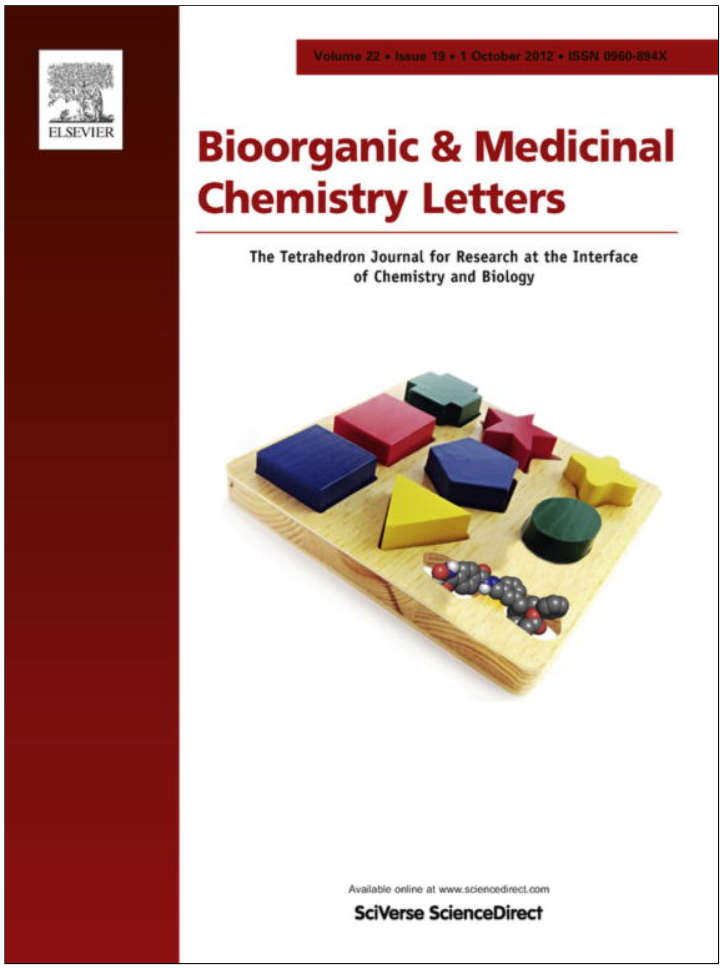

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Synthesis and biological evaluation of a new vitamin D_2 analogue

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ABSTRACT

A new vitamin D_2 analogue was synthesized using the Julia–Kocienski olefination. It has antiproliferative effects on cell lines from squamous cell carcinomas of colon and head and neck, but is also as hypercalcaemic as calcitriol in vivo.

1α,25-Dihydroxyvitamin D_3 (**1b**, calcitriol) (Fig. 1), the active hormone metabolized from vitamin D_3 (**1a**), acts as a regulator in calcium and phosphate homeostasis.[1] Apart from these classic activities, calcitriol has proved to inhibit cellular proliferation and to induce cellular differentiation.[2] However, therapeutic use of these latter effects of **1b** is hampered by the hypercalcaemic side effects of effective doses. This has stimulated a search for analogues that combine a relatively weak systemic effect on calcium metabolism with potent regulatory effects on cell differentiation and proliferation.

The metabolism and biological activity of the non-natural vitamin ergocalciferol (**2a**, vitamin D_2) appear to parallel those of vitamin D_3,[3] and it is in fact marketed as a treatment for refractory rickets. In particular, **2a** undergoes double hydroxylation to 1α,25-dihydroxyvitamin D_2 (**2b**),[4] which like **1b** induces cell differentiation and inhibits the proliferation of a number of tumour cell lines, including leukaemia cells[5] (in spite of which, very few syntheses of either 1α,25-$(OH)_2$-D_2 or 25-(OH)-D_2 have been reported to date).[6]

The vitamin D_2 analogue paricalcitol has been shown to be less hypercalcaemic than calcitriol in vitro[7] and to have antiproliferative activity,[8,9] and in clinical trials it has proved to be partially effective against certain cancers.[10,11] These findings imply that the search for members of the vitamin D family with low hypercalcaemic activity and a high therapeutic index should not overlook

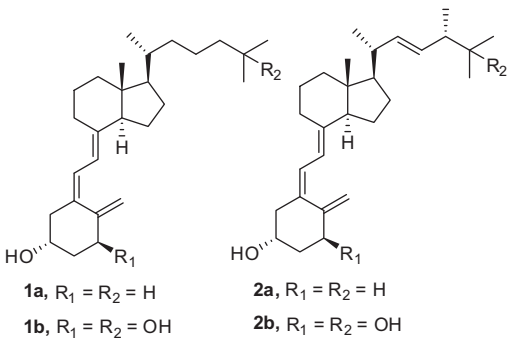

Figure 1. Structures of vitamine D_3 (**1a**) and vitamine D_2 (**2a**) and their metabolites **1b** and **2b**.

vitamin D_2 analogues. Here we describe the synthesis of vitamin D_2 analogue **3** (Fig. 2), the 22Z side chain of which was recently obtained serendipitously by means of a Julia–Kocienski olefination,[12] together with assays of its antiproliferative activities in various cancer cell lines and its hypercalcaemic effects in vivo.

For the synthesis of **3** we started from alcohol **4** (Scheme 1), which is readily obtained in large quantities from vitamin D_2 using the procedures originally described by Calverley[13] and later modified by Choudhry.[14] TPAP oxidation of **4** afforded aldehyde **5** in 95% yield, and Julia–Kocienski olefination of **5** with sulfone **6** gave a 65% yield of ester **7**, which upon reaction with ethyl magnesium iodide in ether at 0°C yielded alcohol **8**. Removal of the silyl protecting

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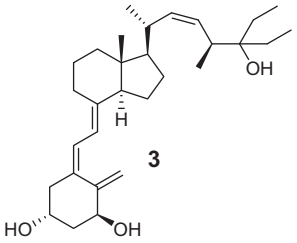
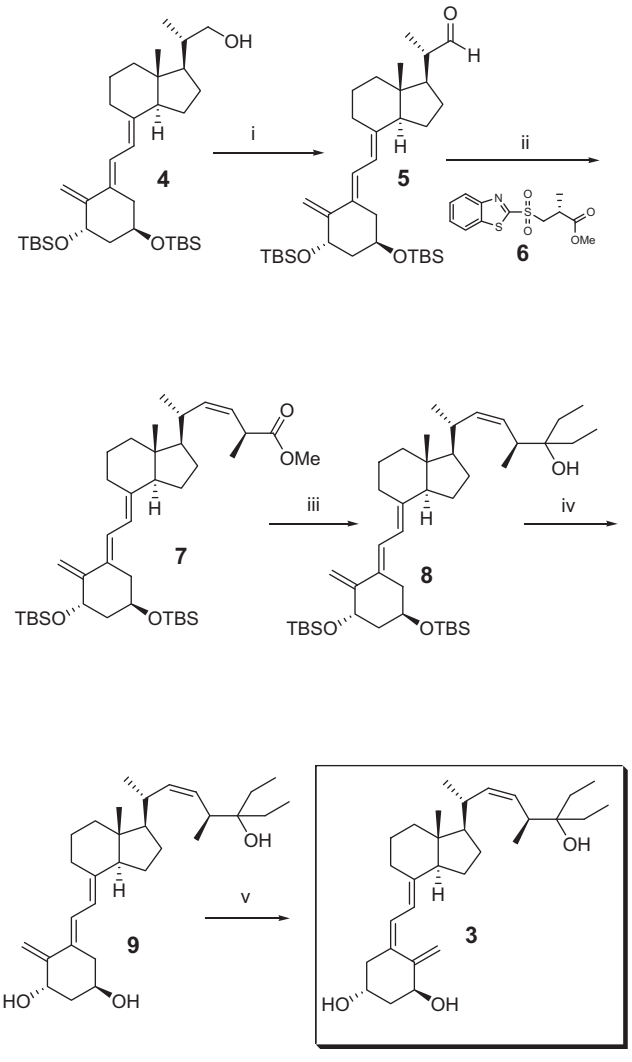


Figure 2. Structure of vitamine D_2 analogue **3**.

groups of **8** with TBAF in THF afforded a 98% yield of triol **9**, and photoisomerization of **9** using anthracene as sensitizer finally gave the target analogue **3** in 90% yield. The overall yield of this five-step synthetic sequence was an excellent 44%.

In order to test the biological activity of analogue **3,** we first analyzed the antiproliferative effects of this compound in several cancer cell lines. For this purpose we performed cell count following treatment of human squamous cell carcinoma (HN12), glioblastoma (T98G), mammary carcinoma (human T47D and murine LM3) and colorectal cancer (HCT116) cell lines with analogue **3**, calcitriol or vehicle. We performed 120 h dose-response analyses comparing



Scheme 1. Reagents and conditions: (i) TPAP, NMO, CH_2Cl_2, molecular sieves (95%); (ii) **6**, LiHMDS, THF, −78 °C (65%); (iii) EtMgI, Et_2O, 0 °C (80%); (iv) TBAF, THF (98%); (v) anthracene, Et_3N, hν, CH_2Cl_2, MeOH (90%).

the effects of analogue **3** with those elicited by the natural hormone calcitriol. As shown in Figure 3, we observed a significant decrease in cell count after treatment with analogue **3,** in the human squamous cell carcinoma (A) and the human colorectal cancer (B) cell lines. The antiproliferative effect exerted by analogue **3** in HN12 cells was similar to that elicited by calcitriol. The half maximal inhibitory concentration (IC_{50}) was 5.05 nM and 4.68 nM for analogue **3** and calcitriol, respectively. In HCT116 cells, the IC_{50} was 0.76 nM and 0.01 nM for analogue **3** and calcitriol, respectively. No effect on cell proliferation was observed in the mammary cancer (C and D) and the glioma (E) cell lines.

It is known that cancer cell lines display a range of sensitivities to the anti-proliferative effects of calcitriol and its derivatives; the reason for this is largely unknown and could result from defects in any component in the VDR signaling pathway including VDR and 24-hydroxylase (CYP24A1).[15] Calcitriol action is limited by its catabolism, which occurs mainly by CYP24A1 resulting in 1α,24,25 $(OH)_3$-D_3, a metabolite with substantially lower affinity for the vitamin D receptor VDR. Although this enzyme is located primarily in liver, it has been demonstrated to be expressed by many tissues.[15] Augmented expression of CYP24A1 has been shown to be detrimental to calcitriol antiproliferative effects. In prostate cancer cell lines, it has been demonstrated that enzyme expression was inversely correlated to the antiproliferative effects displayed by the cells.[16] In this regard, analogue **3** may be degraded by cells displaying high expression of CYP24A1, which could account for the lack of activity observed in some of the cell lines, similarly to what occurs with calcitriol treatment. It has also been reported that splice variants of the enzyme may have implications for the antitumorigenic effects of the hormone and analogues.[17]

As already stated, vitamin D and many of its analogues induce hypercalcemia, a fact that limits the therapeutic efficacy of these compounds for cancer treatment. Therefore, analyses of the calcemic effects need to be performed for each novel analogue synthesized. Because of its significant in vitro antiproliferative activity in human squamous cell carcinoma and in colorectal carcinoma, analogue **3** was then evaluated for its calcemic effects in vivo. Previous pharmacokinetic studies performed in normal mice indicated that calcitriol at 0.125 μg/mouse (approximately 5 μg/kg body weight) results in a C_{max} > 10.0 ng/mL and AUC > 40.0 ng h/mL[18], exceeding the concentration needed for calcitriol anti-tumor activity in vitro.[19] Other studies using either calcitriol or vitamin D analogues at doses lower than 5 μg/kg body weight showed a reduction in tumor burden in animal models of cancer.[20–22] Therefore we chose the dose of 5 μg/kg body weight that have antitumor effects in vivo as a starting concentration for the studies of calcemia, with the idea of increasing the doses in escalating experiments, provided no toxic effects were observed. Mice were divided into three groups (n = 5/group) and given a daily intraperitoneal injection of calcitriol, analogue **3** or vehicle at 5 μg/kg body weight for 4 days. Blood was collected prior to dose administration, then at 24, 48, 72 and 96 h post-treatment. The health condition of the animals was recorded. Plasma calcium levels were measured by reading the absorbance of metallochromic indicator arsenazo III, as previously described.[23] As observed in Figure 4, analogue **3** induced hypercalcemia starting at 24 h of compound delivery, similar to that elicited by calcitriol. Moreover, mice treated with analogue **3** or calcitriol died after 4 days of treatment. Together with the high calcium plasma levels we observed, in the calcitriol- treated mice and the analogue **3**-treated ones, the appearance of conjunctivitis, chills and thirst which are symptoms and signs that have been associated to hypercalcemia.[24] Additionally, an increase in haematocrit was observed (50 at 72 hours; normal levels range from 39 to 47[25]), which is a sign of intoxication.[26]

Vitamin D_2 analogues such as paricalcitol have been shown to be less calcemic than calcitriol in in vitro studies[7] and have been

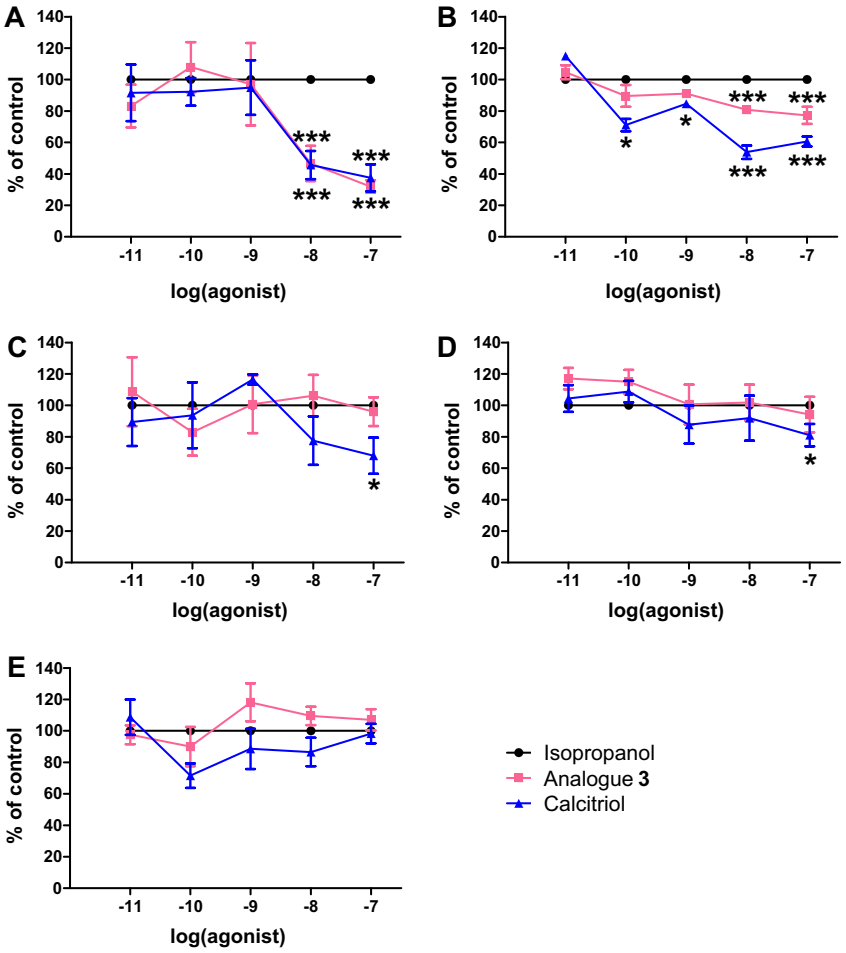


Figure 3. Dose-response effects of analogue **3** on cellular survival and its comparison with calcitriol. (A) Human head and neck squamous cell carcinoma HN12. (B) Human colorectal carcinoma HCT116. (C) Murine mammary adenocarcinoma LM3. (D) Human mammary adenocarcinoma T47D and (E) Human glioma T98G. Cells were exposed to the indicated doses of vehicle (isopropanol), analogue **3** or calcitriol over a total time of 120 h. Cellular survival was expressed as percentage of the vehicle. Data points represent means ± SD from five replicates; $^{*}p \leqslant 0{,}05$ and $^{***}p \leqslant 0001$, with respect to vehicle. The experiments were repeated at least three times for each cell line.

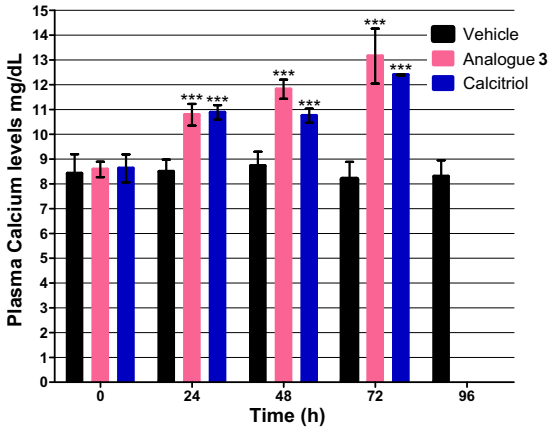


Figure 4. Plasma calcium levels in mice in response to daily intraperitoneal injections of isopropanol (vehicle), analogue **3** or calcitriol during a period of 4 days. Animals were injected with 5 μg/kg body weight of compounds, and plasma calcium was measured before the injection (0 h, basal levels) and at 24, 48, 72 and 96 h. Normal levels range from 8.8 to 10.4 mg/dL.[27] Values for calcitriol and analogue **3** at 96 h were not available because animals died following 3 days of treatment due to hypercalcemia. Values are means ± SDs from five animals in each group. The experiment was repeated two times. $^{***}p \leqslant 0.0001$, with respect to vehicle.

demonstrated to have antiproliferative activity.[8,9] Clinical trials showed a partial response of paricalcitol in cancer[10,11] and therefore new vitamin D_2 analogues should be assayed for antitumoral activity with the aim to identify one with low hypercalcemic effects and high therapeutic index. The study of the biologic effects of analogue **3** showed that although it exerts antiproliferative effects in colon and head and neck squamous cell carcinomas, it displays similar hypercalcemic effects to those elicited by calcitriol.

Acknowledgments

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