield). Mp: 112–114 °C; IR (KBr): υ3344, 2972, 1709, 1691, 1529, 1501, and 1359 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 0.68 (s, 3H, H_{18}), 0.93 (d, 3H, H_{21}, $^3J_{HH}$ 6.4 Hz), 1.20 (s, 6H, $H_{26} + H_{27}$), 1.43 (s, 18H, 2 Me_3CO), 1.89 (s, 3H, H_{19}), 1.0–2.3 (m, 21H, $2H_2 + H_4 + 2H_{11} + 2H_{12} + H_{14} + 2H_{15} + 2H_{16} + H_{17} + H_{20} + 2H_{22} + 2H_{23} + 2H_{24}$ + OH), 2.58 (d, 1H, H_4, $^2J_{HH}$ 14.4 Hz), 3.71 (m, 1H, H_3), 4.25 (m, 1H, H_1), 4.52 (d, 1H, NH, $^3J_{HH}$ 6.7 Hz), 4.67 (d, 1H, NH, $^3J_{HH}$ 7.6 Hz) and 5.94 (d, 1H, H_9, $^3J_{HH}$ 2.8 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 10.9, 18.6, 18.8, 20.7, 24.1, 25.1, 27.9, 28.3, 29.1, 29.2, 35.8, 36.0, 36.3, 36.8, 41.7, 42.9, 44.2, 49.3, 49.9, 54.6, 70.9, 79.4, 87.0, 93.3, 116.8, 122.2, 133.7, 138.1, 155.1, and 155.3; MS (ES^+, *m*/*z*): 635 [$(M+Na)^+$, 100%]; Anal. Calcd (%) for $C_{37}H_{60}N_2O_5$: C, 72.51; H, 9.87; N, 4.57. Found: C, 72.2; H, 9.6; N, 4.7.

5.20. Synthesis of 1α,3β-bis[(*tert*-butoxycarbonyl)amino]-25-hydroxy-3-deoxyprevitamin D_3 (28)

A flask containing Lindlar catalyst (74 mg) was exposed to a positive pressure of hydrogen gas (balloon). Deoxygenated MeOH (1 mL) was added, and to this suspension of quinoline (0.205 mL, 0.17 M in hexane) and **26** (35 mg, 0.058 mmol) in MeOH (4 mL) was added. After 20 min, the mixture was filtered on Celite and concentrated to afford a crude, which was sufficiently pure for the next step.

5.21. Synthesis of 1β,3α-bis[(*tert*-butoxycarbonyl)amino]-25-hydroxy-3-deoxyprevitamin D_3 (29)

A similar procedure as that described for **28** afforded **29**.

5.22. Synthesis of 1α,3β-bis[(*tert*-butoxycarbonyl)amino]-25-hydroxy-3-deoxyvitamin D_3 (30)

A solution of the crude previtamin **28** (0.058 mmol) in anhydrous acetone (4 mL) was placed in a screw-capped vial and heated at 80 °C for 4 h. Then, solvent was evaporated under reduced pressure lo leave a residue, which was purified by flash chromatography (20% EtOAc/hexane). The compound was purified further by HPLC (2.7% ^{i}PrOH/hexane, 3 mL/min, Spherisorb W, 5 μm, 250 × 10 mm) to afford a white solid (43% yield). Mp: 105–107 °C; IR (KBr): υ3446, 2953, 1692, 1502, 1363 and 1243 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 0.54 (s, 3H, H_{18}), 0.92 (d, 3H, H_{21}, $^3J_{HH}$ 6.3 Hz), 1.20 (s, 6H, $H_{26} + H_{27}$), 1.44 (s, 18H, 2 Me_3CO), 1.0-2.2 (m, 22H, $2H_2 + 2H_9 + 2H_{11} + 2H_{12} + H_{14} + 2H_{15} + 2H_{16} + H_{17} + H_{20} + 2H_{22} + 2H_{23} + 2H_{24}$ + OH), 2.59 (d, 1H, H_4, $^2J_{HH}$ 12.5 Hz), 2.79 (d, 1H, H_4, $^2J_{HH}$ 12.5 Hz) 3.88 (m, 1H, H_3), 4.30 (m, 1H, H_1), 4.60 (m, 2H, 2NH), 4.92 (m, 1H, H_{19}), 5.23 (m, 1H, H_{19}) 5.94 (d, 1H, H_7, $^3J_{HH}$ 11.1 Hz) and 6.34 (d, 1H, H_6, $^3J_{HH}$ 11.1 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 11.9, 18.7, 20.7, 22.1, 23.5, 27.5, 28.3, 29.0, 29.1, 29.3, 36.0, 36.3, 38.8, 40.4, 43.0, 44.3, 45.9, 46.2, 51.6, 56.3, 56.4, 71.0, 79.4, 113.2, 116.9, 124.9, 132.4, 143.4, 144.4, 154.9 and 155.0; MS (ES^+, *m*/*z*): 637 [$(M+Na)^+$, 100%] and 653 [$(M+K)^+$, 5%]; Anal. Calcd (%) for $C_{37}H_{62}N_2O_5$: C, 72.27; H, 10.16; N, 4.56. Found: C, 72.4; H, 10.4; N, 4.3.

5.23. Synthesis of 1β,3α-bis[(*tert*-butoxycarbonyl)amino]-25-hydroxy-3-deoxyvitamin D_3 (31)

A similar procedure as that described for **30** afforded **31** as a white solid (40% yield). Mp: 112–114 °C; IR (KBr): 3344, 2972, 1709, 1691, 1529, 1501, and 1359 cm^{-1}; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 0.68 (s, 3H, H_{18}), 0.93 (d, 3H, H_{21}, $^3J_{HH}$ 6.4 Hz), 1.20 (s, 6H, $H_{26} + H_{27}$), 1.43 (s, 18H, 2 Me_3CO), 1.89 (s, 3H, H_{19}), 1.0–2.3 (m, 21H, $2H_2 + H_4 + 2H_{11} + 2H_{12} + H_{14} + 2H_{15} + 2H_{16} + H_{17} + H_{20} + 2H_{22} + 2H_{23} + 2H_{24}$ + OH), 2.58 (d, 1H, H_4, $^2J_{HH}$ 14.4 Hz), 3.71 (m, 1H, H_3), 4.25 (m, 1H, H_1), 4.52 (d, 1H, NH, $^3J_{HH}$ 6.7 Hz), 4.67 (d, 1H, NH, $^3J_{HH}$

7.6 Hz) and 5.94 (d, 1H, H_9, $^3J_{HH}$ 2.8 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 10.9, 18.6, 18.8, 20.7, 24.1, 25.1, 27.9, 28.3, 29.1, 29.2, 35.8, 36.0, 36.3, 36.8, 41.7, 42.9, 44.2, 49.3, 49.9, 54.6, 70.9, 79.4, 87.0, 93.3, 116.8, 122.2, 133.7, 138.1, 155.1 y 155.3; MS (ES^+, m/z): 635 [$(M+Na)^+$, 100%]; Anal. Calcd (%) for $C_{37}H_{62}N_2O_5$: C, 72.27; H, 10.16; N, 4.56. Found: C, 72.5; H, 10.3; N, 4.4.

6. In vitro and in vivo biological evaluation

6.1. Cell proliferation assays

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.[20]

6.2. Cell differentiation assays

Differentiation of promyeolocytic HL 60 leukemia cells (ATCC) was measured by the nitro blue tetrazolium (NBT) reduction assay after a 72 h incubation period in the presence of 1α,25-$(OH)_2$-D_3, analogues or vehicle.[20]

6.3. In vivo calcemic activity

NMRI mice were obtained from the Proefdierencentrum of Leuven (Belgium) and fed with a vitamin D-replete diet (1% calcium, 1% phosphate, and 2500 U vitamin D/kg; Hope Farms, Woerden, The Netherlands). The calcemic effects of the analogues were tested by daily injections intraperitoneally of 1α,25-$(OH)_2$-D_3 (0.1 μg/kg/d), analogues (10 μg/kg/d) or vehicle (arachis oil) for seven consecutive days. Serum and urinary calcium were measured as calcemic parameters using commercially available kit (Sigma Diagnostics).

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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.2005.09.009.

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