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Design, Synthesis and Biological Action of 20*R*-Hydroxyvitamin D₃

Yan Lu[†], Jianjun Chen[†], Zorica Janjetovic[‡], Phillip Michaels[‡], Edith K Y Tang[§], Jin Wang[†], Robert C. Tuckey[§], Andrzej T. Slominski[‡], Wei Li^{†,*}, and Duane D. Miller^{†,*}

[†]Department of Pharmaceutical Sciences, University of Tennessee, Health Science Center, Memphis, TN 38163

[‡]Department of Pathology and Laboratory Medicine, Center for Cancer Research, and Division of Dermatology, Department of Medicine, University of Tennessee, Health Science Center, Memphis, TN, 38163

[§]School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, Crawley, WA, Australia

Abstract

Non-naturally occurring 20*R* epimer of 20-hydroxyvitamin D₃ is synthesized based on chemical design and hypothesis. The 20*R* isomer is separated from semi-preparative HPLC and its structure is characterized. The comparison of 20*R* isomer to its 20*S* counterpart in biological evaluation demonstrates they have different behaviours in both antiproliferative and metabolic studies.

Keywords

20*R*-hydroxyvitamin D₃; 20*S*-hydroxyvitamin D₃; chemical synthesis; NMR; antiproliferative activity; metabolism; CYP11A1; CYP27B1

Introduction

Vitamin D hormone carries out essential biological functions required for human health. Besides its critical role in regulating bone formation and calcium homeostasis, vitamin D hormone also regulates cell growth, differentiation, proliferation, apoptosis, and immune responses through the activation of the nuclear vitamin D receptor (VDR).¹² Vitamin D₃ is derived from a cholesterol precursor in the skin, 7-dehydrocholesterol (7-DHC). After absorption of ultraviolet B (UVB) radiation (315 nm to 280 nm in wavelength), 7-DHC is converted to previtamin D₃, which undergoes further thermally induced transformation to vitamin D₃ (cholecalciferol). Vitamin D₃ from the diet or produced in the epidermis is biologically inactive and requires enzymatic conversion to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, calcitriol), the active form of vitamin D₃. This activation involves sequential 25- and 1 α -hydroxylation in the liver and kidney, respectively.³ Unfortunately, the toxicity (hypercalcemia) induced by high levels of vitamin D largely prevents the clinical use of pharmacological doses of 1,25(OH)₂D₃. More than 40 metabolites of vitamin D have been reported⁴ and understanding the activity of these metabolites can assist in the development of new vitamin D₃ analogues with beneficial actions. Searching for vitamin D analogs that retain biological efficacy and display minimal or no hypercalcemia has been pursued for

*To whom correspondence should be addressed. Tel: (901)448-6026. dmiller@uthsc.edu; or Tel: (901)448-7532, wli@uthsc.edu.
Supporting Information Available: Preparations and spectra of intermediates are available via the internet at <http://pubs.acs.org>.

several decades. As a result, several vitamin D analogs that exhibit reduced hypercalcemia are entering clinical trials and are showing great promise as potential therapeutic agents for the treatment of cancer and other diseases.⁵

20*S*-hydroxyvitamin D3 (20*S*(OH)D3, Figure 1) is a newly isolated metabolite of vitamin D3 produced by the action of the enzyme cytochrome P450_{scc} (CYP11A1).^{6,7} It inhibits cell proliferation and differentiation without inducing hypercalcemia at a dose of 30 µg/kg on mice (unpublished results), stimulates VDR gene expression and also inhibits NFκB activity with similar potency to 1,25(OH)₂D3.⁸ We have chemically synthesized 20*S*(OH)D3,^{6b} which exhibited similar biological properties to those of 20*S*(OH)D3 generated enzymatically using P450_{scc}. The *S* configuration of C-20 in chemically synthesized 20*S*(OH)D3 was determined by both NMR and HPLC characterizations, and was found to be identical to the enzymatically generated metabolite. Many reported potent D3 analogs have 21β-Me configuration which corresponds to 20*R*(OH)D3 configuration.⁹ These analogs are very potent VDR-activators with very low hypercalcemic side effect.¹⁰ It will be very interesting to see if 20*R*(OH)D3 have different activity compared with 20*S*(OH)D3. Hansen *et al.* have reported the preparation of 20*R*(OH)D3 analogs with 22-alkynyl side chains from secosteroids.¹¹ In this report, we designed an efficient synthetic route to prepare 20*R*(OH)-7DHC from pregnenolone, which following UVB irradiation and HPLC purification yielded the 20*R*(OH)D3 epimer. Analysis of its NMR spectra and comparison to that of 20*S*(OH)D3 confirmed the formation of the 20*R*-epimer without detectable amounts of the other.

A noticeable phenomenon was reported in the preparation of 20*S*(OH)-7DHC that only 20*S* epimer was produced from the Grignard reaction, while theoretically both 20*R* and 20*S*-(OH)-7DHC should be obtained. This result suggested that the attack of Grignard agent is highly stereo-selective at the 20-carbonyl position (Figure 2). A similar phenomenon was also reported in the synthesis of 20*S*-hydroxycholesterol and other 20-hydroxysteroids.¹² Both theoretical calculation and NMR spectra characterization^{6b} of synthesized 20*S*(OH)-7DHC clearly indicate that there is a preference for the orientation of the 17-acetyl group. It is conceivable that this conformational preference dictated the outcome of the bulky side chain addition to form 20*S* epimer. As shown in Figure 2A, the attack of the nucleophilic Grignard reagent takes place predominantly from the less hindered “bottom” side of the carbonyl group to form 20*S*(OH)-7DHC, while the attack from the “top” side to form 20*R*(OH)-7DHC is sterically prohibited.

The above observations encourage us to design a synthesis approach to exclusively produce 20*R*(OH)-7DHC. We predict that if the bulky side chain at the 20-carbonyl position is introduced in earlier synthesis steps, followed by introducing the 21-methyl group with smaller Grignard reagent CH₃MgI, the 20*R* conformation should be obtained as the only epimer product (Figure 2B). To validate our hypothesis, we employed chemical synthesis to access material for biological evaluation. The synthesis route of 20*R*(OH)D3 is shown in Scheme 1.

Results and Discussion

Chemistry

The synthesis of 20*R*(OH)-7DHC started with high yield (> 95%) conversion of pregnenolone acetate to 3β-acetoxy-steroid **1** under oxidation with *in situ* prepared NaOBr from bromine and sodium hydroxide solution.¹³ We tried the coupling conditions employing EDCI/HOBt/NMM to introduce Weinreb amide, but the major product separated was the intermediate active ester of benzotriazolyl carboxylate, which is close to desired compound **2** on the TLC plate but showed UV absorption. We therefore changed coupling

conditions to HBTU/DIPEA, HNMeOMe-HCl salt which was reacted with 17 α carboxylic acid to yield Weinreb amide in 75.9 % yield. We chose introducing silicon ether protection of the 3 β -hydroxyl in the next step to reduce the consumption of (4-methylpentyl)magnesium bromide **4**, which was prepared from 1-bromo-4-methylpentane and magnesium turnings in anhydrous THF. Three silyl chlorides (TMSCl, TBDMSCl, and TBDPSCl) were used in the presence of various bases such as triethylamine, imidazole, and N-methylimidazole. After comparing and examination of the above conditions, we chose TBDP-SCl as the silylation agent for introducing chromophore to compound **3** and using N-methylimidazole as the base with iodine as the catalyst to accelerate the reaction (89.1% yield).¹⁴ Grignard reaction of compound **3** and (4-methylpentyl) magnesium bromide yielded the bulky side chain ketone **5** with 47.6% yield.

We designed the synthesis route by introducing a 5, 7-diene to compound **5** under 1,3-dibromo-5,5-dimethylhydantoin (dibromantin)/AIBN/TBAB/TBAF condition, thus the deprotection of silicon ether can be used in the same step under TBAF treatment. However, we found from TLC analysis that there was a more complex set of reaction products with silyl protected substrate compared with a similar reaction from acetyl protected substrate in preparation of 20S(OH)-7DHC. Neither the dibromantin condition nor the NBS/ γ -collidine reaction afforded satisfactorily pure 5,7-diene product. Moreover, 5, 7-diene structures are known to be unstable under light and acidic conditions. Thus we chose to postpone the formation of the 5, 7-diene and replace TBDPS protection with acetyl protection. The deprotection of silicon ether with TBAF afforded the 3 β -hydroxyl compound **6** in satisfactory yield (quantitative). Introducing the acetyl protecting group at 3 β -OH with acetyl chloride and pyridine gave compound **7** in an 88.7% yield.

Transformation of **7** into the 5, 7-diene **9** was carried out by dibromantin/AIBN employed in the synthesis of 20S(OH)-7DHC using hexane and benzene as the solvent in the bromination step. Treatment of TBAB/TBAF in THF yielded the diene via dehydrobromination. The purification of the product **9** (34 % overall yield) was carried out through silver nitrate impregnated silica gel chromatography to remove other impurities such as 4, 6-diene by-products. Grignard reaction of 5, 7-diene substrate **9** with CH₃MgI afforded the precursor 20R(OH)-7DHC **10** (yield 85.6 %).

For B-ring opening, 20R(OH)-7DHC **10** was subjected to UVB irradiation for 20 min in a quartz tube at 65°C to achieve the maximum conversion to pre-20R(OH)D3 (**11**). The reaction mixture was incubated at room temperature for one week, which produced a mixture of 20R(OH)D3 (**12**), 20R(OH)-tachysterol (**13**), 20R(OH)-lumisterol (**14**), and other minor 20R(OH)-products. The photolysis reaction and subsequent time-dependent conversion to 20R(OH)D3 was analyzed by HPLC. The final 20R(OH)D3 was purified by a Gilson Semi-preparative HPLC system.

Different epimers of both 20(OH)-7DHC precursors and final 20-hydroxyvitamin D3 analogs have characteristic HPLC retention times (RT). The RT of precursor 20R(OH)-7DHC appeared at 16.0 min and its 20S counterpart could be eluted earlier at 13.8 min under the same HPLC conditions. The final 20R(OH)D3 showed a slight difference as compared to the 20S(OH)D3 isomer peak at RT of 12.3 min vs. 12.2 min, respectively. (see Supplemental files p6-9).

¹H NMR comparisons of both 20(OH)-7DHC epimer precursors and final 20(OH)D3 products are effective methods to identify 20R and 20S isomers of vitamin D3 analogs. It is established that in the pregnane compounds, the ¹H NMR chemical shifts for the 21-Me in the 20S-OH isomers are downfield relative to 20R-OH isomers.^{12a} They have distinct values and are the basis used to assign the absolute configuration at C₂₀ of these two epimers. The

R-configuration at C₂₀ can be deduced from comparisons of ¹H NMR spectra of 21-methyls in both precursor 20*R*/*S*(OH)-7DHC and final 20*R*/*S*(OH)D₃. An upfield chemical shift (1.16 ppm) was obtained for the 21-methyl in 20*R*(OH)-7DHC, while in 20*S*(OH)-7DHC a downfield chemical shift at 1.27 ppm was observed. Similarly, we found that the 21-Me showed a chemical shift at 1.13 ppm in 20*R*-isomer of 20(OH)D₃, while a downfield chemical shift of 1.24 ppm was observed for 21-Me in 20*S*(OH)D₃ (As show in Figure 3, ¹H NMR figures of 20*R*/*S*(OH)-7DHC are shown in supplemental files p1-3).

Biological Evaluations

Previously we documented that 20*S*(OH)D₃ and related 20*S*(OH)D₂ inhibit proliferation and stimulate differentiation of human epidermal keratinocytes (HEKn) in a dose dependent manner similar to that of 1,25(OH)₂D₃ with the highest potency at concentrations >10⁻¹⁰M.^{6b, 8b, 15} In the present studies we compared the antiproliferative activity of 20*S*(OH)D₃ with its epimer, 20*R*(OH)D₃. Figure 4 shows that 20*S*(OH)D₃ and 1,25(OH)₂D₃ (positive control) exhibited similar dose-dependent inhibition of proliferation, as expected, with an EC₅₀ of 7.28×10⁻¹⁰ M and 4.54×10⁻¹⁰ M, respectively. In contrast, 20*R*(OH)D₃ had a biphasic effect, slightly stimulating DNA synthesis at a concentration of 0.1 nM, with significant (p<0.001) inhibition of cell proliferation at concentrations ≥10 nM. This inhibitory effect was similar to that of 20*S*(OH)D₃ (<50% of control), however, with higher EC₅₀=4.09×10⁻⁹M. These data show a clear difference between the *R* and *S* isomers at low concentrations of the ligand (0.1 nM) and similar effects at the higher concentrations, however, with lower potency for the *R* form. In separate experiment we demonstrated that 20*S*(OH)D₃ and 1,25(OH)₂D₃ inhibit colony formation by HaCaT epidermal keratinocytes with similar potency, while 25(OH)D₃ has no- or a small effect (SI S30).

Metabolism of 20(OH)D₃ by CYP11A1 and CYP27B1

Since biologically generated 20*S*(OH)D₃ is derived from the action of P450scc on vitamin D₃ and can be further metabolized to dihydroxy- (e.g. 17,20(OH)₂D₃; 20,22(OH)₂D₃; 20,23(OH)₂D₃) and trihydroxy- (e.g. 17,20,23(OH)₃; 20,22,X(OH)₃D₃) metabolites by this enzyme,^{6a, 7, 16} we compared the ability of P450scc to metabolize 20*S*(OH)D₃ and 20*R*(OH)D₃ (Figure 5A). Due to the formation of many products, metabolites were characterized in the case of the 20*S* isomer¹⁶ but not for the 20*R*-isomer, data are presented as the amount of substrate metabolized. 20*R*(OH)D₃ proved to be a much better substrate than 20*S*(OH)D₃ for P450scc with 58% being consumed in the first 2 min of incubation for the *R*-isomer, but only 13% for the *S*-isomer. While the structures of the products from 20*R*(OH)D₃ remain to be elucidated, HPLC retention times are consistent with formation of di- and tri-hydroxy derivatives analogous to those produced from the 20*S*-isomer.¹⁶

CYP27B1 catalyzes the 1α-hydroxylation of a range of vitamin D derivatives including 20(OH)D₃ and 20(OH)D₂.^{15, 17} In the case of 20*S*(OH)D₃, the 1α,20*S*-dihydroxyvitamin D₃ product exhibits calcemic activity which is in contrast to the parent 20*S*(OH)D₃ which lacks this activity.^{8a} We therefore compared the ability of CYP27B1 to metabolize 20*S*(OH)D₃ and 20*R*(OH)D₃ (Figure 5B). Each isomer was metabolized to a single product at comparable initial rates but subsequently the rate declined more rapidly for the *R*-isomer than the *S*-isomer. This phenomenon could suggest that the 20*R*(OH)D₃ isomer could show less calcemic toxicity than 20*S*(OH)D₃ due to its lower efficiency of 1α-hydroxylation. The hypercalcemic effects of the 1α-hydroxy-metabolites will be examined in future studies.

Conclusion

We chemically synthesized the *R* epimer of 20(OH)D₃ based on the preferred conformation of the reactant and the associated strong steric preference for the formation of this isomer.

NMR characterization of the chemically synthesized compound and comparisons with 20*S*(OH)-7DHC and 20*S*(OH)D3 confirmed the *R* chirality at the C20 position. Biological studies demonstrated the antiproliferative activity of *R*-epimer on keratinocytes, similar to that of the *S*-epimer and 1,25(OH)₂D3 at concentrations of 10 nM and 100 nM, with a noted difference (opposite effect) at lower concentrations. This overlapping but different behavior was further demonstrated by the ability of P450_{scc} and CYP27B1 to metabolize both the *R* and *S*-epimers but with the different efficiencies. 20*R*(OH)D3 was a better substrate for P450_{scc} than 20*S*(OH)D3, while 20*S*(OH)D3 was a better substrate for CYP27B1 than for 20*R*(OH)D3. The latter could explain the higher potency of 20*S*(OH)D3 in the inhibition of HEK293 cell proliferation. Alternatively, P450_{scc} which is present in keratinocytes¹⁸, may cause a more rapid inactivation of the *R*-epimer than the *S*-epimer, however it remains to be established if the products of P450_{scc} action on 20*R*(OH)D3 are more or less potent than the parent compound. In-depth investigations of the biological activity of 20*R/S*(OH)D3 epimers for anti-inflammatory and hypercalcemic effects, the nature of signal transduction pathways induced by these compounds and the potential role of 1-hydroxylation in these processes will be carried out in the future.

Experimental Procedures

All reagents for the synthesis were purchased from commercial sources and were used without further purification. Moisture-sensitive reactions were carried out under an argon atmosphere. NMR spectra were obtained on a Bruker ARX-300 MHz (Billerica, MA) or an Agilent Inova-500 MHz spectrometer (Santa Clara, CA). Mass spectral data was collected on a Bruker ESQUIRE-LC/MS system equipped with an ESI source. High resolution mass spectra were recorded on a Waters Xevo G2 QTOF LCMS using ESI. The purity of the final compounds was analyzed by an Agilent 1100 HPLC system (Santa Clara, CA). Purities of the compounds were established by careful integration of areas for all peaks detected and ≥ 95%.

(1*S,Z*)-3-((*E*)-2-((7*αS*)-1-((*R*)-2-hydroxy-6-methylheptan-2-yl)-7*α*-methylhexahydro-1*H*-inden-4(2*H*)-ylidene)ethylidene)-4-methylenecyclohexanol (20*R*(OH)D3, **12**)

A methanol solution of 20*R*(OH)-7DHC (**10**) (5 mg, 2 mg/mL) was subjected to UVB irradiation for 20 min in a quartz tube at 65°C, using a Rayonet RPR-100 photochemical reactor (Branford, CT). The reaction mixture was incubated at room temperature for one week to allow the conversion from pre-20*R*(OH)D3 (**11**) to 20*R*(OH)D3 (**12**). The reaction mixture was analyzed using an Agilent 1100 HPLC system (Santa Clara, CA) to confirm the production of 20*R*(OH)D3 and to optimize the condition for the separation using a semi-preparative HPLC system. The reaction mixture (5 µL) of irradiated 20*R*(OH)-7DHC was injected by an autosampler onto a 5 µM Phenomenex Luna-PFP column (250 × 4.6 mm) (Torrance, CA) with mobile phase of 85% methanol–water at a flow rate of 1.0 mL/min. The separation of reaction mixture was conducted using a Gilson semi-preparative HPLC System with a 5 µM Phenomenex Luna-PFP semi-preparative column (250 × 10 mm), and a mobile phase as 85% methanol–water at a flow rate of 6.0 mL/min. Fractions were collected based on a pre-setup UV threshold and were reanalyzed by RP-HPLC. Fractions containing above 95% of pure compound **12** (for 240 nm and 265 nm spectra) were pooled, freeze dried, and characterized by NMR and Mass Spectrometry. ¹H NMR (500 MHz, MeOH-*d*₄): δ 6.22 (d, 1 H, *J* = 10.0 Hz), 6.02 (d, 1 H, *J* = 10.0 Hz), 5.04 (s, 1 H), 4.74 (s, 1 H), 3.78–3.74 (m, 1 H), 2.87–2.84 (m, 1 H), 2.54–2.52 (m, 1 H), 2.42–2.39 (m, 1 H), 2.21–2.09 (m, 2 H), 2.01–1.96 (m, 1 H), 1.74–1.14 (m, 26 H), 1.13 (s, 3 H), 0.91 (d, 6 H, *J* = 5.0 Hz), 0.71 (s, 3 H). MS (ESI) *m/z* 423.4 [M+Na]⁺, HPLC purity 100%, HRMS calculated for C₂₇H₄₅O₂ [M+H]⁺ 401.3420. found 401.3423.

Metabolic studies of 20(OH)D3

To test enzymatic metabolism, the 20S(OH)D3 and 20R(OH)D3 substrates were incorporated into phospholipid vesicles prepared from dioleoyl phosphatidylcholine and cardiolipin as before,¹⁹ with the ratio of substrate to phospholipid being 0.025 mol/mol phospholipid. Vesicles were incubated with 2 μ M bovine CYP11A1 or 0.06 μ M human CYP27B1 at 37°C for up to 10 min. Products and remaining substrate were extracted with dichloromethane, and measured by reverse phase HPLC as described before.^{16, 17b}

Inhibition of proliferation by different epimers of 20(OH)D3 in comparison to Calcitriol

Neonatal human epidermal keratinocytes (HEKn) were isolated from neonatal fore-skin of African-American donors and grown in HKM (Lonza) medium supplemented with HKGF (Lonza) as described previously.^{8b, c} For the cell proliferation assay the cells from a third passage were seeded into 24-well plates (TPP, Switzerland) and grown to ~80% confluence. Secosteroids were dissolved in ethanol and then diluted in keratinocyte medium containing 0.1% BSA (Sigma). Cells were incubated for 24 h then 1 μ Ci/ml [³H]-thymidine (Moravsek Biochemicals Inc., Brea, CA) was added and cells were incubated for a further 4 h. Excess of unbound thymidine was removed by washing cells with PBS. Cells were precipitated with 10% trichloroacetic acid (TCA) (Sigma) and then the precipitate dissolved with 1N NaOH. The solution was collected in vials and thymidine incorporation was determined using a liquid scintillation counter (Beckman LS 6000, Santa Clara, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

D3	Vitamin D3
7-DHC	7-dehydrocholesterol
1,25(OH)₂D3	1 α ,25-dihydroxyvitamin D3
VDR	vitamin D receptor
P450_{scc}	CYP11A1
HBTU	O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
TMSCl	Trimethylsilyl chloride
DIPEA	N,N-Diisopropylethylamine
TBDMSCl	<i>tert</i> -butyldimethylsilyl chloride
AIBN	2-2'-azobisisobutyronitrile
TBAF	tetrabutylammonium fluoride
TBDPSCl	<i>tert</i> -butylchlorodiphenylsilane
RT	Room temperature

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Figure 1.
Structures of Calcitriol, Vitamin D₃, and new VD₃ metabolite 20S(OH)D₃.

Figure 2.

Hypothesis to obtain 20*R*-epimer. Molecular models were constructed using the MM2 energy minimization algorithm in ChemBioOffice 2010 Chem 3D Pro (Version 12.0). (A). It was proved that “bottom” side attack by the bulky Grignard reagent to form 20*S* epimer is favoured, while attack from the “top” is prohibited due to the steric hindrance from both 18-CH₃ and the steroid scaffold. (B). We hypothesize that “bottom” side attack of the small sized CH₃MgI is favored by the pre-exist bulky side chain, while it is not inclined to attack from “top” side to form the 20*R* epimer.

Figure 3. Comparison of ^1H NMR Chemical shifts for 21-Me in $20R$ and $20S(\text{OH})\text{D}_3$. 21-Methyl showed a chemical shift at 1.13 ppm in $20R(\text{OH})\text{D}_3$, while the downfield chemical shift 1.24 ppm was observed for 21-Me in $20S(\text{OH})\text{D}_3$.

Figure 4.

20(OH)D₃ isomers inhibit growth of human keratinocytes. HEKn cells were treated for 24 h with 1,25(OH)₂D₃, or *R*, or *S* epimers of 20(OH)D₃ at the concentrations listed. The rate of ³H-thymidine incorporation into DNA served as a measure of proliferative activity. Data are presented as mean $\pm$ SD, n=4. Incorporation into DNA is shown as a percentile (%) of control (ethanol treated cells). Statistical significance was measured using Student t-test (*) and one-way ANOVA (*) presented as **p<0.05, ***p<0.01, and ****p<0.001.

Figure 5. Metabolism of 20(OH)D3 isomers by CYP11A1 and CYP27B1. Substrates were incorporated into phospholipid vesicle and incubated with CYP11A1 (A) or CYP27B1 (B) then substrate and products extracted with dichloromethane and analyzed by reverse phase HPLC

Scheme 1.
Chemical Synthesis of 20R(OH)D3