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26- and 27-Methyl groups of 2-substituted, 19-nor-1α,25-dihydroxylated vitamin D compounds are essential for calcium mobilization *in vivo*

Pawel Grzywacz, Lori A. Plum, Margaret Clagett-Dame, Hector F. DeLuca *

Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706, USA

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ABSTRACT

Twelve new analogs of 19-nor-1α,25-dihydroxyvitamin D_3 **6**–**17**, were prepared by a multi-step procedure from known alcohols **18** and **19**. We have examined the influence of removing two methyl groups located at C-25, as well as the 25-hydroxy group, on the biological *in vitro* and *in vivo* biological activity. Surprisingly, removal of the 26- and 27-methyl groups from either the 2α-methyl or 2-methylene-19-nor-1α,25-dihydroxyvitamin D_3 reduced vitamin D receptor binding, HL-60 differentiation, and 25-hydroxylase transcription *in vitro* only slightly to moderately (compounds **6**–**13**). However, these compounds were devoid of *in vivo* bone mobilization activity and had markedly reduced activity on intestinal calcium transport. The analogs **14**–**17** with a 2β-methyl substitution had little or no activity *in vitro* and *in vivo* as expected from previous work.

1. Introduction

1α,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, calcitriol, **1**; Fig. 1), the hormonally active form of vitamin D_3, possesses a broad spectrum of biological activities. Its function consists of regulation of calcium homeostasis [1–4], immunomodulation [5,6], cell differentiation and antiproliferation [7–10]. Potential therapeutic applications of 1,25-$(OH)_2D_3$, which are associated with the inhibition of cell growth and with the stimulation of cell differentiation, are however hampered by the danger of hypercalcemia. This has resulted in attempts at modification of its structure to separate calcemic activities from other biological responses [11,12]. We have focused on side chain modifications [13,14]. In 2010, we reported the biological properties of 2-methyl and 2-methylene-19,26-dinor analogs **2**–**5** (Fig. 1) with a fixed 25-hydroxy group [15]. The study clearly shows that removal of one methyl from carbon-25 of the 19-norvitamin D_3 compounds markedly reduces the calcemic activity from either bone or intestine. The lack of that methyl group had little impact on VDR binding, HL-60 differentiation and *in vitro* transcription. These results suggest that *in vivo* calcemic activity might be further reduced by the elimination of both 26- and 27-methyl groups. We now report several new analogs (**6**–**17**) produced by removing two 26,27-methyl groups, as well as 25-hydroxy group, from the 2-carbon-substituted 19-nor-1,25-$(OH)_2D_3$ and its 20*S* isomer.

2. Results and discussion

2.1. Chemistry

The convergent synthesis of new analogs **6**–**17** was based on the Lythgoe type Horner-Wittig olefination reaction [16], which was successfully utilized by us earlier for preparation of the vitamin D compounds **2**–**5** [15]. This approach required using new Grundmann type ketones **31**–**34** (Scheme 1), which we decided to prepare by attaching a three-carbon fragment to iodides (**20** and **21**) in one step, based on an efficient method [17] introduced by Mourino in the vitamin D field [18]. The needed compounds (**20** and **21**) were easily obtained from the known alcohols **18** and **19** [13]. The 22-hydroxy group of the compound **18** was selectively tosylated and then converted into the corresponding iodide **20**. To improve the efficacy of the synthesis of iodide **21** the primary alcohol **19** was treated with triphenylphosphine, iodine and imidazole [19]. The introduction of side chains was realized via Ni(0) catalyzed oxidative addition [17] of iodides (**20** and **21**) to *iso*-propyl acrylate, which provided *iso*-propyl esters **22** and **23**. The removal of the *tert*-butyldimethylsilyl protective group from the ether **23** under acidic conditions gave the secondary alcohol **24**. The *iso*-propyl esters (**22** and **24**) were treated with lithium aluminum hydride to produce diols **25** and **26**. The primary hydroxy groups of compounds (**25** and **26**) were selectively protected as *tert*-butyldimethylsilyl ethers **27** and **28**. The same 25-hydroxy groups of diols (**25** and **26**) were selectively converted into tosylates, which were further reduced with lithium aluminum hydride to afford the C,D-ring fragments **29** and **30**, possessing alkyl side chains. The secondary alcohols **27**–**30** were oxidized with pyridinium dichromate in the presence of pyridinium *para*-toluenesulfonate to the corresponding ketones **31**–**34**. The Horner-Wittig

* Corresponding author. Fax: +1 608 262 7122.
E-mail address: deluca@biochem.wisc.edu (H.F. DeLuca).

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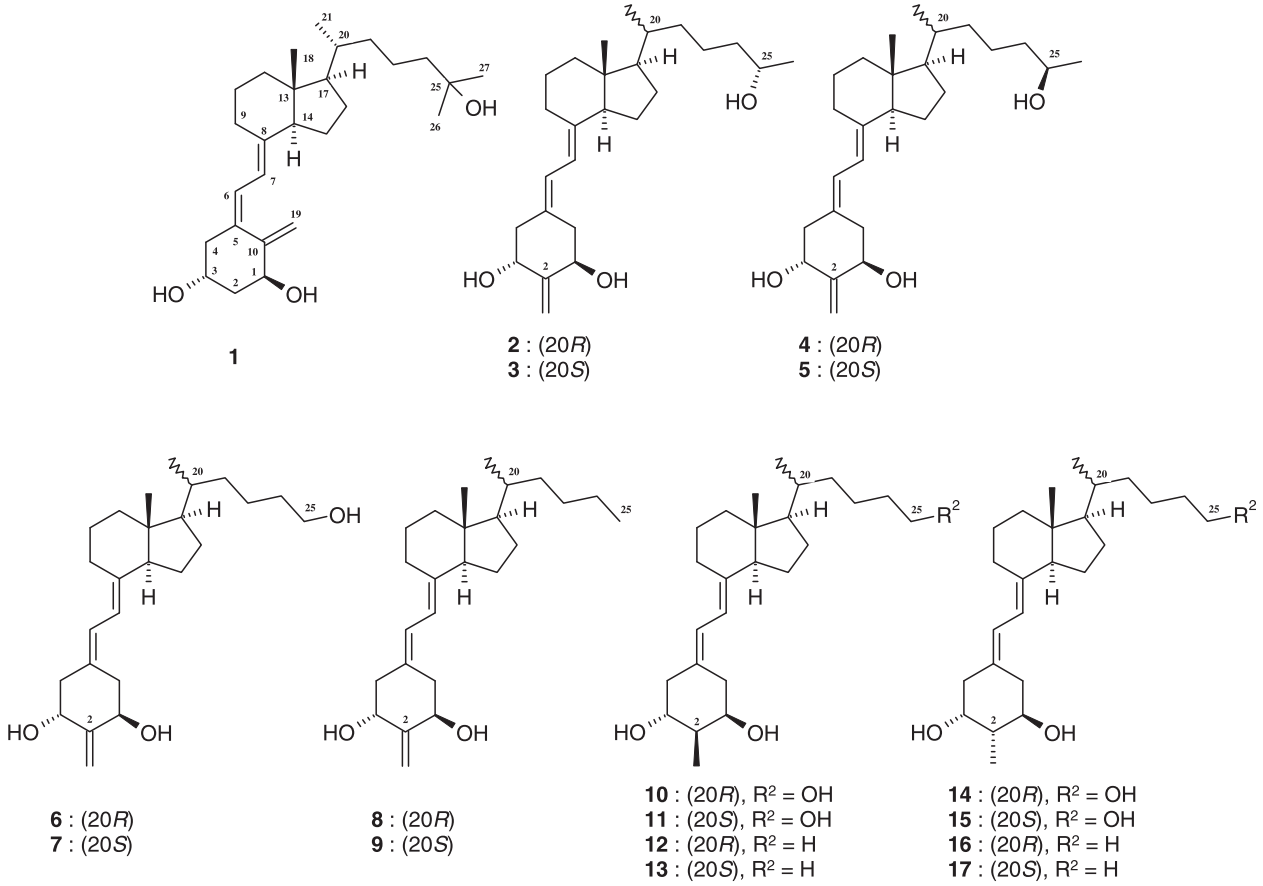
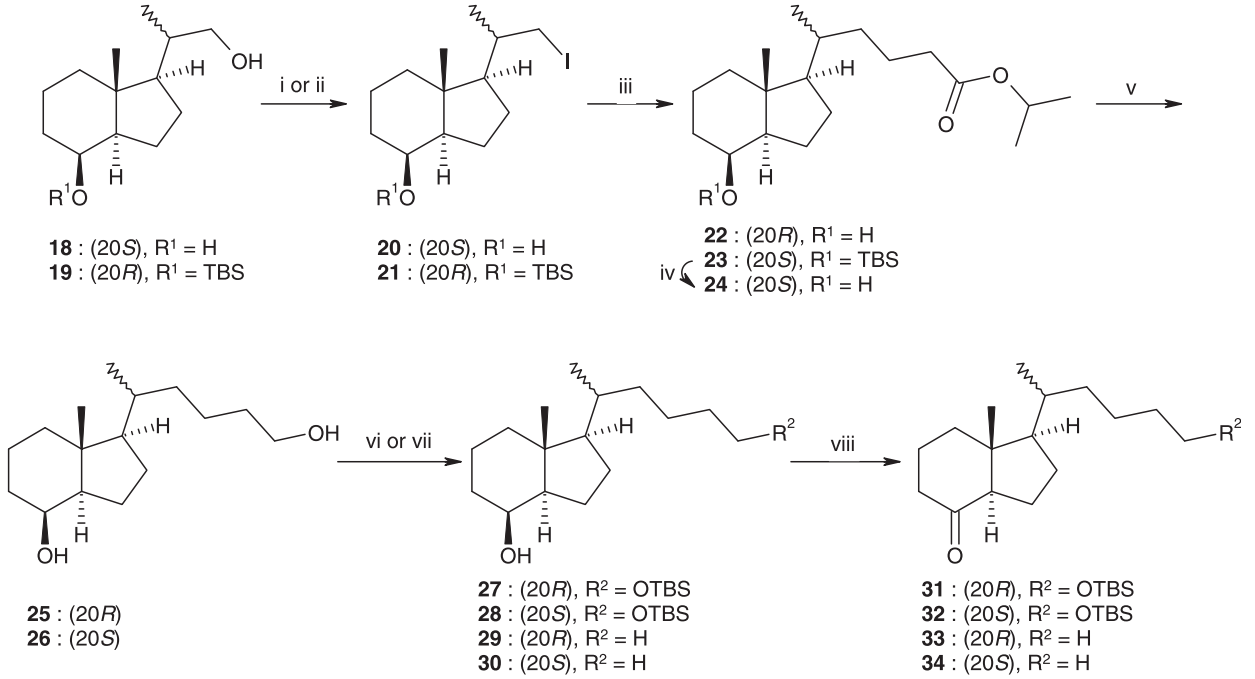
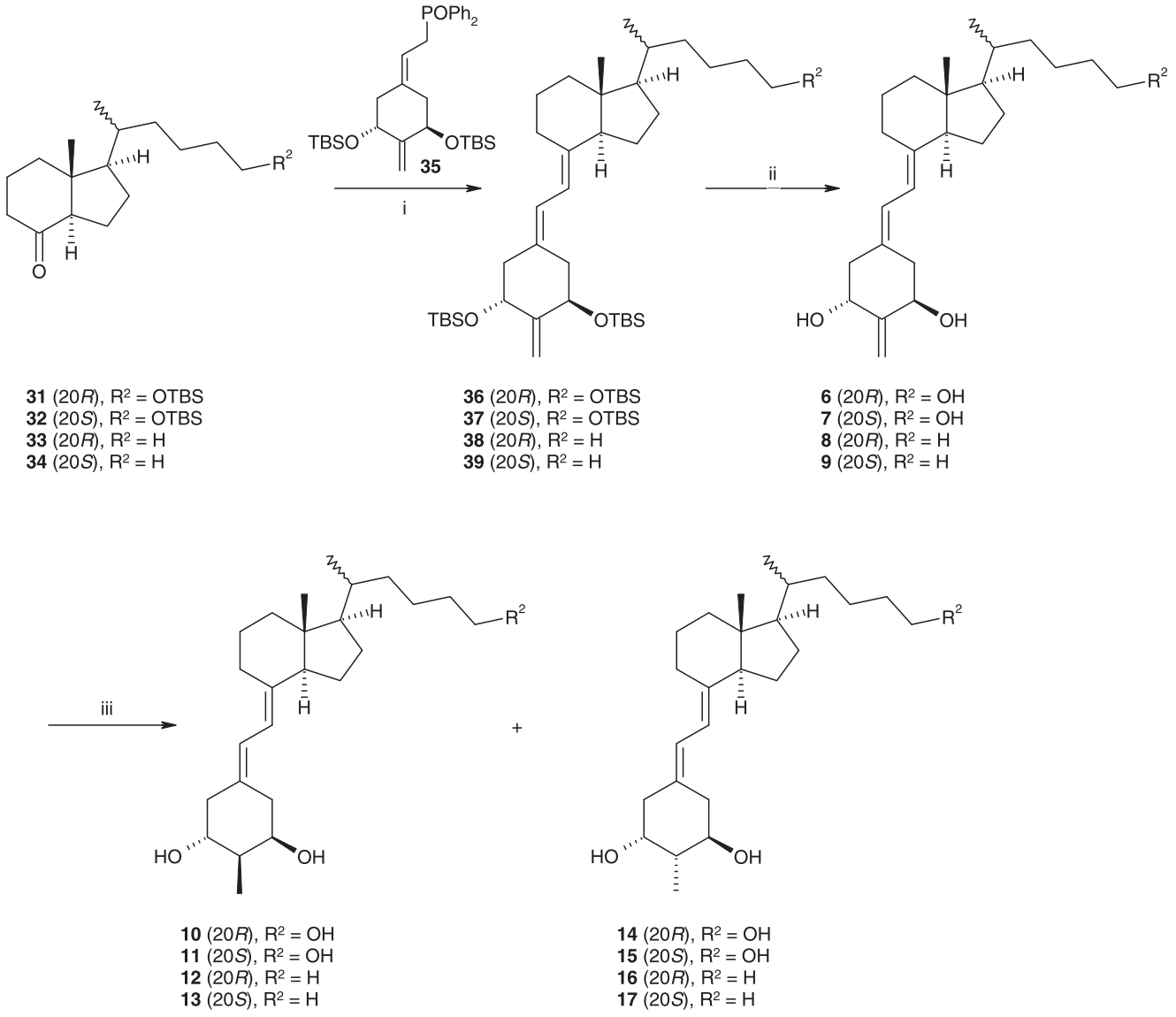


Fig. 1. Chemical structures of 1α,25-dihydroxyvitamin D_3 (calcitriol, **1**) and 19-nor analogs **2**–**17**.



Scheme 1. Synthesis of the Grundmann ketones **31**–**34**. Reagents and yields: (i) (1) TsCl, DMAP, Et3N, CH2Cl2; (2) KI, acetone, (**20** 85%); (ii) (1) I2, Ph3P, imidazole, CH2Cl2; (2) **19**, CH2Cl2, (**21** 100%); (iii) (1) Zn, pyridine, *iso*-propyl acrylate, NiCl2.6H2O; (2) **20** or **21**, pyridine, (**22** 83%, **23** 82%); (iv) aqueous HF, THF, MeCN, (**24** 82%); (v) (1) LiAlH4, THF; (2) aqueous KNa tartrate, (**25** 94%, **26** 96%); (vi) TBSCl, Et3N, CH2Cl2, (**27** 95%, **28** 94%); (vii) (1) TsCl, DMAP, Et3N, CH2Cl2; (2) LiAlH4, THF; (3) aqueous KNa tartrate, (**29** 90%, **30** 84%); (viii) PDC, PPTS, CH2Cl2, (**31** 89%, **32** 90%, **33** 95%, **34** 82%).

reaction between the corresponding C,D-fragments (**31**–**34**) and the anion, generated from the phosphine oxide **35** by phenyllithium, produced the protected vitamin D compounds **36**–**39** (Scheme 2). The silyl-protective groups were cleaved in the presence of hydrofluoric acid and after the final purification by HPLC the target vitamin D analogs **6**–**9** were obtained. The homogenous catalytic hydrogena-



31 (20*R*), R^2 = OTBS
32 (20*S*), R^2 = OTBS
33 (20*R*), R^2 = H
34 (20*S*), R^2 = H

36 (20*R*), R^2 = OTBS
37 (20*S*), R^2 = OTBS
38 (20*R*), R^2 = H
39 (20*S*), R^2 = H

6 (20*R*), R^2 = OH
7 (20*S*), R^2 = OH
8 (20*R*), R^2 = H
9 (20*S*), R^2 = H

10 (20*R*), R^2 = OH
11 (20*S*), R^2 = OH
12 (20*R*), R^2 = H
13 (20*S*), R^2 = H

14 (20*R*), R^2 = OH
15 (20*S*), R^2 = OH
16 (20*R*), R^2 = H
17 (20*S*), R^2 = H

Scheme 2. Syntheses of the vitamin D analogs **6**–**17**. Reagents and yields: (i) (1) **35**, PhLi, THF; (2) **31**, **32**, **33** or **34**, THF, (**36** 88%, **37** 80%, **38** 91%, **39** 79%); (ii) aqueous HF, THF, MeCN, (**6** 79%, **7** 87%, **8** 75%, **9** 77%); (iii) H2, (Ph3P)3RhCl, PhH, (**10** 36%, **11** 26%, **12** 38%, **13** 40%, **14** 31%, **15** 22%, **16** 34%, **17** 35%).

tion of 2-methylene compounds **6**–**9**, in the presence of tris(triphenylphosphine)rhodium(I) chloride, provided approximately an equimolar mixture of 2-methyl-19-norvitamins **10**–**17**, which were easily separated by HPLC.

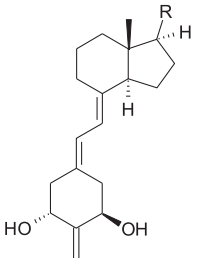
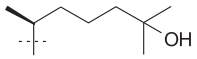
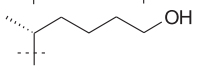
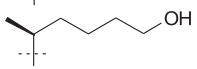
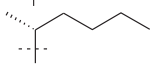
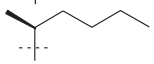
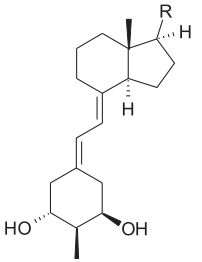
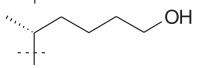
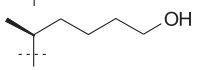
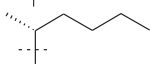
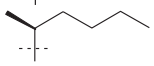
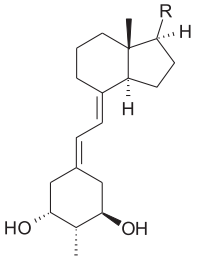
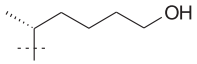
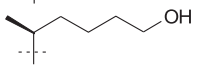
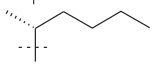
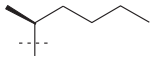
2.2. Biological evaluation

The removal of the 26- and 27-methyl groups from the side chain of 19-nor-1α,25-dihydroxyvitamin D_3 compounds having a 2-methylene (**6**–**9**) or 2α-methyl (**10**–**13**) substitution had little or moderate impact on binding to the vitamin D receptor (VDR) (Table 1). However, in two cell-based assays (differentiation of HL-60 cells and transactivation of the CYP24A1 gene in rat bone cells), activity levels were reduced up to 200× (Table 1). As expected, 2β-methyl substitution of all derivatives (**14**–**17**) resulted in a drastic reduction in all *in vitro* activities as previously noted [13–15,20–24]. The 20*S*-configuration increased *in vitro* activity of all analogs (**7**, **9**, **11**, **13**, **15**, **17**) by 1 or 2 orders of magnitude above the 20*R* counterparts (**6**, **8**, **10**, **12**, **14**, **16**). This effect has been noted previously with other analogs [13–15,24–26]. However, the ability of analogs (**6**–**13**) to support the mobilization of calcium from bone was essentially eliminated by removal of the 26- and 27-methyl groups (Fig. 2). Although intestinal calcium transport was not completely eliminated by removal of the two terminal methyl groups, it was only significant at the higher doses (100× or more that of 1α,25-$(OH)_2D_3$ **1**) (Fig. 3). Of some interest is the fact that removal of the 25-hydroxy from the analogs that are devoid of the 26- and 27-methyl groups (**8**, **9**, **12**, **13**) actually increases *in vivo* activity on intestine. The decrease in activity by a hydroxyl on analogs of 1,25-$(OH)_2D_3$ having a shortened side chain has been noted previously [27] but remains unexplained. Since binding of the VDR appeared unchanged by the 25-hydroxyl in this series, while *in vivo* activity on intestinal calcium transport is decreased, we suggest that the hydroxyl on the side chain is more easily conjugated for excretion-reducing biological activity.

3. Conclusions

Removal of the 26- and 27-methyl groups of either 2α-methyl (**6**–**9**) or 2-methylene-19-nor-1,25-$(OH)_2D_3$ (**10**–**13**) has little or moderate effect on *in vitro* binding to the VDR. However, *in vivo* the absence of these methyl groups eliminated the ability to mobi-

Table 1
VDR binding properties[a], HL-60 differentiating activities[b], and transcriptional activities[c] of the vitamin D analogs **6–17.**

Compound	Structure of a side chain (R)	Compd no.	VDR binding		HL-60 differentiation		24-OHase transcription	
			K_i	Ratio	EC_{50}	Ratio	EC_{50}	Ratio
1α,25-$(OH)_2D_3$	–	**1**	1×10^{-10} M	1	2×10^{-9} M	1	2×10^{-10} M	1
		–	1×10^{-10} M	1	8×10^{-11} M	0.04	7×10^{-12} M	0.035
		**6**	2×10^{-10} M	2	2×10^{-8} M	10	4×10^{-9} M	20
		**7**	3×10^{-10} M	3	3×10^{-9} M	1.5	5×10^{-10} M	2.5
		**8**	3×10^{-10} M	3	1×10^{-7} M	50	4×10^{-8} M	200
		**9**	3×10^{-10} M	3	5×10^{-9} M	2.5	3×10^{-9} M	15
		**10**	1×10^{-9} M	10	3×10^{-8} M	15	6×10^{-9} M	30
		**11**	1×10^{-10} M	1	7×10^{-9} M	3.5	7×10^{-10} M	3.5
		**12**	2×10^{-9} M	20	2×10^{-7} M	100	5×10^{-8} M	250
		**13**	2×10^{-10} M	2	2×10^{-8} M	10	1×10^{-8} M	50
		**14**	5×10^{-8} M	500	8×10^{-7} M	400	6×10^{-7} M	3000
		**15**	5×10^{-8} M	500	1×10^{-6} M	500	6×10^{-8} M	300
		**16**	$\sim 4 \times 10^{-6}$ M	~40,000	2×10^{-5} M	10,000	2×10^{-6} M	~10000
		**17**	1×10^{-7} M	1000	5×10^{-7} M	250	3×10^{-7} M	1500

[a] Competitive binding of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized vitamin D analogs to the full-length recombinant rat vitamin D receptor. The K_i values are derived from dose–response curves and represent the inhibition constant when radiolabeled 1α,25-$(OH)_2D_3$ is present at 1 nM and a K_d of 0.2 nM is used. The binding ratio is the average ratio of the analog K_i to the K_i for 1α,25-$(OH)_2D_3$.

[b] Induction of differentiation of HL-60 promyelocytes to monocytes by 1α,25-$(OH)_2D_3$ and the synthesized vitamin D analogs. Differentiation state was determined by measuring the percentage of cells reducing nitro blue tetrazolium (NBT). The EC_{50} values are derived from dose–response curves and represent the analog concentration capable of inducing 50% maturation. The differentiation activity ratio is the average ratio of the analog EC_{50} to the EC_{50} for 1α,25-$(OH)_2D_3$.

[c] Transcriptional assay in rat osteosarcoma cells stably transfected with a 24-hydroxylase gene reporter plasmid. The EC_{50} values are derived from dose–response curves and represent the analog concentration capable of increasing the luciferase activity by 50%. The luciferase activity ratio is the average ratio of the EC_{50} for the analog to the EC_{50} for 1α,25-$(OH)_2D_3$. All the experiments were carried out in duplicate on at least two different occasions.

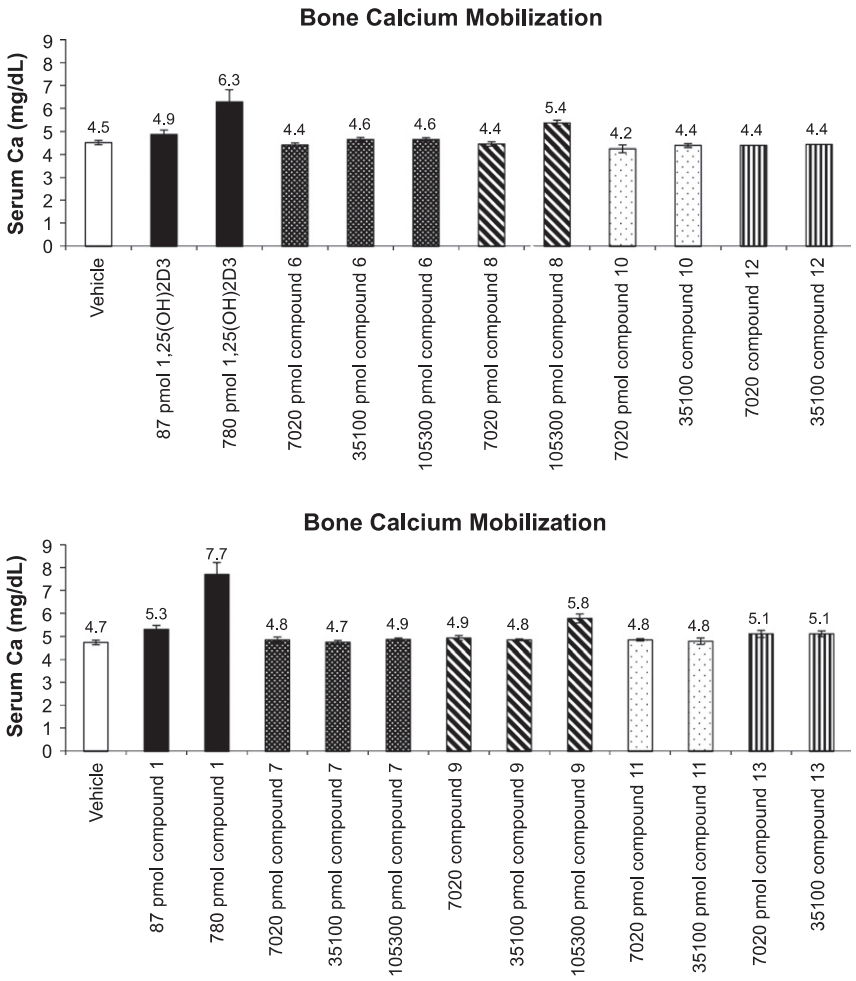


Fig. 2. Those compounds lacking a 25-hydroxy group can mobilize calcium from bone stores, but only at levels more than 1000 times that of the natural hormone. Rats were made vitamin D-deficient, fed a diet nearly devoid of calcium and administered four intraperitoneal injections of the listed analogs prior to collecting blood for serum calcium analysis.

lize bone calcium. Although this did not completely eliminate the stimulation of intestinal calcium transport, it did decrease potency by at least two orders of magnitude.

Converting the 2α-methyl to a 2β-methyl configuration in this series of compounds (**14**–**17**) drastically reduces activity both *in vitro* and *in vivo*. This has been noted in a previously prepared group of both 19-nor compounds [28]. The 20*S*-modification increases potency both *in vivo* and *in vitro* as previously noted in other series of vitamin D analogs [29,30]. However, increased potency is not the result of improved binding to the receptor. No explanation can be provided for this finding but is significant in designing analogs of 1α,25-$(OH)_2D_3$. The 25-hydroxyl group traditionally improves both binding to the receptor and both *in vivo* and *in vitro* potency. However, if the 26- and 27-methyl groups are absent, the 25-hydroxyl actually reduces potency *in vivo*. This suggests that the 25-hydroxyl requires surrounding hydrophobic groups for expression of potency [31].

Overall this series (**6**–**17**) illustrates the importance of the 26- and 27-methyls (and their hydrophobic properties) of the vitamin D analogs for a range of biological activities but especially its calcemic activities.

4. Experimental section

4.1. Chemistry

Melting points (uncorrected) were determined on a Thomas-Hoover capillary melting-point apparatus. Optical rotations were measured in chloroform using a Perkin–Elmer Model 343 polarimeter at 22 °C. Ultraviolet (UV) absorption spectra were recorded with a Perkin–Elmer Lambda 3B UV–Vis spectrophotometer in ethanol or hexane. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in deuteriochloroform at 400 and 500 MHz with Bruker Instruments DMX-400 and DMX-500 Avance console spectrometers. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in deuteriochloroform at 100 and 125 MHz with the same Bruker Instruments. Chemical shifts (δ) in parts per million are quoted relative to internal Me_4Si (δ 0.00). Numbers in parentheses following the chemical shifts in the ^{13}C NMR spectra refer to the number of attached hydrogens as revealed by DEPT experiments. Electron impact (EI) mass spectra were obtained with a Micromass AutoSpec (Beverly, MA) instrument. High-performance liquid chromatography (HPLC) was performed on a Waters Associates liquid chromatograph equipped with a Model 6000A solvent delivery system, Model U6K Universal injector and Model 486 tunable absorbance detector. The known alcohols **18** and **19** were prepared according to the published procedures [13]. Solvents were dried and distilled following standard procedures. A designation "(volume + volume)", which appears in general procedures, refers to an original volume plus a rinse volume.

All final vitamin D analogs synthesized by us gave single sharp peaks on HPLC and they were judged at least 99% pure. Two HPLC systems (straight- and reversed-phase) were employed as indicated in Table 2 (Supporting Information).

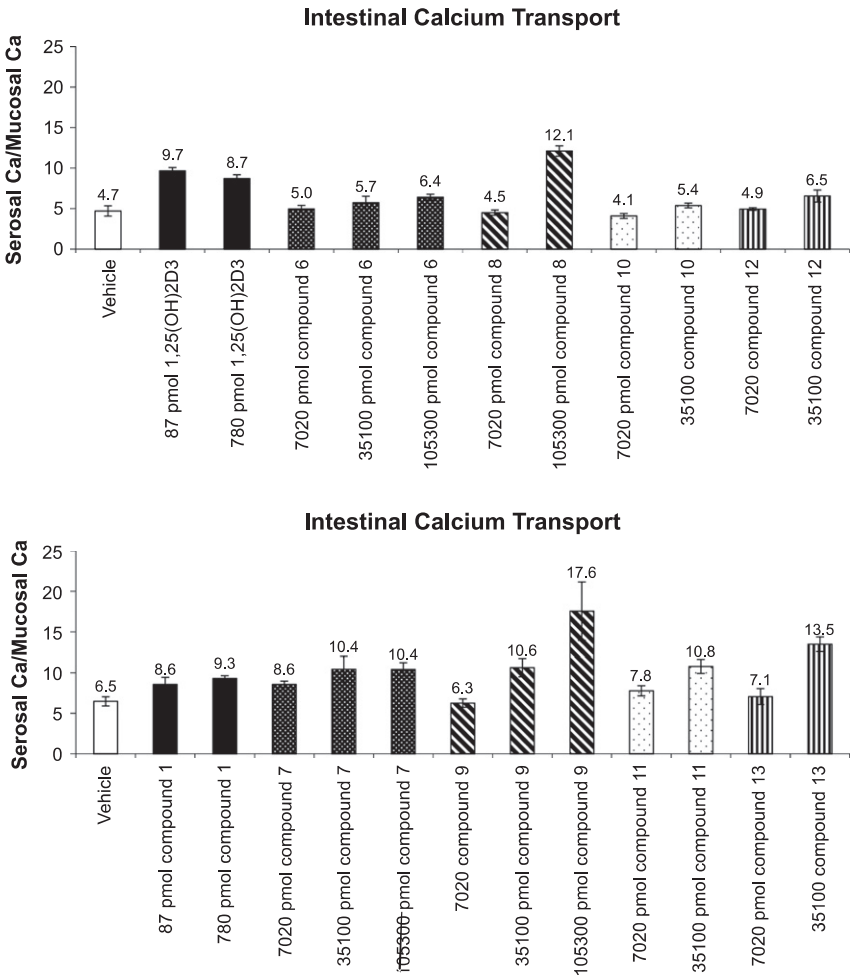


Fig. 3. All vitamin D analogs with an abbreviated side chain possess lower intestinal calcium transport activity compared to $1,25(OH)_2D_3$. Ex-vivo measurements of gut transport were made by preparing everted gut sacs from vitamin D-deficient rats given four intraperitoneal injections of the described compounds.

*4.2. Procedure for the synthesis of compound **20***

To a stirred solution of the diol **18** (133 mg, 0.63 mmol), DMAP (10 mg, 0.08 mmol) and triethylamine (250 μL, 182 mg, 1.8 mmol) in anhydrous methylene chloride (5 mL), *p*-toluenesulfonyl chloride (180 mg, 0.94 mmol) was added at 0 °C. A cooling bath was removed and the reaction mixture was stirred at room temperature for 22 h. Methylene chloride was added and the mixture was washed with water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica (6 → 12% ethyl acetate/hexane) to give the primary tosylate (224 mg, 97% yield).

To a stirred solution of the tosylate (224 mg, 0.61 mmol) in anhydrous acetone (20 mL), potassium iodide (3 g, 18 mmol) was added. The reaction mixture was refluxed for 18 h. Water (30 mL) was added to dissolve salts and the mixture was extracted with ethyl acetate. Combined organic phases were washed with a saturated aqueous $Na_2S_2O_3$ solution, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica (4 → 6% ethyl acetate/hexane) to give the iodide **20** (172 mg, 88% yield).

*4.3. Procedure for the synthesis of compound **21***

To a solution of triphenylphosphine (482 mg, 1.84 mmol) and imidazole (250 mg, 3.68 mmol) in anhydrous methylene chloride (15 mL), a solution of iodine (471 mg, 1.85 mmol) in anhydrous methylene chloride (30 mL) was added at 0 °C. The reaction mixture was stirred under argon at 0 °C for 15 min and then a solution of the alcohol **19** (149 mg, 0.46 mmol) in anhydrous methylene chloride (3 + 1 mL) was added. The reaction mixture was stirred at 0 °C for 20 min and at room temperature for 18 h. It was washed with water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica (0 → 3% ethyl acetate/hexane) to give the product **21** (201 mg, >96% yield).

*4.4. General procedure for the synthesis of compounds **22** and **23***

To a stirred mixture of zinc powder (3.7–5.0 equiv), *iso*-propyl acrylate (3.7–4.8 equiv) and anhydrous pyridine (4 mL), nickel (II) chloride hexahydrate (1.0–1.2 equiv) was added at 50 °C. The resulting mixture was warmed to 65 °C and stirred for 2 h. After cooling to 0 °C, a solution of the iodide **20** or **21** (1 equiv) in anhydrous pyridine (2 + 1 mL) was added and the reaction mixture was stirred at room temperature for 17 h. It was diluted with ethyl acetate and the precipitate was filtered off through a pad of Celite. The filtrate was washed with aqueous 5% solution of HCl and water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica (4 → 6% ethyl acetate/hexane) to give the ester **22** or **23**.

*4.5. Procedure for the synthesis of compound **24***

To a solution of the silyl ether **23** (94 mg, 0.22 mmol) in THF (3 mL) and acetonitrile (3 mL), a solution of aqueous 48% HF in ace-

tonitrile (1:9 ratio, 2 mL) was added and the resulting mixture was stirred at room temperature for 2 days. The reaction was quenched with a saturated aqueous $NaHCO_3$ solution and extracted with ethyl acetate. Combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, concentrated under reduced pressure. The residue was purified by column chromatography on silica (3 → 5% ethyl acetate/hexane) to give the recovered substrate **23** (9 mg, 0.02 mmol) and the secondary alcohol **24** (51 mg, 82% yield).

4.6. General procedure for the synthesis of compounds *25* and *26*

To a stirred solution of the ester **22** or **24** (1 equiv) in anhydrous THF (4 mL), lithium aluminum hydride (5.8–11.3 equiv) was added at 0 °C. A cooling bath was removed and the reaction mixture was stirred at room temperature for 30 min. The excess hydride was quenched by careful addition of aqueous 10% solution of potassium sodium tartrate. The mixture was extracted with methylene chloride. Combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica (6 → 60% ethyl acetate/hexane) to give the diol **25** or **26**.

4.7. General procedure for the synthesis of compounds *27* and *28*

To a solution of the diol **25** or **26** (1 equiv) and triethylamine (3–4 equiv) in anhydrous methylene chloride (3 mL), *tert*-butyldimethylsilyl chloride (1.3 equiv) was added at room temperature and the mixture was stirred for 18 h. The reaction was quenched with water and extracted with ethyl acetate. Combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica (2% ethyl acetate/hexane) to give the alcohol **27** or **28**.

4.8. General procedure for the synthesis of compounds *29* and *30*

To a stirred solution of the diol **25** or **26** (1 equiv), DMAP (0.2–0.4 equiv) and triethylamine (3–4 equiv) in anhydrous methylene chloride (3 mL), *p*-toluenesulfonyl chloride (1.4–1.5 equiv) was added at 0 °C. A cooling bath was removed and the reaction mixture was stirred at room temperature for 22 h. Methylene chloride was added and the mixture was washed with a saturated aqueous $NaHCO_3$ solution, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was dissolved in anhydrous THF (4 mL) and lithium aluminum hydride (60 mg, 1.6 mmol) was added at 0 °C. A cooling bath was removed and the reaction mixture was stirred at room temperature for 18 h. The excess hydride was quenched by careful addition of aqueous 10% solution of potassium sodium tartrate and the mixture was extracted with methylene chloride. Combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica (5% ethyl acetate/hexane) to give the alcohol **29** or **30**.

4.9. General procedure for the synthesis of compounds *31*–*34*

To a stirred solution of the alcohol **27**, **28**, **29** or **30** (1 equiv), pyridinium *p*-toluenesulfonate (0.13–0.20 equiv) in anhydrous methylene chloride (6 mL), pyridinium dichromate (4–5 equiv) was added and the mixture was stirred at room temperature for 5 h. The resulting suspension filtered through a Waters silica Sep-Pak cartridge (5 g), which was further washed with methylene chloride. Solvents were evaporated under reduced pressure to give the ketone **31**, **32**, **33** or **34**.

4.10. General procedure for the synthesis of compounds *36*–*39*

To a stirred solution of the phosphine oxide **35** (1.4–3.0 equiv) in anhydrous THF (700 μL), a solution of phenyllithium (1.5–5.3 equiv) was added at −20 °C under argon. The mixture was stirred for 30 min and then cooled to −78 °C. A pre-cooled (−78 °C) solution of the Grundmann's type ketone **31**, **32**, **33** or **34** (1 equiv) in anhydrous THF (200 + 100 μL) was added *via* cannula and the reaction mixture was stirred for 5 h at −78 °C. Then the reaction mixture was stirred at 4 °C for 19 h. Ethyl acetate (20 mL) was added and the organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified on a Waters silica Sep-Pak cartridge (0 → 2% ethyl acetate/hexane) to give the protected vitamin D compound **36**, **37**, **38** or **39**.

4.11. General procedure for the synthesis of compounds *6*–*9*

To a solution of the protected vitamin **36**, **37**, **38** or **39** in THF (2 mL) and acetonitrile (2 mL), a solution of aqueous 48% HF in acetonitrile (1:9 ratio, 1 mL) was added at 0 °C and the resulting mixture was stirred at room temperature for 3 h. The reaction was quenched with a saturated aqueous $NaHCO_3$ solution and extracted with ethyl acetate. Combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, concentrated under reduced pressure. The residue was purified on a Waters silica Sep-Pak cartridge (10 → 30% ethyl acetate/hexane) to give the crude products. Final purifications of the vitamin D compounds were performed by straight phase HPLC, followed by reversed-phase HPLC to give the analytically pure 19,26,27-trinorvitamin D analogs **6**, **7**, **8** or **9**.

4.12. General procedure for the synthesis of compounds *10*–*17*

Tris(triphenylphosphine)rhodium (I) chloride (1.0–1.1 equiv) was added to dry benzene (8 mL) presaturated with hydrogen (15 min). The mixture was stirred at room temperature until a homogeneous solution was formed (ca. 25 min). A solution of the analog **6**, **7**, **8** or **9** (1 equiv) in dry benzene (2 + 1 mL) was added and the reaction was allowed to proceed under a continuous stream of hydrogen for 4 h. The solvent was removed under reduced pressure, the residue was redissolved in hexane/ethyl acetate (1:1) and applied on a Waters silica Sep-Pak cartridge (2 g). A mixture of 2-methyl vitamins was eluted with the same solvent system. A mixture of compounds was further purified by straight-phase HPLC. The separation of 2α-methyl analogs **10**, **11**, **12** or **13**, from the 2β-methyl ones **14**, **15**, **16** or **17**, was achieved by reversed-phase HPLC.

5. Biological studies

5.1. In vitro studies

VDR binding, HL-60 differentiation and 24-hydroxylase transcription assays were performed as previously described [28].

5.2. In vivo studies

Bone calcium mobilization and intestinal calcium transport were performed as previously described [29,32]. Briefly, weanling rats were made vitamin D-deficient by housing under lighting conditions that block vitamin D production in the skin. In addition, the animals were fed a diet devoid of vitamin D and alternating levels of calcium. Experimental compounds were administered intraperitoneally once per day for four consecutive days. Twenty-four

hours after the last dose was given, the blood was collected, and everted gut sacs were prepared. Calcium was measured in the blood and two different intestinal compartments using atomic absorption spectroscopy. Each study was comprised of at least 5–6 animals/experimental group and was controlled with a vehicle group (5% ethanol:95% propylene glycol) and one or more positive control groups [1α,25(OH)$_2$D$_3$].

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Appendix A. Supplementary material

Purity criteria, spectral data of the synthesized compounds; representative figures with either the competitive binding curves or dose–response curves derived from the binding, cellular differentiation and transcriptional assays of the vitamin D analogs **6**–**17**. This material is available free of charge via the Internet at http://.pubs.acs.org. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.01.001.

References

[1] D. Feldman, J.W. Pike, F.H. Glorieux (Eds.), In Vitamin D, 2nd ed., Elsevier Academic Press, Burlington, 2005.
[2] G. Jones, S.A. Strugnell, H.F. DeLuca, Physiol. Rev. 78 (1998) 1193–1231.
[3] A.W. Norman, R. Bouillon, M. Thomasset (Eds.), In Vitamin D, a Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications, Walter de Gruyter, Berlin, 1994.
[4] L.A. Plum, H.F. DeLuca, Clin. Rev. Bone Miner. Metab. 7 (2009) 20–41.
[5] J.M. Lemire, J. Cell. Biochem. 49 (1992) 26–31.
[6] G.K. Schwalfenberg, Mol. Nutr. Food Res. 55 (2011) 96–108.
[7] T. Suda, T. Shinki, N. Takahashi, Annu. Rev. Nutri. 10 (1990) 195–211.
[8] T. Suda, Proc. Soc. Exp. Biol. Med. 191 (1989) 214–220.
[9] V.K. Ostrem, H.F. DeLuca, Steroids 49 (1987) 73–102.
[10] E. Abe, C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, T. Suda, Proc. Natl. Acad. Sci. USA 78 (1981) 4990–4994.
[11] A.J. Brown, E. Slatopolsky, Mol. Aspects Med. 29 (2008) 433–452.
[12] G.H. Posner, M. Kahraman, Eur. J. Org. Chem. (2003) 3889–3895.
[13] P. Grzywacz, L.A. Plum, R.R. Sicinski, M. Clagett-Dame, H.F. DeLuca, Arch. Biochem. Biophys. 460 (2007) 274–284.
[14] P. Grzywacz, L.A. Plum, W. Sicinska, R.R. Sicinski, J.M. Prahl, H.F. DeLuca, J. Steroid Biochem, Mol. Biol. 89–90 (2004) 13–17.
[15] P. Grzywacz, G. Chiellini, L.A. Plum, M. Clagett-Dame, H.F. DeLuca, J. Med. Chem. 53 (2010) 8642–8649.
[16] B. Lythgoe, T.A. Moran, M.E.N. Nambudiry, S. Ruston, J. Chem. Soc. Perkin Trans. I (1976) 2386–2390.
[17] R. Sustmann, P. Hopp, P. Holl, Tetrahedron Lett. 30 (1989) 689–692.
[18] J.L. Mascarenas, J. Perez-Sestelo, L. Castedo, A. Mourino, Tetrahedron Lett. 32 (1991) 2813–2816.
[19] P.J. Garegg, B. Samuelsson, J. Chem. Soc. Perkin Trans. I (1980) 2866–2869.
[20] T. Fujishima, Z. Liu, D. Miura, M. Chokki, S. Ishizuka, K. Konno, H. Takayama, Bioorg. Med. Chem. Lett. 8 (1998) 2145–2148.
[21] K. Konno, S. Maki, T. Fujishima, Z. Liu, D. Miura, M. Chokki, H. Takayama, Bioorg. Med. Chem. Lett. 8 (1998) 151–156.
[22] K. Nakagawa, M. Kurobe, K. Ozono, K. Konno, T. Fujishima, H. Takayama, T. Okano, Biochem. Pharmacol. 59 (2000) 691–702.
[23] T. Fujishima, L. Zhaopeng, K. Konno, K. Nakagawa, T. Okano, K. Yamaguchi, H. Takayama, Bioorg. Med. Chem. 9 (2001) 525–535.
[24] K. Konno, T. Fujishima, S. Maki, Z. Liu, D. Miura, M. Chokki, S. Ishizuka, K. Yamaguchi, Y. Kan, M. Kurihara, N. Miyata, C. Smith, H.F. DeLuca, H. Takayama, J. Med. Chem. 43 (2000) 4247–4265.
[25] F.J. Dilworth, M.J. Calverley, H.L.J. Makin, G. Jones, Biochem. Pharmacol. 6 (1994) 987–993.
[26] T. Fujishima, K. Konno, K. Nakagawa, M. Kurobe, T. Okano, H. Takayama, Bioorg. Med. Chem. 8 (2000) 123–134.
[27] M.F. Holick, M. Garabedian, H.K. Schnoes, H.F. DeLuca, J. Biol. Chem. 250 (1975) 226–230.
[28] R.R. Sicinski, J.M. Prahl, C.M. Smith, H.F. DeLuca, J. Med. Chem. 41 (1998) 4662–4674.
[29] A. Glebocka, R.R. Sicinski, L.A. Plum, M. Clagett-Dame, H.F. DeLuca, J. Med. Chem. 49 (2006) 2909–2920.
[30] A. Flores, R.R. Sicinski, P. Grzywacz, J.B. Thoden, L.A. Plum, M. Clagett-Dame, H.F. DeLuca, J. Med. Chem. 55 (2012) 4352–4366.
[31] S. Yamada, M. Shimizu, K. Yamamoto, Med. Res. Rev. 23 (2003) 89–115.
[32] G. Chiellini, P. Grzywacz, L.A. Plum, R. Barycki, M. Clagett-Dame, H.F. DeLuca, Bioorg. Med. Chem. 16 (2008) 8563–8573.