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1-Desoxy analog of 2MD: Synthesis and biological activity of (20*S*)-25-hydroxy-2-methylene-19-norvitamin D_3 ☆

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ABSTRACT

During our ongoing structure–activity studies in the vitamin D area, we obtained (20*S*)-1α,25-dihydroxy-2-methylene-19-norvitamin D_3 (**5**). This analog, designated 2MD, is characterized by a significantly enhanced calcemic activity and is currently evaluated as a potential drug for osteoporosis. Therefore, it was of interest to synthesize also its 1-desoxy analog and to evaluate its biological action. These studies were aimed at solving an intriguing problem: can such a vitamin also be hydroxylated *in vivo* at the allylic C-1 position despite lack of the exomethylene moiety at C-10? The Wittig-Horner coupling of the known protected (20*S*)-25-hydroxy Grundmann ketone **17** and the phosphine oxides **16** and **33**, differing in their hydroxyls protection, provided the target 1-desoxy-2MD (**6**) after removal of the silyl protecting groups. Two synthetic paths have been elaborated leading to the desired A-ring synthons and starting from commercially available compounds: 1,4-cyclohexanedione monoethylene acetal (**7**) and (−)-quinic acid (**19**). The biological activity *in vitro* of the synthesized 1-desoxy-2MD (**6**) was evaluated and this analog was found to have an affinity for the vitamin D receptor (VDR) similar as its parent compound 2MD (**5**) while being much less active in the transcriptional assay. The results of the biological tests *in vivo* are also discussed.

1. Introduction

1α,25-Dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$, calcitriol, **1**; Fig. 1] is the active, hormonal form of vitamin D_3, involved in the regulation of the serum calcium and phosphorus homeostasis as well as in the wide array of other biological functions [1,2]. Formation of calcitriol in mammals includes two steps. In the liver, vitamin D_3 (**2**) undergoes its first metabolic activation in which it is converted to 25-hydroxyvitamin D_3 (**3**), the major circulating metabolite of vitamin D [3]. Second step occurs in the kidney [4] where compound **3** is hydroxylated at 1α-position to form the final vitamin D hormone, 1α,25-$(OH)_2D_3$ (**1**). Compared to its biological precursor **3**, calcitriol has proved to be about three orders of magnitude more active then 25-OH-D_3 in binding to the VDR and in the differentiation of human promyelocytic leukemia (HL-60) cells, respectively [5,6]. In our continued investigation of the structure–activity relationship in the vitamin D area, we obtained an analog of 1α,25-$(OH)_2D_3$ possessing the inverted (20*S*)-configuration and the A-ring exocyclic methylene group transposed from C-10 to C-2 [7,8]. This compound, called 2MD (**5**), is characterized by a significantly enhanced activity on bone [9] and is currently in clinical development as a potential drug for osteoporosis. Taking into account a very high calcemic activity of 2MD, we synthesized the related compound **4** (25-desoxy-2MD), that can be "activated" in a living organism by 25-hydroxylation process [10]. Such a "prodrug" turned out to be a highly potent analog, with calcemic activities approaching these of the corresponding 25-hydroxylated counterpart **5**. The above-mentioned results, suggesting *in vivo* side chain hydroxylation of the analog **4** in the liver, encouraged us to undertake the studies directed towards the synthesis of the respective analog of 2MD lacking the 1α-hydroxyl group (**6**, 1-desoxy-2MD). We expected that biological testing of such an analog could provide indication whether the enzymatic 1α-hydroxylation process was also possible in the 2-methylene-19-norvitamin D derivatives.

2. Materials and methods

2.1. Preparation of (20*S*)-25-hydroxy-2-methylene-19-norvitamin D_3 (*6*)

(20*S*)-25-Hydroxy-2-methylene-19-norvitamin D_3 was synthesized at the Department of Biochemistry, University of Wisconsin-Madison and at the Department of Chemistry, Uni-

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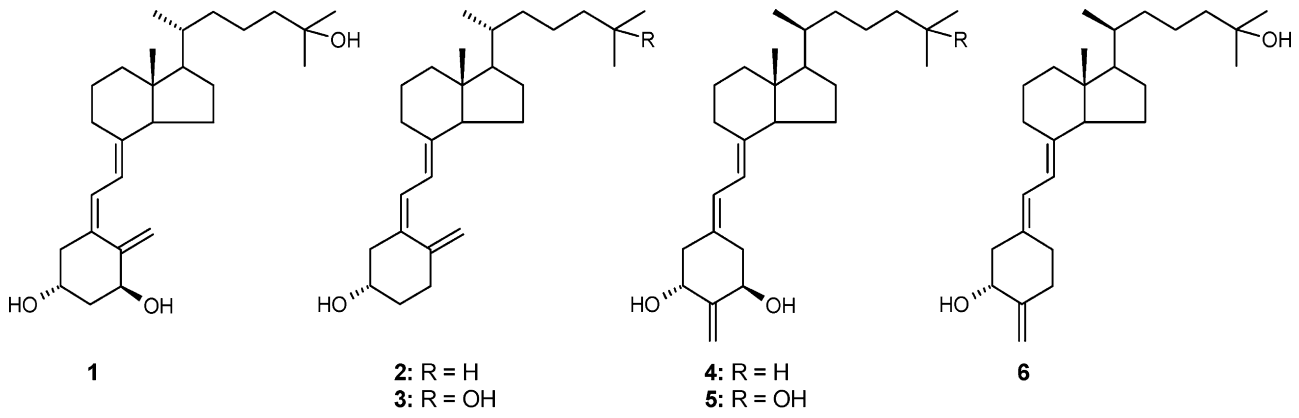


Fig. 1. Chemical structure of 1α,25-dihydroxyvitamin D_3 (calcitriol, **1**) and its analogs.

versity of Warsaw, according to the synthetic route presented in Schemes 1 and 2. All prepared compounds exhibited spectroscopic and analytical data consistent with their structures. Full details of their synthesis will be reported elsewhere.

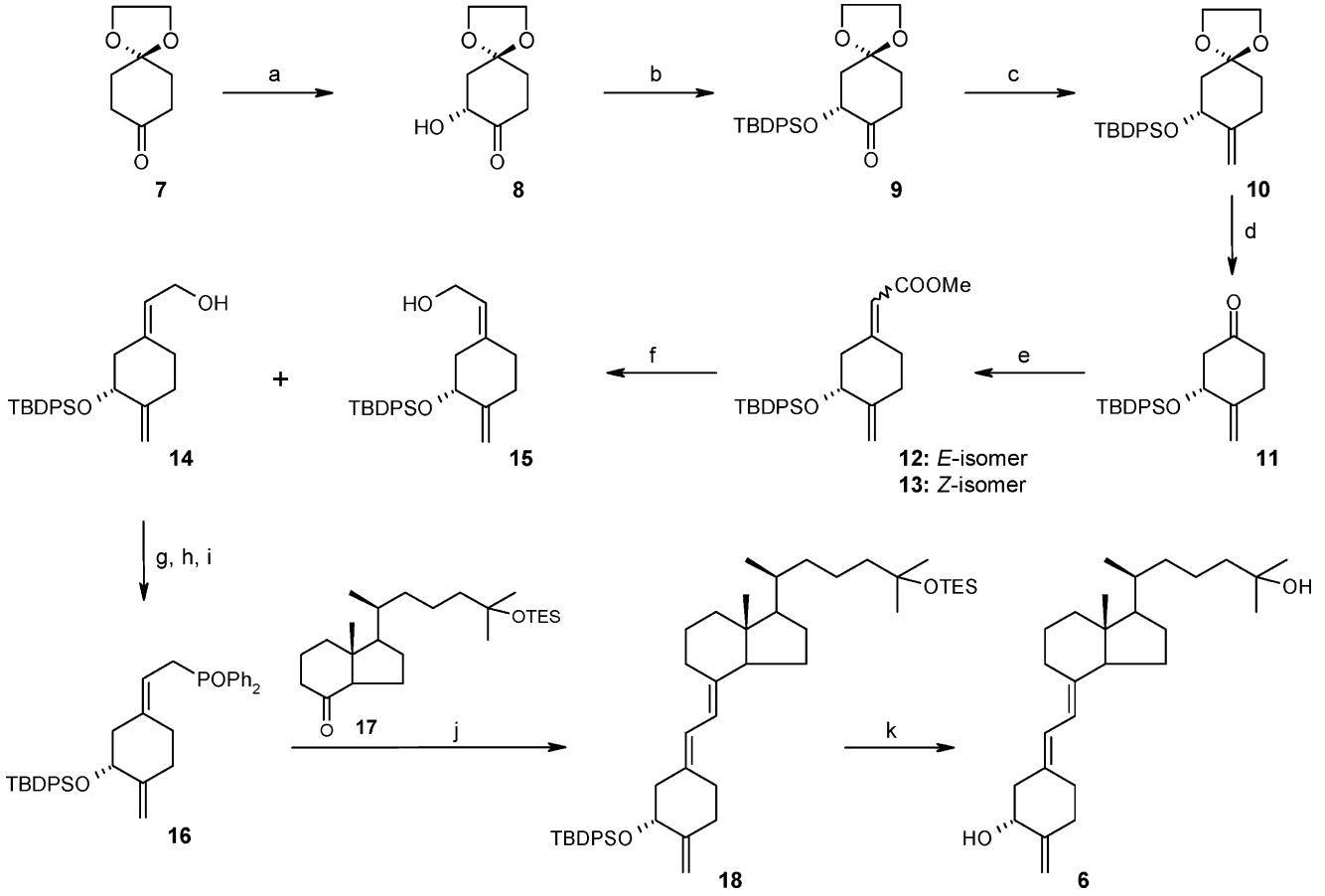
2.2. In vitro studies

2.2.1. Measurement of binding to the rat recombinant vitamin D receptor

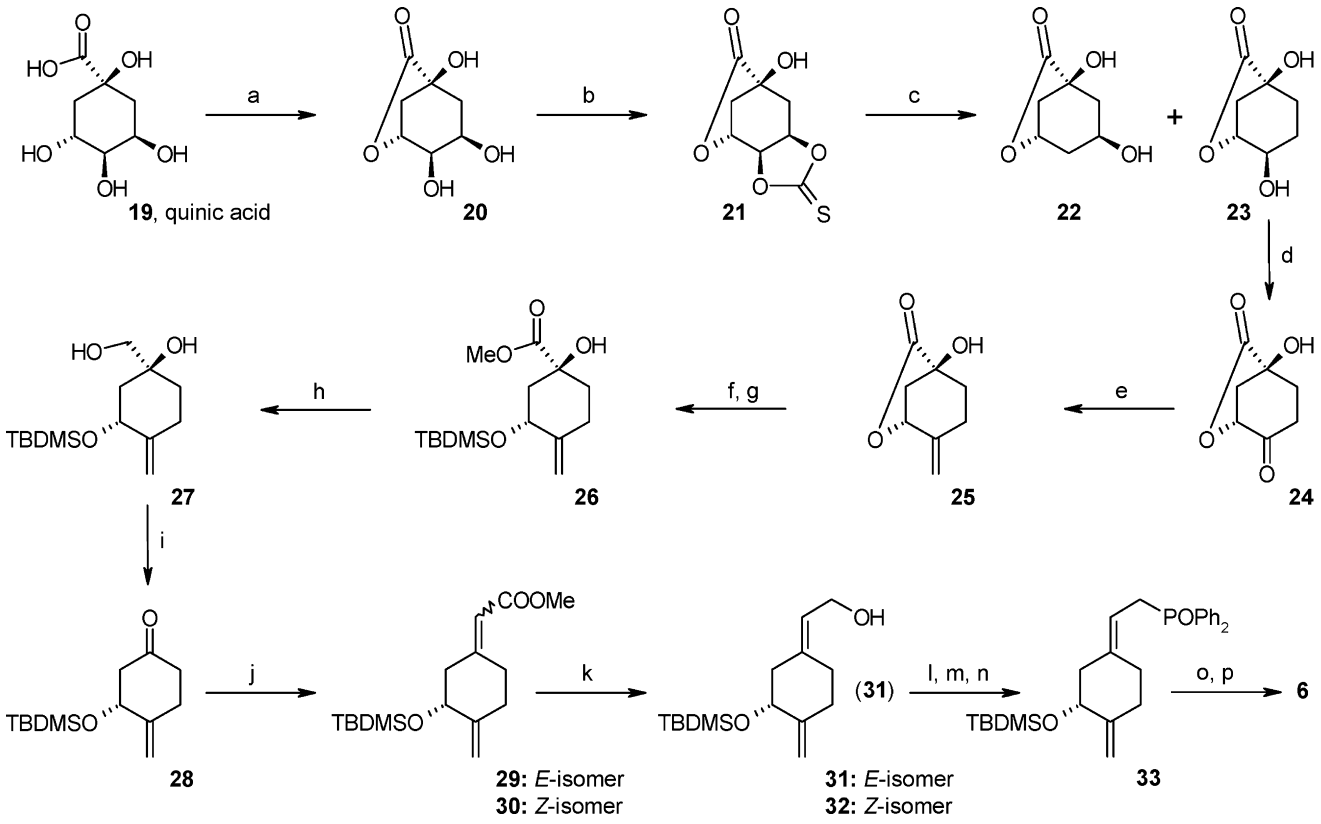
The procedure for obtaining the purified rat recombinant vitamin D receptor used in the binding studies will be reported in detail elsewhere. Competition binding assays were performed using 1α,25-$(OH)_2$[26,27-^{3}H]D_3 as previously described [11]. The experiments were carried out in duplicate, on two or three different occasions.

2.2.2. Measurement of cellular differentiation

Human leukemia HL-60 cells (obtained from ATTC) were plated at 2×10^5 cells per plate and incubated. Then the test compounds were added and, after 4 days, superoxide production was measured by nitro blue tetrazolium (NBT) reduction. The experiment was repeated in duplicate, two times. This method is described in detail elsewhere [6].



Scheme 1. (a) PhNO, L-Pro, $CHCl_3$, 44% (e.e. > 97%); (b) TBDPSCl, Im, DMF, 88%; (c) $Ph_3P^+CH_3Br^-$, *n*-BuLi, THF, 93%; (d) $FeCl_3 \times H_2O$, CH_2Cl_2, 99%; (e) $TMSCH_2COOMe$, LDA, THF, 90%; (f) DIBALH, PhMe, CH_2Cl_2, chrom. sep., 92% (ratio of **14**:**15** = 0.74:1); (g) *n*-BuLi, *p*-TsCl, THF; (h) *n*-BuLi, Ph_2Ph, THF; (i) H_2O_2, H_2O, CH_2Cl_2, 79% (over three steps); (j) *n*-BuLi, **17**, THF, 70%; (k) *n*-Bu_4NF, THF, 85%.



Scheme 2. (a) *p*-TsOH, PhH, DMF, 72%; (b) TDCI, MeCN, 63%; (c) Bu_3SnH, AIBN, PhH, THF, 88% (ratio of **22**:**23** = 0.06:1; (d) PDC, 4 Å mol. sieves, MeCN, 82%; (e) $Ph_3P^+CH_3Br^-$, *t*-BuOK, THF, 80%; (f) MeOH, 4 Å mol. sieves, 59%; (g) TBDMSOTf, 2,6-lutidine, CH_2Cl_2, 80% (over two steps); (h) DIBALH, CH_2Cl_2, 94%; (i) $NaIO_4$, MeOH, H_2O, 95%; (j) Ph_3P=CHCOOMe, PhH, 89%; (k) DIBALH, CH_2Cl_2, chrom. sep., 97% (ratio of **31**:**32** = 0.67:1); (l) *n*-BuLi, *p*-TsCl, THF; (m) *n*-BuLi, Ph_2Ph, THF; (n) H_2O_2, H_2O, CH_2Cl_2, 69% (over three steps); (o) PhLi, **17**, THF, 70%; (p) CSA, MeOH, 14% (over two steps).

2.2.3. Transcriptional assay

Transcription activity was measured in ROS 17/2.8 (bone) cells that were stably transfected with a 24-hydroxylase (24OHase) gene promoter upstream of a luciferase reporter gene [12]. The cells were given a range of doses. Sixteen hours after dosing, the cells were harvested and luciferase activities were measured using a luminometer. Each experiment was performed in duplicate, two or three times.

2.3. In vivo studies

Bone calcium mobilization. Male, weanling Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN). The animals were group housed and placed on Diet 11 (0.47% Ca) + AEK oil for one week followed by Diet 11 (0.02% Ca) + AEK oil for 3 weeks. The rats were then switched to a diet containing 0.47% Ca [13] for 1 week followed by 2 weeks on a diet containing 0.02% Ca. Dose administration began during the last week on 0.02% Ca diet. Four consecutive intraperitoneal doses were given approximately 24 h apart. Twenty-four hours after the last dose, blood was collected from the severed neck and the concentration of serum calcium determined as a measure of bone calcium mobilization.

3. Results and discussion

3.1. Chemical synthesis of *6*

For the preparation of the A-ring fragments, phosphine oxides **16** and **33**, two alternative synthetic routes were established. The first synthesis started from an achiral, commercially available acetal-ketone **7** (Scheme 1), that was enantioselectively α-hydroxylated, using the method elaborated by Hayashi et al. [14] and involving the reaction of a ketone with nitrosobenzene in the presence of a catalytic amount of L-proline. The introduced secondary hydroxyl group in the product **8** was silylated and the protected compound **9** was subjected to the Wittig reaction with an ylide generated from methyltriphenylphosphonium bromide and *n*-butyllithium. In the resulting olefinic compound **10**, the carbonyl group was deprotected in the reaction with the Lewis acid and the formed cyclohexanone **11** was subjected to a Peterson reaction leading to the mixture of α,β-unsaturated esters **12** and **13**. The separation of the geometric isomers, although possible also at this stage, was more easily achieved (by column chromatography) after the reduction step, providing the *E*- and *Z*-allylic alcohols **14** and **15**, respectively. The *E*-isomer **15** was then transformed in a three-step procedure into the corresponding phosphine oxide **16**. The Wittig-Horner coupling of the known Grundmann ketone **17** [7] with a lithium phosphinoxy carbanion generated from the phosphine oxide **16** was subsequently carried out, producing the protected 19-norvitamin D compound **18** which, after deprotection of hydroxyl groups with tetrabutylammonium fluoride, provided the desired (20*S*)-25-hydroxy-2-methylene-19-nor-vitamin D_3 (**6**, 1-desoxy-2MD).

A different synthetic sequence led to the building block **33** and to the final vitamin **6**. The chiral starting compound was a commercially available (−)-quinic acid **19**, which was at first converted to the known lactone **20** [15]. Treatment of this compound with a 1,1′-thiocarbonyldiimidazole resulted in formation of the cyclic thiocarbonate **21** [16]. The Barton-McCombie deoxygenation of **21** with tributyltin hydride and AIBN provided two isomeric products: the known compound **22** [17] and the desired diol **23**. Oxidation of the secondary hydroxyl group in the latter isomer yielded the ketone **24** which was subjected to the Wittig methylenation. The lactone ring in the formed compound **25** was then opened and the

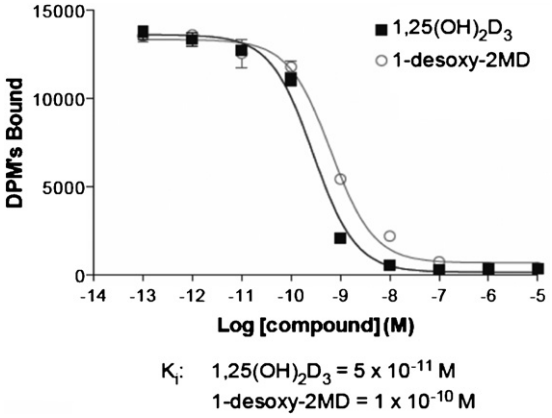


Fig. 2. Competitive binding of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized 1-desoxy-2MD (**6**) to the rat recombinant vitamin D receptor. This experiment was carried out in duplicate on two different occasions.

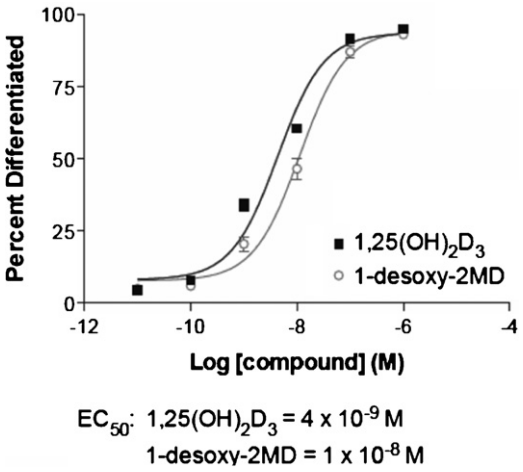


Fig. 3. Differentiation activity of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized 1-desoxy-2MD (**6**). The differentiation state was determined by measuring the percentage of cells reducing nitro blue tetrazolium (NBT). The experiment was repeated in duplicate, two times.

secondary hydroxyl group silylated. The methyl ester moiety in the obtained product **26** was reduced and the diol **27** was subjected to periodate oxidation. The Wittig reaction of the obtained cyclohexanone **28** with methyl (triphenylphosphoranylidene)acetate provided a mixture of α,β-unsaturated esters **29** and **30**. These were reduced with DIBALH and the obtained allylic alcohols separated by column chromatography. The *E*-isomer **31** was then converted into the corresponding allylic phosphine oxide **33**. Its anion, generated by phenyllithium, was coupled with the Grundmann ketone **17** and the final 19-norvitamin **6** was obtained after acidic deprotection of hydroxyls.

3.2. Biological evaluation of the synthesized analog *6*

Interestingly, the synthesized vitamin **6** was almost as active as 1α,25-$(OH)_2D_3$ and its parent 2MD [7], in binding to the full-length recombinant rat vitamin D receptor (Fig. 2). This is a surprising result because removal of the 1α-hydroxyl group from the native hormone reduces the binding activity by three orders of magnitude [5]. The obtained vitamin **6** proved to be slightly less active in the HL-60 assay (Fig. 3) than the natural hormone but ca. 70 times less potent compared to 2MD [7]. When the ability of the analog **6** to induce transcription of vitamin D-responsive genes was examined using the 24-hydroxylase (CYP-24) luciferase reporter gene system (Fig. 4), the synthesized compound appeared to be less potent by three orders of magnitude than 2MD; a similar relationship was observed for 25-OH-D_3 and 1α,25-$(OH)_2D_3$ [12]. The *in vivo* tests performed in rats (Fig. 5) showed that removal of the 1α-hydroxyl group from the native hormone (**1**) does not appear to affect bone calcium mobilization of its 1-desoxy form **3** (25-OH-D_3) because the 25-OH-D_3 is readily 1-hydroxylated *in vivo*. However, in the case of 2MD, elimination of its 1α-hydroxyl group dramatically reduced (by ca. two orders of magnitude) the activity of its 1-desoxy analog **6** in the bone [7]. Thus it is likely that analog **6** in clear contrast to 25-OH-D_3 is not significantly hydroxylated *in vivo*.

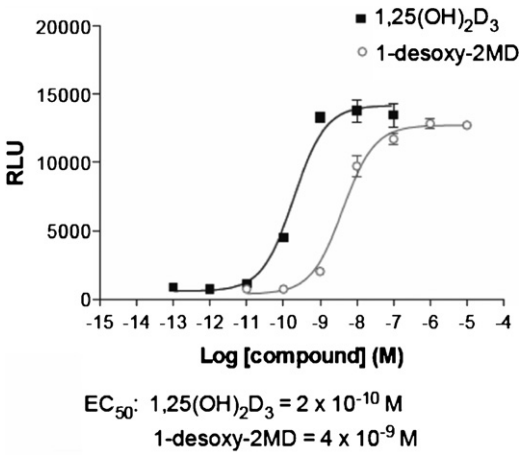


Fig. 4. Transcriptional activity of 1α,25-$(OH)_2D_3$ (**1**) and the synthesized 1-desoxy-2MD (**6**). Transcriptional assay was carried out in rat osteosarcoma cells stably transfected with a 24-hydroxylase gene reporter plasmid. Each experiment was performed in duplicate, two or three separate times.

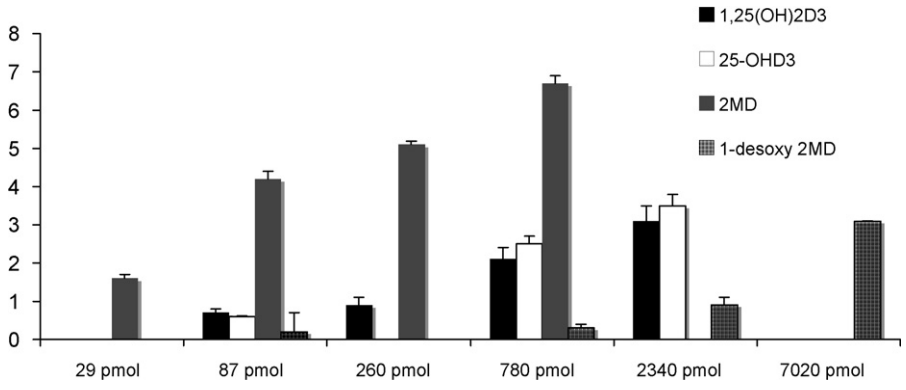


Fig. 5. Bone calcium mobilization of 1α,25-$(OH)_2D_3$ (**1**), 25-OH-D_3 (**3**), 2MD (**5**) and the synthesized 1-desoxy-2MD (**6**).

3.3. Conclusion

It has been commonly accepted that the presence of 1α-hydroxyl group in the vitamin D molecules is of crucial importance for their binding to the VDR and exertion of other biological activities. It was, therefore, rather surprising that 1-desoxy analog of 2MD, whose synthesis is described above, retained good affinity for the VDR. However, in other *in vitro* assays, the analog **6** showed the expected much lower activity compared to its parent compound 2MD. Lack of 1α-OH had also a detrimental effect *in vivo*, proving a significantly decreased calcemic potency of **6**. These results seem to indicate that 2-methylene-19-norvitamins with a (20*S*)-configuration are not enzymatically hydroxylated (at least, not efficiently) at C-1 despite the fact that this position is allylic, similarly to the case of the "normal" vitamin D compounds with a preserved C(10) = C(19) moiety and (20*R*)-configuration.

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