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Novel A-ring homodimeric C-3-carbamate analogues of 1α,25-dihydroxyvitamin D_3: Synthesis and preliminary biological evaluation

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Abstract—The synthesis of a new class of vitamin D_3 analogues in which two units of 1α,25-dihydroxyvitamin D_3 are linked at the C-3 position by a dicarbamate functionality of variable length is described. The analogues demonstrated no affinity for the vitamin D receptor and possessed no antiproliferative or transactivating properties.
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1. Introduction

1α,25-Dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3, **1**, Figure 1], the most active metabolite of vitamin D_3 (**2**), plays a major role in many biological processes including calcium–phosphorus homeostasis, cell differentiation and proliferation, and immune reactions.[1] However, the mechanisms for these differential actions have not been clearly defined. In the last two decades, various analogues[2] of 1α,25-$(OH)_2$-D_3 have been developed to improve the biological profile of the natural hormone for a potential therapeutic application.[3] Some of these derivatives have similar or more potent antiproliferative, yet reduced hypercalcemic actions, than the natural hormone. An increasing number of synthetic vitamin D derivatives are currently in use as drugs for treatment of various human diseases and new candidates are in human clinical trials.[2a,4]

The genomic actions of 1α,25-$(OH)_2$-D_3 are mediated by the vitamin D nuclear receptor (n-VDR), which is a member of the nuclear steroid hormone receptor superfamily.[5] Structure-function analysis of the n-VDR protein reveals distinct domains involved in nuclear localization, DNA binding, ligand binding, receptor dimerization, and gene transactivation.[6] It is believed that n-VDR-mediated gene expression is initiated by binding of 1α,25-$(OH)_2$-D_3 to the ligand binding domain of n-VDR which is assumed to cause conformational changes in the receptor protein. Thus, a detailed understanding of the complementarity of the ligand shape with that of the interior surface of the nuclear VDR receptor ligand domain is the key to understand not only the structural basis of receptor action, but also to design new analogues of 1α,25-$(OH)_2$-D_3. The n-VDR is a ligand-dependent transcription factor that regulates target gene expression by interacting with response elements in gene promoters.

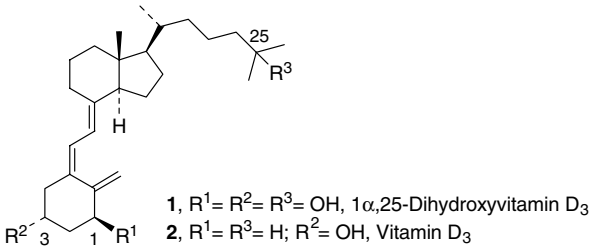

Figure 1. 1α,25-Dihydroxyvitamin D_3, the most active metabolite of vitamin D_3.

Keywords: Vitamin D_3 analogues; Enzymatic catalysis; Biological evaluation.
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The vitamin D receptor binds to the vitamin D response elements as a homodimer or as a heterodimer with retinoic acid receptors (RARs) or retinoic X receptors (RXRs).[7]

It is known that small differences in structure of ligand may elicit different biological profiles. Crystal structure of the VDR reveals that the relatively large ligand-binding cavity can accommodate much structural variation in potential calcitriol analogue ligands.[8] To the best of our knowledge, there are only three research groups that reported on the synthesis of vitamin D dimers. The first, by Mouriño et al.[9] describes the generation of dimeric vitamin D_3 and 1α,25-$(OH)_2$-D_3 comprising two units linked through the C-11 position by a carbon side chain of modulated size. In the second, Posner et al.[10] report the synthesis of a vitamin D dimeric analogue in which the monomeric vitamin D units are linked via a side-chain heteroaromatic ring. Finally, Fall et al.[11] show the preparation of a vitamin D dimer containing a disulfide bridge in the side-chain moiety.

Taking into account the efficient synthesis of A-ring carbamate precursors of 1α,25-$(OH)_2$-D_3 through a chemoenzymatic pathway,[12] we describe here an efficient convergent strategy to newly designed A-ring homodimeric derivatives of 1α,25-$(OH)_2$-D_3 (**3**, Fig. 2). In these dimers two units of 1α,25-$(OH)_2$-D_3 are linked at the C-3 position by a dicarbamate functionality of variable length, while leaving the two hydroxyl groups at C-1 and C-25 unchanged. The crucial role of both groups for the binding to VDR and DBP proteins has been established.[13] In addition, results of preliminary evaluation of their biological properties are reported to investigate the structure–activity relationships of the natural hormone.

2. Results and discussion

The homodimeric derivatives **3** were synthesized according to the enyne approach, originally developed by Lythgoe.[14] This method, based on the coupling between an A-ring synthon enyne and an enol triflate of the CD-ring/side-chain fragment, has become one of the most convenient methods for the synthesis of 1α,25-$(OH)_2$-D_3 analogues. For that, key A-ring precursors **8** are constructed as indicated in Scheme 1.

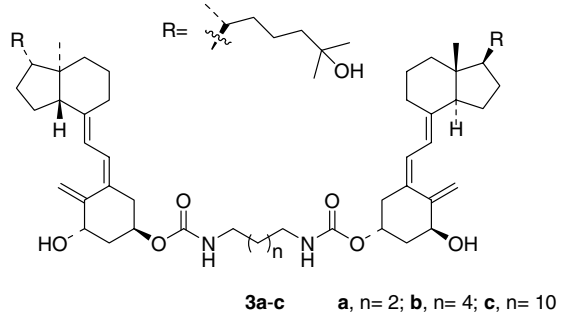
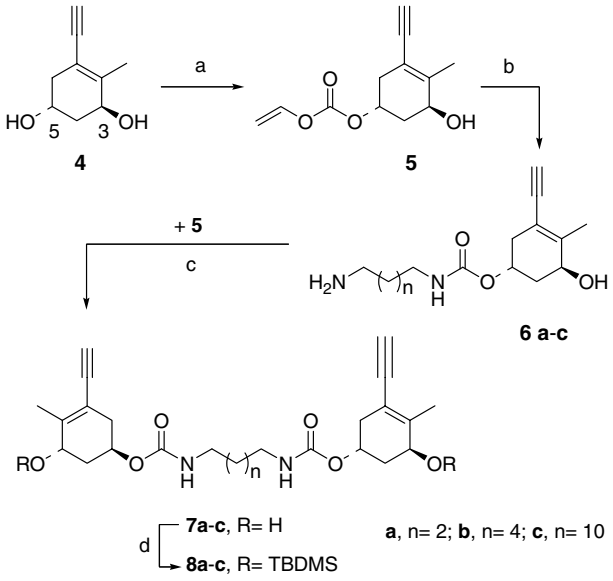


Figure 2. Homodimeric C-3-carbamate derivatives of 1α,25-dihydroxyvitamin D_3.



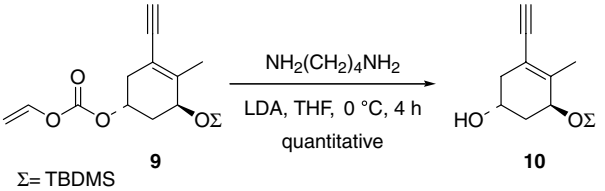
Scheme 1. Reagents and conditions: (a) CH_2=$CHOCO_2N$=CMe_2, CAL-B, toluene, 30 °C, 4 h (quantitative); (b) $NH_2(CH_2)_nNH_2$, THF, 65 °C, overnight (**6a**, 94%; **6b**, 91%; and **6c**, 93%); (c) 5, DMAP, 1,4-dioxane, 85 °C, 48 h (**7a**, 39%; **7b**, 41%; and **7c**, 40%); (d) TBDMSCl, imidazol, DMAP, DMF, rt, 14 h (**8a**, 93%; **8b**, 84%; and **8c**, 79%).

1α,25-$(OH)_2$-D_3 A-ring fragment possesses two hydroxyl groups which can be selectively modified by means of biocatalysts. The preparation of optically active compounds via enzyme-mediated reactions has demonstrated the usefulness of these catalysts.[15] Thus, *Candida antarctica* lipase B (CAL-B) catalyzes the regioselective alkoxycarbonylation at the C-5 hydroxyl group[16] of diol **4**.[12b] The reaction was carried out at 30 °C in toluene using acetone *O*-[(vinyloxy)carbonyl]oxime as acylating agent. It is noteworthy that not C-3 alkoxycarbonylation product was observed being isolated exclusively, after 4 h, vinyl carbonate **5** in quantitative yield.

Subsequent reaction of **5** with linear diamines of various lengths between the two amino functionalities afforded carbamates **6**. Next, these derivatives were allowed to react with vinyl carbonate **5** in 1,4-dioxane and DMAP as catalyst. Conversion of the carbamates to the dimers was relatively low and the reaction yielded **7** in only moderate yields (39–41%). In addition, starting materials were recovered.

The resulting homodimeric A-ring synthons **7** were conveniently protected as silyl ethers with *tert*-butyldimethylsilyl chloride.

All attempts to obtain **8** directly from **5** were unsuccessful. The reaction of **9**, the protected silyl derivative of **5**,



Scheme 2.

with the lithium diamidate of butan-1,4-diamine afforded alcohol **10** as sole product (Scheme 2). Unproductive results were also obtained in the treatment of silyl ether of **6** with LDA and subsequent treatment with **9**.

The synthesis of the homodimeric derivatives of 1α,25-$(OH)_2$-D_3 starts with the coupling[17] of A-ring precursors **8a–c** and vinyl triflate **11**,[18] prepared according to the published procedure, in the presence of catalytic amount of bis(triphenylphosphine)palladium (II) acetate-copper (I) iodide (Scheme 3). The reaction crude was subjected to desilylation with tetrabutylammonium fluoride (TBAF) to afford dienynes **12a–c** in 70–89% yield (for coupling and desilylation steps). Catalytic hydrogenation of tetraols **12** in methanol, in the presence of Lindlar catalyst and quinoline poison, generated previtamins **13**. A critical factor is the state of the catalyst, since it should be previously predried at 50 °C in vacuum. Thermolysis of the latter at 80 °C for 4 h afforded the dimers **3a–c** in 50–60% yield from **12**.

3. Biological evaluation

We evaluated the potencies of the C-3-carbamate vitamin D_3 analogs in terms of their ability to bind to the pig intestinal vitamin D receptor in comparison to the natural hormone. In addition, the transactivating capacity of 1α,25-$(OH)_2$-D_3 and its analogues was measured as well as their potency to inhibit MCF-7 breast cancer cell proliferation.

The affinity of 1α,25-$(OH)_2$-D_3 for the pig duodenal mucosa vitamin D receptor was 1.9×10^{10} M^{-1}, whereas the analogues possessed no affinity at all for the vitamin D receptor (Fig. 3).

Next, the ability of the C-3-carbamate vitamin D_3 analogues to transactivate a reporter construct, which contained a vitamin D responsive element, was determined and compared to the transactivation potential of the parent compound (Fig. 4). A 10^{-8} M concentration of 1α,25-$(OH)_2$-D_3 led to a half-maximal transactivation. However, the compounds **3a**, **3b**, and **3c** were almost unable to transactivate this reporter construct (a 1.5- to 1.9-fold induction at 10^{-6} M).

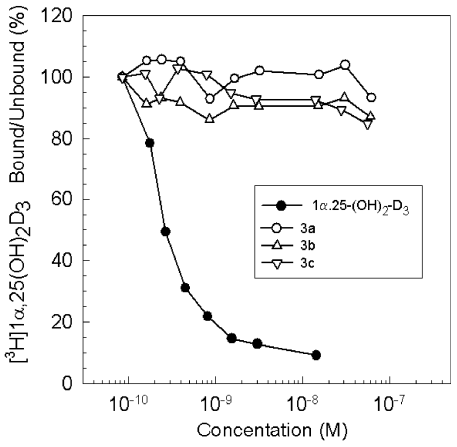


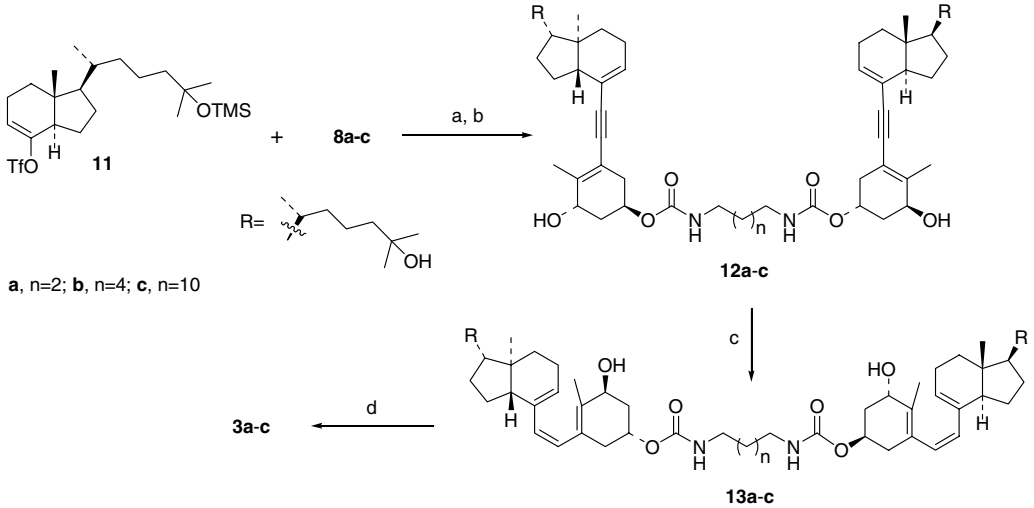
Figure 3. Affinity of 1α,25-$(OH)_2$-D_3 and its analogues for pig vitamin D receptor.

These results were paralleled by the findings on cell proliferation. The EC_{50} value of 1α,25-$(OH)_2$-D_3 to inhibit the proliferation of MCF-7 cells was 7×10^8 M^{-1} (Fig. 5).

However, none of the three C-3-carbamate analogues were able to inhibit the proliferation of MCF-7 breast cancer cells.

4. Conclusions

A facile synthetic strategy for the preparation of new dimeric 1α,25-$(OH)_2$-D_3 derivatives was developed through a chemoenzymatic pathway that allows the selective functionalization of the C-3 position. Our ini-



Scheme 3. Reagents and conditions: (a) $Pd(PPh_3)_2(OAc)_2$, CuI, Et_2NH, DMF, rt, 2 h; (b) Bu_4NF, THF, rt, overnight (**12a**, 70%; **12b**, 89%; and **12c**, 72%, 2 steps); (c) H_2, Lindlar cat., quinoline, MeOH, rt, 20 min; (d) acetone, 80 °C, 4 h (**3a**, 52%; **3b**, 50%; and **3c**, 60%, 2 steps).

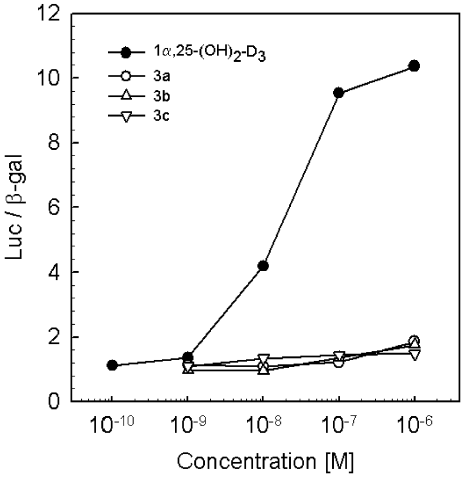


Figure 4. Transactivating potency of 1α,25-$(OH)_2$-D_3 and its analogues in COS-1 cells. Luciferase activity was normalized to β-galactosidase activity and expressed relative to the normalized activity of transfected, vehicle-stimulated cells.

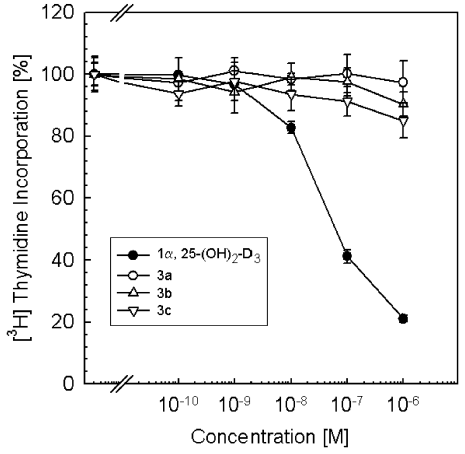


Figure 5. In vitro antiproliferative effects of 1α,25-$(OH)_2$-D_3 and its analogues in MCF-7 cells. [^{3}H]thymidine incorporation was determined in cells which were incubated during a 72 h incubation period with 1α,25-$(OH)_2$-D_3 or with the compounds **3a**, **3b** or **3c**.

tial aim was directed at the incorporation of linkers of different length. Unfortunately the analogues were not able to bind to the vitamin D receptor and demonstrated no transactivating or antiproliferative activity.

5. Experimental

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 NaCl plates or KBr pellets. Flash chromatography was performed using silica gel 60 (230–400 mesh). ^{1}H, ^{13}C NMR, and DEPT were obtained using AC-300 (^{1}H, 300.13 MHz and ^{13}C, 75.5 MHz) spectrometer for routine experiments. An AMX-400 spectrometer operating at 400.13 and 100.61 MHz for ^{1}H and ^{13}C, respectively, was used for the adquisition of ^{1}H–^{1}H and ^{1}H–^{13}C correlation experiments. The chemical shifts are given in delta (δ) values and the coupling constants (J) in Hertz (Hz). ESI$^+$ and EI (70 eV) were 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, 5μm silica gel column, 250 × 10 mm. Compound **5** has been previously synthesized.[12b]

5.2. Synthesis of 3,3′-[Alkan(1,*n*-dicarbamoyloxy)]bis-1α,25-dihydroxyvitamin D_3 (3)

A flask containing Lindlar catalyst (88 mg) was exposed to a positive pressure of hydrogen gas (balloon). Deoxygenated MeOH (3 mL) was added, and to this suspension quinoline (0.240 mL, 0.17 M in hexane) and **12** (0.0342 mmol) in MeOH (4 mL) were added. After 20 min, the mixture was filtered on Celite and concentrated to afford a crude, which was sufficiently pure for the next step. A solution of the crude previtamin **13** (0.0237 mmol) in anhydrous acetone (4 mL) was placed in a screw-capped vial and heated at 80 °C for 4 h. The solvent was evaporated to leave a residue which was purified by flash chromatography (gradient eluent 80–100% EtOAc/hexane). The compound was purified further by HPLC (18% ^{i}PrOH/hexane, 2.5 mL/min, Spherisorb W, 5μ, 250× 10 mm).

5.2.1. 3,3′-[Butan(1,4-dicarbamoyloxy)]bis-1α,25-dihydroxyvitamin D_3 (3a). Yield 52%. Mp 96–98 °C (decompose); IR (KBr): ν 3413, 2945, 2870, 1701, 1527 and 1377 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.55 (s, 6H, $6H_{18}$), 0.94 (d, 6H, $6H_{21}$, $^3J_{HH}$ 6.3 Hz), 1.23 (s, 12H, $6H_{26} + 6H_{27}$), 1.2–2.6 (remaining ring and side-chain hydrogens, series of m), 2.82 (d, 2H, $^2J_{HH}$ 9.4 Hz), 3.16 (m, 4H, 2CH_2–NH), 4.38 (m, 2H, $2H_1$), 4.80 (m, 2H, 2NH), 5.0 (s, 2H, $2H_{19}$), 5.12 (m, 2H, $2H_3$), 5.4 (s, 2H, $2H_{19}$), 6.04 (d, 2H, $2H_7$, $^3J_{HH}$ 11.0 Hz) and 6.33 (d, 2H, $2H_6$, $^3J_{HH}$ 11.0 Hz);[13]C NMR ($CDCl_3$, 75.5 MHz): δ 11.9, 18.7, 20.7, 22.2, 23.6, 27.1, 27.6, 29.0, 29.1, 29.3, 36.0, 36.3, 40.2, 40.4, 41.7, 44.3, 45.9, 56.3, 56.4, 70.0, 71.1, 111.1, 117.0, 124.3, 132.9, 143.0, 147.5 and 156.0; (ESI$^+$, m/z): 995 [(M+Na)$^+$, 85%], 1011 [(M+K)$^+$, 10%]; Anal. Calcd (%) for $C_{60}H_{96}N_2O_8$: C, 74.03; H, 9.94; N, 2.88. Found: C, 74.3; H, 10.1; N, 3.0.

5.2.2. 3,3′-[Hexan(1,6-dicarbamoyloxy)]bis-1α,25-dihydroxyvitamin D_3 (3b). Yield 50%. Mp 100–102 °C (decompose); IR (KBr): ν 3414, 2941, 2867, 1701, 1618, 1527 and 1377 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.47 (s, 6H, $6H_{18}$), 0.86 (d, 6H, $6H_{21}$, $^3J_{HH}$ 6.3 Hz), 1.14 (s, 12H, $6H_{26} + 6H_{27}$), 1.0–2.5 (remaining ring and side-chain hydrogens, series of m), 2.74 (d, 2H, $^2J_{HH}$ 12.6 Hz), 3.06 (m, 4H, 2CH_2–NH), 4.30 (m, 2H, $2H_1$), 4.67 (m, 2H, 2NH), 4.92 (s, 2H, $2H_{19}$), 5.04 (m, 2H, $2H_3$), 5.3 (s, 2H, $2H_{19}$), 5.97 (d, 2H, $2H_7$, $^3J_{HH}$ 11.0 Hz) and 6.25 (d, 2H, $2H_6$, $^3J_{HH}$ 11.0 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 11.9, 18.7, 20.6, 22.2, 23.5, 26.0, 27.6, 29.1, 29.3, 29.6, 36.0, 36.3, 40.1, 40.4, 40.5, 41.7, 44.3, 45.9, 56.3, 56.4, 69.9, 71.1, 111.0, 117.0, 124.2, 133.0, 143.0, 147.5 and 155.9; (ESI$^+$, m/z): 1023 [(M+Na)$^+$, 100%];

Anal. Calcd (%) for $C_{62}H_{100}N_2O_8$: C, 74.36; H, 10.06; N, 2.80. Found: C, 74.6; H, 10.3; N, 2.6.

5.2.3. 3,3′-[Dodecan(1,12-dicarbamoyloxy)]bis-1α, 25-dihydroxyvitamin D_3 (3c). Yield 60%. Mp 103–105 °C (decompose); IR (KBr): *ν* 3411, 2928, 2854, 1700, 1528, 1466 and 1377 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): *δ* 0.56 (s, 6H, $6H_{18}$), 0.95 (d, 6H, $6H_{21}$, $^3J_{HH}$ 6.1 Hz), 1.23 (s, 12H, $6H_{26} + 6H_{27}$), 1.27 (s, 20H, $10CH_2$), 1.2–2.6 (remaining ring and side-chain hydrogens, series of m), 3.16 (m, 4H, $2CH_2$–NH), 4.38 (m, 2H, $2H_1$), 4.68 (m, 2H, 2NH), 5.03 (s, 2H, $2H_{19}$), 5.13 (m, 2H, $2H_3$), 5.37 (s, 2H, $2H_{19}$), 6.05 (d, 2H, $2H_7$, $^3J_{HH}$ 11.2 Hz) and 6.33 (d, 2H, $2H_6$, $^3J_{HH}$ 11.2 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): *δ* 11.9, 18.7, 20.7, 22.2, 23.5, 26.7, 27.6, 29.0, 29.1, 29.2, 29.3, 29.4, 29.9, 36.0, 36.3, 40.2, 40.4, 40.9, 41.8, 44.3, 45.9, 56.3, 56.4, 67.0, 69.8, 70.1, 71.0, 111.0, 117.0, 124.3, 133.0, 143.0, 147.6 and 155.8; (ESI^+, *m/z*): 1085 [$(M+H)^+$, 10%], 1107 [$(M+Na)^+$, 23%]; Anal. Calcd (%) for $C_{68}H_{112}N_2O_8$: C, 75.23; H, 10.40; N, 2.58. Found: C, 75.5; H, 10.4; N, 2.5.

5.3. Synthesis of (3*S*,5*R*)-5-[*N*-(aminoalkyl)carbamoyloxy]-1-ethynyl-3-hydroxy-2-methylcyclohex-1-ene (6)

In a typical procedure, a solution of **5** (0.675 mmol) and corresponding diamine (3 equiv of 1,4-butanodiamine, 1,6-hexanediamine or 1,12-dodecadiamine) in THF (6 mL) was heated at 65 °C for 16 h. Then, solvent was evaporated under reduced pressure, and the residue was subjected to flash chromatography (1% NH_3/MeOH) to afford a colorless oil.

5.3.1. (3*S*,5*R*)-5-[*N*-(4-Aminobutyl)carbamoyloxy]-1-ethynyl-3-hydroxy-2-methylcyclohex-1-ene (6a). Yield 94%. IR (neat): *ν* 3288, 2933, 2855, 2091, 1711, 1552 and 1441 cm^{-1}; ^1H NMR (MeOH-d_4, 300 MHz): *δ* 1.69 (m, 4H, $2CH_2$), 2.17 (s, 3H, $3H_9$), 2.07–2.2 (m, 2H, $2H_4$), 2.33 (dd, 1H, H_6, $^2J_{HH}$ 17.1 Hz, $^3J_{HH}$ 6.5 Hz), 2.75 (d, 1H, H_6, $^2J_{HH}$ 17.1 Hz), 2.85 (apparent t, 2H, CH_2–NH_2, $^3J_{HH}$ 6.8 Hz), 3.29 (m, 2H, CH_2–NH), 3.67 (s, 1H, H_8), 4.38 (apparent t, 1H, H_3, $^3J_{HH}$ 4.8 Hz) and 5.17 (m, 1H, H_5); ^{13}C NMR (MeOH-d_4, 75.5 MHz): *δ* 18.5 (C_9), 28.1, 30.4 ($C_{12} + C_{13}$), 36.5 (C_6), 37.6 (C_4), 41.3, 41.9 ($C_{11} + C_{14}$), 68.1, 68.5 ($C_5 + C_3$), 81.9 (C_8), 83.8 (C_7), 114.7 (C_1), 144.2 (C_2) and 158.3 (C=O); MS (ESI^+, *m/z*): 267 [$(M+H)^+$, 100%], 289 [$(M+Na)^+$, 90%]; Anal. Calcd (%) for $C_{14}H_{22}N_2O_3$: C, 63.13; H, 8.33; N, 10.52. Found: C, 63.4; H, 8.5; N, 10.6.

5.3.2. (3*S*,5*R*)-5-[*N*-(6-Aminohexyl)carbamoyloxy]-1-ethynyl-3-hydroxy-2-methylcyclohex-1-ene (6b). Yield 91%. IR (neat): *ν* 3287, 2921, 2091, 1699, 1565, 1443 and 1359 cm^{-1}; ^1H NMR (MeOH-d_4, 200 MHz): *δ* 1.4–1.8 (m, 8H, $4CH_2$), 2.08 (m, 2H, $2H_4$), 2.15 (s, 3H, $3H_9$), 2.28 (dd, 1H, H_6, $^2J_{HH}$ 18 Hz, $^3J_{HH}$ 6.8 Hz), 2.71 (dd, 1H, H_6, $^2J_{HH}$ 18 Hz), 2.87 (apparent t, 2H, CH_2–NH_2, $^3J_{HH}$ 6.8 Hz), 3.24 (apparent t, 2H, CH_2–NH, $^3J_{HH}$ 6.6 Hz), 3.64 (s, 1H, H_8), 4.34 (m, 1H, H_3) and 5.2 (m, 1H, H_5); ^{13}C NMR (MeOH-d_4, 50.3 MHz): *δ* 18.5 (C_9), 27.4, 30.6, 31.8 ($C_{12} + C_{13} + C_{14} + C_{15}$), 36.5 ($C_6$), 37.5 ($C_4$), 41.4, 41.7 ($C_{11} + C_{16}$), 68.1 ($C_5$), 68.5 ($C_3$), 81.9 ($C_8$), 83.8 ($C_7$), 114.8 ($C_1$), 144.1 ($C_2$) and 158.3 (C=O); ($ESI^+$, *m/z*): 295 [$(M+H)^+$, 100%]; Anal. Calcd (%) for $C_{16}H_{26}N_2O_3$: C, 65.28; H, 8.90; N, 9.52. Found: C 65.1; H, 9.1; N, 9.3.

5.3.3. (3*S*,5*R*)-5-[*N*-(12-Aminododecyl)carbamoyloxy]-1-ethynyl-3-hydroxy-2-methylcyclohex-1-ene (6c). Yield 93%. IR (neat): *ν* 3345, 3282, 2923, 2859, 2093, 1667, 1534 and 1468 cm^{-1}; ^1H NMR (MeOH-d_4, 300 MHz): *δ* 1.4–1.8 (m, 20H, 10 CH_2), 2.17 (s, 3H, $3H_9$), 2.1–2.17 (m, 2H, $2H_4$), 2.33 (dd, 1H, H_6, $^2J_{HH}$ 17.1 Hz, $^3J_{HH}$ 6.8 Hz), 2.74 (d, 1H, H_6, $^2J_{HH}$ 17.1 Hz), 2.96 (apparent t, 2H, CH_2–NH_2, $^3J_{HH}$ 7.4 Hz), 3.26 (apparent t, 2H, CH_2–NH, $^3J_{HH}$ 7.1 Hz), 3.66 (s, 1H, H_8), 4.38 (m, 1H, H_3) and 5.17 (m, 1H, H_5); ^{13}C NMR (MeOH-d_4, 75.5 MHz): *δ* 18.5 (C_9), 27.7, 27.7, 30.3, 30.6, 30.8, 31.0 ($C_{12} + C_{13} + C_{14} + C_{15} + C_{16} + C_{17} + C_{18} + C_{19} + C_{20} + C_{21}$), 36.5 ($C_6$), 37.6 ($C_4$), 41.5, 41.6 ($C_{11}$+$C_{22}$), 68.1 ($C_5$), 68.6 ($C_3$), 81.8 ($C_8$), 83.8 ($C_7$), 114.8 ($C_1$), 144.1 ($C_2$) and 158.4 (C=O); ($ESI^+$, *m/z*): 379 [$(M+H)^+$, 100%]; Anal. Calcd (%) for $C_{22}H_{38}N_2O_3$: C, 69.80; H, 10.12; N, 7.40. Found: C, 69.6; H, 10.3; N, 7.5.

5.4. Synthesis of (3*S*,5*R*,3′*S*,5′*R*)-1,1′-Diethynyl-3,3′-dihydroxy-2,2′-dimethyl-5,5′-[alkan(1,*n*-dicarbamoyloxy)]dicyclohex-1-ene (7)

A mixture of carbonate **5** (0.765 mmol), carbamate **6** (0.765 mmol), and DMAP (catalytic amount) in 1,4-dioxane (10 mL) was heated at 85 °C for 60 h. After evaporation of the solvent, the residual mixture was purified by flash chromatography (gradient eluent 70–100% EtOAc/hexane) to give compound **7** as a white solid.

5.4.1. (3*S*,5*R*,3′*S*,5′*R*)-1,1′-Diethynyl-3,3′-dihydroxy-2,2′-dimethyl-5,5′-[butan(1,4-dicarbamoyloxy)]dicyclohex-1-ene (7a). Yield 39%. Mp 206–208 °C (decompose); IR (KBr): *ν* 3488, 3336, 2963, 2092, 1669, 1542 and 1451 cm^{-1}; ^1H NMR (MeOH-d_4, 300 MHz): *δ* 1.67 (m, 4H, $2CH_2$), 2.16 (s, 6H, $3H_9 + 3H'_9$), 2.05–2.2 (m, 4H, $2H_4 + 2H'_4$), 2.33 (dd, 2H, $H_6 + H'_6$, $^2J_{HH}$ 16.7 Hz, $^3J_{HH}$ 4.9 Hz), 2.74 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 16.7 Hz), 3.28 (m, 4H, $2CH_2$–NH), 3.65 (s, 2H, $H_8 + H'_8$), 4.37 (m, 2H, $H_3 + H'_3$) and 5.17 (m, 2H, $H_5 + H'_5$); ^{13}C NMR (MeOH-d_4, 75.5 MHz): *δ* 19.3 ($C_9 + C'_9$), 28.9 ($C_{12} + C'_{12}$), 37.4, 38.5 ($C_4 + C'_4 + C_6 + C'_6$), 42.1 ($2CH_2$–NH), 69.1, 69.5 ($C_3 + C'_3 + C_5 + C'_5$), 82.7 ($C_8 + C'_8$), 84.7 ($C_7 + C'_7$), 115.7 ($C_1 + C'_1$), 145.0 ($C_2 + C'_2$) and 159.3 (2C=O); (ESI^+, *m/z*): 467 [$(M+Na)^+$, 100%]; Anal. Calcd (%) for $C_{24}H_{32}N_2O_6$: C, 64.85; H, 7.26; N, 6.30. Found: C, 65.0; H, 7.2; N, 6.4.

5.4.2. (3*S*,5*R*,3′*S*,5′*R*)-1,1′-Diethynyl-3,3′-dihydroxy-2,2′-dimethyl-5,5′-[hexan(1,6-dicarbamoyloxy)]dicyclohex-1-ene (7b). Yield 41%. Mp 170–172 °C (decompose); IR (KBr): *ν* 3459, 3346, 3294, 2934, 2092, 1667, 1543, 1455 and 1370 cm^{-1}; ^1H NMR (MeOH-d_4, 300 MHz): *δ* 1.52 (m, 4H, $2CH_2$), 1.67 (m, 4H, $2CH_2$), 2.12 (m, 4H, $2H_4 + 2H'_4$), 2.16 (s, 6H, $3H_9 + 3H'_9$), 2.33 (dd, 2H, $H_6 + H'_6$, $^2J_{HH}$ 17.1 Hz, $^3J_{HH}$ 6.3 Hz), 2.74 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 17.1 Hz), 3.27 (apparent t, 4H, $2CH_2$–NH, $^3J_{HH}$ 6.8 Hz), 3.66 (s, 2H, $H_8 + H'_8$), 4.38 (m, 2H, $H_3 + H'_3$) and 5.17 (m, 2H, $H_5 + H'_5$); ^{13}C NMR

(MeOH-d_4, 75.5 MHz): δ 18.5 ($C_9 + C'_9$), 27.3, 30.7 ($C_{12} + C'_{12} + C_{13} + C'_{13}$), 36.5, 37.5 ($C_4 + C'_4 + C_6 + C'_6$), 41.4 ($2CH_2$–NH), 68.1, 68.5 ($C_3 + C'_3 + C_5 + C'_5$), 81.8 ($C_8 + C'_8$), 83.8 ($C_7 + C'_7$), 114.8 ($C_1 + C'_1$), 141.1 ($C_2 + C'_2$) and 158.3 (2C=O); (ESI$^+$, *m/z*): 495 [(M+Na)$^+$, 100%]; Anal. Calcd (%) for $C_{26}H_{36}N_2O_6$: C, 66.08; H, 7.68; N, 5.93. Found: C, 66.2; H, 7.8; N, 6.0.

5.4.3. (3*S*,5*R*,3′*S*,5′*R*)-1,1′-Diethynyl-3,3′-dihydroxy-2,2′-dimethyl-5,5′-[dodecan(1,12-dicarbamoyloxy)]dicyclohex-1-ene (7c). Yield 40%. Mp 123–125 °C (decompose); IR (KBr) *v* 3347, 2925, 2093, 1686, 1534 and 1466 cm^{-1}; ^{1}H NMR (MeOH-d_4, 300 MHz): δ 1.49 (m, 16H, $8CH_2$), 1.66 (m, 4H, $2CH_2$), 2.17 (s, 6H, $3H_9 + 3H'_9$), 2.05–2.2 (m, 4H, $2H_4 + 2H'_4$), 2.33 (dd, 2H, $H_6 + H'_6$, $^2J_{HH}$ 16.5 Hz, $^3J_{HH}$ 6 Hz), 2.73 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 16.5 Hz), 2.25 (m, 4H, $2CH_2$–NH), 3.66 (s, 2H, $H_8 + H'_8$), 4.37 (m, 2H, $H_3 + H'_3$) and 5.16 (m, 2H, $H_5 + H'_5$); ^{13}C NMR (MeOH-d_4, 75.5 MHz): δ 18.4 ($C_9 + C'_9$), 27.7, 30.3, 30.6, 30.8 ($C_{12} + C'_{12} + C_{13} + C'_{13} + C_{14} + C'_{14} + C_{15} + C'_{15} + C_{16} + C'_{16}$), 36.5, 37.6 ($C_4 + C'_4 + C_6 + C'_6$), 41.6 ($2CH_2$-NH), 68.1, 68.6 ($C_3 + C'_3 + C_5 + C'_5$), 81.8 ($C_8 + C'_8$), 83.8 ($C_7 + C'_7$), 114.8 ($C_1 + C'_1$), 144.1 ($C_2 + C'_2$) and 158.4 (2C=O); (ESI$^+$, *m/z*): 579 [(M+Na)$^+$, 100%]; Anal. Calcd (%) for $C_{32}H_{48}N_2O_6$: C, 69.04; H, 8.69; N, 5.03. Found: C, 69.2; H, 8.8; N, 4.9.

5.5. Synthesis of (3*S*,5*R*,3′*S*,5′*R*)-3,3′-Di[(*tert*-butyldimethylsilyl)oxy]-1,1′-diethynyl-2,2′-dimethyl-5,5′-[alcan(1,n-dicarbamoyloxy)]dicyclohex-1ene (8)

Imidazol (93 mg, 1.404 mmol), DMAP (catalytic amount), and *tert*-butyldimethylsilyl chloride (140 mg, 0.936 mmol) were added to a stirred solution of dimer **7** (0.234 mmol) in DMF (3 mL) under nitrogen. The solution was stirred at room temperature for 12 h, and then evaporated under reduced pressure to leave a residue which was purified by flash chromatography (30% EtOAc/hexane). Colorless oil.

5.5.1. (3*S*,5*R*,3′*S*,5′*R*)-3,3′-Di[(*tert*-butyldimethylsilyl)oxy]-1,1′-diethynyl-2,2′-dimethyl-5,5′-[butan(1,4-dicarbamoyloxy)]dicyclohex-1ene (8a). Yield 93%. IR (neat) *v* 3500, 2956, 2940, 1700, 1549 and 1521 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.11 (s, 12H, $2SiMe_2$), 0.91 (s, 18H, $2CMe_3$), 1.53 (m, 4H, $2CH_2$), 1.95 (s, 6H, $3H_9 + 3H'_9$), 1.8–2.0 (m, 4H, $2H_4 + 2H'_4$), 2.18 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 15 Hz), 2.58 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 15 Hz), 3.06 (s, 2H, $H_8 + H'_8$), 3.18 (m, 4H, $2CH_2$–NH), 4.24 (m, 2H, $H_3 + H'_3$), 4.71 (m, 2H, 2NH) and 5.06 (m, 2H, $H_5 + H'_5$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ -4.9 (2Si*Me*), −4.4 (2Si*Me*), 18.0 ($2SiCMe_3$), 18.5 ($C_9 + C'_9$), 25.7 ($2CMe_3$), 27.2 ($C_{12} + C'_{12}$), 35.2, 36.8 ($C_4 + C'_4 + C_6 + C'_6$), 40.4 ($2CH_2$-NH), 67.4, 68.4 ($C_3 + C'_3 + C_5 + C'_5$), 79.8 ($C_8 + C'_8$), 83.2 ($C_7 + C'_7$), 112.8 ($C_1 + C'_1$), 144.1 ($C_2 + C'_2$) and 155.8 (2C=O); (ESI$^+$, *m/z*): 695 [(M+Na)$^+$, 100%]; Anal. Calcd (%) for $C_{36}H_{60}N_2O_6Si_2$: C, 64.24; H, 8.99, N, 4.16. Found: C, 64.0; H, 9.2; N, 3.9.

5.5.2. (3*S*,5*R*,3′*S*,5′*R*)-3,3′-Di[(*tert*-butyldimethylsilyl)oxy]-1,1′-diethynyl-2,2′-dimethyl-5,5′-[hexan(1,6-dicarbamoyloxy)]dicyclohex-1ene (8b). Yield 84%. IR (neat) *v* 3313, 2931, 2094, 1709, 1529, 1472 and 1362 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.13 (s, 12H, $2SiMe_2$), 0.93 (s, 18H, $2CMe_3$), 1.36 (m, 4H, $2CH_2$), 1.53 (m, 4H, $2CH_2$), 1.98 (s, 6H, $3H_9 + 3H'_9$), 1.8–2.0 (m, 4H, $2H_4 + 2H'_4$), 2.22 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 18 Hz), 2.63 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 18 Hz), 3.09 (s, 2H, $H_8 + H'_8$), 3.19 (m, 4H, $2CH_2$-NH), 4.27 (m, 2H, $H_3 + H'_3$), 4.68 (m, 2H, 2NH) and 5.09 (m, 2H, $H_5 + H'_5$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ -4.9 (2Si*Me*), -4.4 (2Si*Me*), 17.9 ($2SiCMe_3$), 18.5 ($C_9 + C'_9$), 25.7 ($2CMe_3$), 26.2, 29.8 ($C_{12} + C'_{12} + C_{13} + C'_{13}$), 35.2, 36.8 ($C_4 + C'_4 + C_6 + C'_6$), 40.7 ($2CH_2$-NH), 67.3, 68.4 ($C_3 + C'_3 + C_5 + C'_5$), 79.8 ($C_8 + C'_8$), 83.2 ($C_7 + C'_7$), 112.8 ($C_1 + C'_1$), 144.1 ($C_2 + C'_2$) and 155.8 (2C=O); (ESI$^+$, *m/z*): 723 [(M+Na)$^+$, 100%]; Anal. Calcd (%) for $C_{38}H_{64}N_2O_6Si_2$: C, 65.10; H, 9.20; N, 4.00. Found: C, 65.3; H, 9.6; N, 3.8.

5.5.3. (3*S*,5*R*,3′*S*,5′*R*)-3,3′-Di[(*tert*-butyldimethylsilyl)oxy]-1,1′-diethynyl-2,2′-dimethyl-5,5′-[dodecan(1,12-dicarbamoyloxy)]dicyclohex-1ene (8c). Yield 79%. IR (neat) *v* 3317, 2932, 2095, 1701, 1528, 1460 and 1360 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.13 (s, 12H, $2CMe_3$), 0.93 (s, 18H, $2CMe_3$), 1.29 (m, 16H, $8CH_2$), 1.51 (m, 4H, $2CH_2$), 1.98 (s, 6H, $3H_9 + 3H'_9$), 1.85-2.05 (m, 4H, $2H_4 + 2H'_4$), 2.23 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 17.3 Hz), 2.63 (d, 2H, $H_6 + H'_6$, $^2J_{HH}$ 17.3 Hz), 3.09 (s, H, $H_8 + H'_8$), 3.19 (m, 4H, $2CH_2$-NH), 4.27 (m, 2H, $H_3 + H'_3$), 4.66 (m, 2H, 2NH), and 5.10 (m, 2H, $H_5 + H'_5$); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ −4.9 (2Si*Me*), −4.4 (2Si*Me*), 17.9 ($2SiCMe_3$), 18.5 ($C_9 + C'_9$), 25.7 ($2CMe_3$), 26.7, 29.2, 29.4, 29.9 ($C_{12} + C'_{12} + C_{13} + C'_{13} + C_{14} + C'_{14} + C_{15} + C'_{15} + C_{16} + C'_{16}$), 35.6, 36.8 ($C_4 + C'_4 + C_6 + C'_6$), 40.9 ($2CH_2$-NH), 67.2, 68.4 ($C_3 + C'_3 + C_5 + C'_5$), 79.8 ($C_8 + C'_8$), 83.2 ($C_7 + C'_7$), 112.8 ($C_1 + C'_1$), 144.1 ($C_2 + C'_2$) and 155.7 (2C=O); (ESI$^+$, *m/z*): 807 [(M+Na)$^+$, 100%]; Anal. Calcd (%) for $C_{44}H_{76}N_2O_6Si_2$: C, 67.30; H, 9.76; N, 3.57. Found: C, 67.2; H, 9.7; N, 3.3.

5.6. Synthesis of (3*S*,5*R*)-3-[(*tert*-Butyldimethylsilyl)oxy]-1-ethynyl-2-methyl-5-[(vinyloxy)carbonyloxy]cyclohex-1-ene (9)

To a solution of **10** (1.73 mmol) in CH_2Cl_2 (10 mL) and pyridine (0.4 mL) at 0 °C was added dropwise vinyl chloroformate (4.33 mmol). The progress of the reaction was followed by TLC (40% EtOAc/hexane). After consumption of the starting material (4 h), the reaction mixture was extracted with CH_2Cl_2 and brine. The organic layer was dried, evaporated under reduced pressure, and the residue was subjected to flash chromatography (15% EtOAc/hexane). White solid. Yield 87%. mp 63–65 °C; IR (KBr) *v* 3500, 2956, 2096, 1748, 1648, 1470 and 1364 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.12 (s, 6H, 2*Me*Si), 0.91 (s, 9H, $SiCMe_3$), 1.96 (s, 3H, $3H_9$), 1.95-2.1 (m, 2H, $2H_4$), 2.30 (dd, 1H, H_6, $^2J_{HH}$16.4, $^3J_{HH}$ 6.9 Hz), 2.68 (dd, 1H, H_6, $^2J_{HH}$16.4, $^3J_{HH}$ 3.8 Hz), 3.08 (s, 1H, H_8), 4.27 (apparent t, $^3J_{HH}$4.6 Hz), 4.58 (dd, 1H, H_2C=*cis*, $^3J_{HH}$6.4, $^2J_{HH}$1.8 Hz), 4.93 (dd, 1H, H_2C=*trans*, $^3J_{HH}$13.8, $^2J_{HH}$1.8 Hz), 5.10 (m, 1H, H_5) and 7.10 (dd, 1H, =CH, $^3J_{HH}$13.8, $^3J_{HH}$6.4 Hz); ^{13}C NMR ($CDCl_3$, 50 MHz): δ −4.9 (Si*Me*), −4.5 (Si*Me*), 17.9 ($SiCMe_3$), 18.7 (C_9), 25.6 (CMe_3), 34.7, 36.6 ($C_4 + C_6$), 68.4, 71.7 ($C_3 + C_5$), 80.2 (C_8), 82.8 (C_7), 97.6 (CH_2=), 112.5 (C1), 142.5, 143.8 (C_2 + CH=) and 151.9

(C=O); (EI, *m/z*): 336 (M^+, 2%), 279 (M-^{t}Bu, 20), 249 (45), 248 (40), 233 (14), 192 (16), 145 (91), 117 (59), 101 (53), 75 (100); Anal. Calcd (%) for $C_{18}H_{28}O_4Si$: C, 64.25; H, 8.39. Found: C, 64.1; H, 8.6.

5.7. Synthesis of (3*S*,5*R*)-3[(*tert*-Butyldimethylsilyl)oxy]-1-ethynyl-5-hydroxy-2-methylcyclohex-1-ene (10)

To a solution of 1,4-butanodiamine (0.044 mmol) in THF (3 mL) at −78 °C was added dropwise LDA (0.45 mmol). The reaction mixture was stirred at 0 °C for 15 min and then cooled to −78 °C. Compound **9** was added (0.089 mmol) and the reaction was followed by TLC. After 4 h, the reaction mixture was extracted with Et_2O/brine, the organic layer was dried and evaporated under reduced pressure, and the crude was subjected to flash chromatography to obtain **10** in quantitative yield. This compound has been previously described.[19] ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.10 (s, 6H, 2*Me*Si), 0.89 (s, 9H, *Me*$_3$CSi), 1.75 (ddd, 1H, H_4, $^2J_{HH}$13.4, $^3J_{HH}$9.8, $^3J_{HH}$4.6 Hz), 1.85–2.0 (m, 3H$_9$ + H$_4$), 2.0 (dd, 1H, H_6, $^2J_{HH}$16.5, $^3J_{HH}$7.8 Hz), 2.55 (m, 1H, H_6), 3.06 (s, 1H, H_8), 4.15 (m, 1H, H_5) and 4.24 (m, 1H, H_3).

5.8. Synthesis of 3,3′-[Alkan(1,n-dicarbamoyloxy)]bis-1α,25-dihydroxy-6,7-dehydroprevitamin D_3 (12)

CuI (5 mg, 0.026 mmol), $Pd(PPh_3)_2(OAc)_2$ (7 mg, 0.009 mmol), and Et_2NH (2.5 mL) were added to a solution of **8** (0.148 mmol) and **11** (137 mg, 0.282 mmol) in DMF (2.5 mL). The reaction mixture was stirred at room temperature for 2 h under nitrogen and then poured into water and extracted with diethyl ether (3× 10 mL). The combined ether fractions were dried over Na_2SO_4, filtered, 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 (5 mL of 1.0 M in THF, 5 mmol) was added to a solution of the crude in THF (10 mL) at 0 °C and the reaction mixture was stirred at room temperature for 18 h. 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 over Na_2SO_4 and evaporated in a vacuum. The crude was purified by flash chromatography (EtOAc) to afford **12** as a white solid.

5.8.1. 3,3′-[Butan(1,4-dicarbamoyloxy)]bis-1α,25-dihydroxy-6,7-dehydroprevitamin D_3 (12a). Yield 70%. Mp 99–101 °C; IR (KBr): *v* 3393, 2951, 1699, 1541, 1458 and 1377 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.68 (s, 6H, 6H$_{18}$), 0.94 (d, 6H, 6H$_{21}$, $^3J_{HH}$ 6.5 Hz), 1.20 (s, 12H, 6H$_{26}$+6H$_{27}$), 1.97 (s, 6H, 6H$_{19}$), 1.0–2.3 (remaining ring and side-chain hydrogens, series of m), 2.57 (d, 2H, $^2J_{HH}$ 13.4 Hz), 3.15 (m, 6H, 2CH_2–NH+2OH), 4.20 (m, 2H, 2H$_1$), 5.01 (br s, 2H, 2NH), 5.14 (m, 2H, 2H$_3$) and 5.94 (d, 2H, 2H$_9$, $^3J_{HH}$ 3.1 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 10.9, 18.4, 18.6, 20.7, 24.0, 25.1, 27.0, 27.9, 29.0, 29.2, 35.7, 36.0, 36.2, 40.3, 41.7, 44.2, 49.9, 54.5, 67.1, 68.2, 71.0, 87.3, 93.1, 114.9, 122.3, 133.5, 140.2, and 156.1; (ESI$^+$, *m/z*): 991 [(M+Na)$^+$, 100%], 1007 [(M+K)$^+$, 15%]; Anal. Calcd (%) for $C_{60}H_{92}N_2O_8$: C, 74.34; H, 9.57; N, 2.89. Found: C, 74.2; H, 9.7; N, 2.6.

5.8.2. 3,3′-[Hexan(1,6-dicarbamoyloxy)]bis-1α,25-dihydroxy-6,7-dehydroprevitamin D_3 (12b). Yield 89%. Mp 105–107 °C; IR (KBr): *v* 3413, 2948, 2874, 1701, 1528 and 1466 cm^{-1}; ^{1}H NMR (MeOH-d_4, 300 MHz): δ 0.92 (s, 6H, 6H$_{18}$), 1.18 (d, 6H, 6H$_{21}$, $^3J_{HH}$ 6.3 Hz), 1.37 (s, 12H, 6H$_{26}$ + 6H$_{27}$), 1.2–2.5 (remaining ring and side-chain hydrogens, series of m), 2.14 (s, 6H, 6H$_{19}$), 2.73 (d, 2H, $^2J_{HH}$ 13.9 Hz), 3.28 (t, 4H, 2CH_2NH, $^3J_{HH}$ 6.6 Hz), 4.38 (m, 2H, 2H$_1$), 5.15 (m, 2H, 2H$_3$) and 6.10 (d, 2H, 2H$_9$, $^3J_{HH}$ 2.8 Hz); ^{13}C NMR (MeOH-d_4, 75.5 MHz): δ 11.4, 18.8, 19.2, 21.8, 25.3, 26.0, 27.3, 29.0, 29.1, 29.2, 30.7, 36.9, 37.1, 37.4, 37.6, 37.7, 41.4, 42.9, 45.1, 51.3, 56.0, 68.3, 68.8, 71.3, 88.4, 94.0, 116.1, 123.7, 134.1, 141.2 and 158.4; (ESI$^+$, *m/z*): 1019 [(M+Na)$^+$, 60%]; Anal. Calcd (%) for $C_{62}H_{96}N_2O_8$: C, 74.66; H, 9.70; N, 2.81. Found: C, 74.5; H, 9.9; N, 2.9.

5.8.3. 3,3′-[Dodecan(1,12-dicarbamoyloxy)]bis-1α,25-dihydroxy-6,7-dehydroprevitamin D_3 (12c). Yield 72 %. Mp 120–122 °C; IR (KBr): *v* 3416, 2928, 1700, 1541, 1466 and 1381 cm^{-1}; ^{1}H NMR (MeOH-d_4, 300 MHz): δ 0.92 (s, 6H, 6H$_{18}$), 1.17 (d, 6H, 6H$_{21}$, $^3J_{HH}$ 6.0 Hz), 1.37 (s, 12H, 6H$_{26}$ + 6H$_{27}$), 1.49 (s, 20H, 10CH_2), 1.4–2.5 (remaining ring and side-chain hydrogens), 2.14 (s, 6H, 6H$_{19}$), 2.73 (d, 2H, $^2J_{HH}$ 16.0 Hz), 3.25 (m, 4H, 2CH_2NH), 4.39 (m, 2H, 2H$_1$), 5.16 (m, 2H, 2H$_3$) and 6.10 (d, 2H, 2H$_9$, $^3J_{HH}$ 3.0 Hz); ^{13}C NMR (MeOH-d_4, 75.5 MHz): δ 11.3, 18.7, 19.1, 21.8, 25.3, 25.9, 27.5, 27.7, 29.0, 29.2, 30.3, 30.5, 30.8, 36.9, 37.1, 37.4, 37.6, 37.7, 41.5, 42.9, 45.1, 51.3, 56.0, 68.3, 68.8, 71.3, 88.4, 93.9, 116.1, 123.7, 134.1, 141.2 and 158.4; (ESI$^+$, *m/z*): 1103 [(M+Na)$^+$, 5%]; Anal. Calcd (%) for $C_{68}H_{108}N_2O_8$: C, 75.51; H, 10.06; N, 2.59. Found: C, 75.8; H, 10.3; N, 2.8.

5.9. In vitro biological evaluation

5.9.1. Affinity for VDR. 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. 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).[20]

5.9.2. Transactivation assay. COS-1 cells (American Type Culture Collection, Manassas, VA) (30,000) were seeded in 24-well dishes. The next day, cells were transfected with 100 ng of a luciferase reporter construct, which contained three copies of the mouse osteopontin vitamin D responsive element, 50 ng pSG5-based expression vectors for VDR and RXR, and 20 ng pcDNA3.1(-)/Myc-His/*lacZ* (Invitrogen, Merelbeke, Belgium). Cells were transfected with FuGENE 6 (Roche, Mannheim, Germany). The day after transfection, cells were stimulated with vehicle (ethanol) or dilu-

tions of 1α,25-$(OH)_2$-D_3 or analogues. The next day, cells were lysed with Reporter Lysis Buffer (Roche Diagnostics), and luciferase activity was measured with the Luciferase Assay System (Promega, Madison, WI) and normalized to β-galactosidase activity, measured with the Galacto-Light Plus System (Applied Biosystems, Foster City, CA). Normalized luciferase activity was expressed relative to transfected vehicle-stimulated cells.

5.9.3. Cell proliferation assays. As a measure of cell proliferation, [^{3}H]thymidine incorporation of breast cancer MCF-7 (ATCC) 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]

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