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Chemical Synthesis and Biological Activities of 20*S*,24*S*/*R*-Dihydroxyvitamin D3 Epimers and Their 1*α*-Hydroxyl Derivatives

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Abstract

Bioactive vitamin D3 metabolites 20*S*,24*S*-dihydroxyvitamin D3 [20*S*,24*S*(OH)₂D3] and 20*S*,24*R*-dihydroxyvitamin D3 [20*S*,24*R*(OH)₂D3] were chemically synthesized and confirmed to be

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00881.

Preparation and characterization of intermediates and final products (PDF)

Molecular formula strings (CSV)

The authors declare no competing financial interest.

identical to their enzymatically generated counterparts. The absolute configurations at C24 and its influence on the kinetics of 1 α -hydroxylation by CYP27B1 were determined. Their corresponding 1 α -hydroxyl derivatives were subsequently produced. Biological comparisons of these products showed different properties with respect to vitamin D3 receptor activation, anti-inflammatory activity, and antiproliferative activity, with 1 α ,20S,24R(OH)₂D3 being the most potent compound.

INTRODUCTION

Vitamin D3 (D3), obtained from photoconversion of 7-dehydrocholesterol (7DHC) in the skin or dietary sources, plays an important role in the regulation of many physiological processes such as mineralization,¹ inflammation,² and cell proliferation and differentiation.³ After entering the bloodstream, D3 is carried by D3 binding protein (DBP) and is transported to the liver where it undergoes initial hydroxylation at C25 by the microsomal cytochrome P450 (CYP) enzyme 2R1 (CYP2R1)⁴ or mitochondrial CYP27A1⁵ to produce 25-hydroxyvitamin D3 [25(OH)D3] (Figure 1). 25(OH)D3, the major circulating form of D3, is further hydroxylated by 1 α -hydroxylase (CYP27B1) in the kidney to yield 1 α ,25-dihydroxyvitamin D3 [1,25(OH)₂D3], the active form of D3. 1,25(OH)₂D3 exerts its effects through the vitamin D receptor (VDR) as a native ligand and modulates the expression of many genes including that encoding CYP24A1. CYP24A1 catalyzes the initial inactivation step of 1,25(OH)₂D3 producing 1 α ,24R,25-trihydroxyvitamin D3 [1,24,25(OH)₃D3], which is further oxidized by CYP24A1 to calcitroic acid which is excreted.⁶⁻⁸ A more recently discovered pathway of D3 activation is catalyzed by CYP11A1, with 20S-hydroxyvitamin D3 [20S(OH)D3] being the initial and major product.⁹⁻¹³ Although initially identified from in vitro studies, there is mounting evidence that this pathway occurs in vivo.¹⁴⁻¹⁶ 20S(OH)D3 acts as a biased agonist on the VDR and thus displays many but not all of the biological activities of 1,25(OH)₂D3.¹¹ Similar properties include promoting the differentiation and suppressing proliferation of keratinocytes, melanocytes, fibroblasts, and melanoma cells.^{11,17}

In contrast to 1,25(OH)₂D3, 20S(OH)D3 is a poor inducer of CYP24A1 and is noncalcemic in mice up to a dose of 60 μ g/kg,^{18,19} suggesting that it might be useful as a therapeutic agent. In addition, 20S(OH)D3 is an antagonist or inverse agonist of ROR α and ROR γ in skin cells.³³ It can exert anti-inflammatory effects through upregulation of I κ B in the NF κ B pathway^{20,21} or directly inhibit interleukin-17 (IL-17) production in different cell lines.¹¹ Rodent models further confirm its anti-inflammatory effects where reduced disease symptoms of scleroderma²² and rheumatoid arthritis are observed after its administration.¹¹ Thus, the metabolism of 20S(OH)D3 by CYP24A1 and other P450s is of great importance in understanding its actions and fate.

Predictably, both rat and human CYP24A1 are able to hydroxylate 20S(OH)D3 producing both 20,24-dihydroxyvitamin D3 [20,24(OH)₂D3] isomers.^{6,23} The major isomer made by CYP24A1 is also produced from 20S(OH)D3 by a different P450, yet to be identified, in mouse liver microsomes.²⁴ Activity tests have shown that this isomer and its 1 α -hydroxyderivative are more potent than the parent 20S(OH)D3, markedly inhibiting colony formation of SKMEL 188 melanoma cells at low concentrations, suggesting enhanced

therapeutic potential as antiproliferative agents.⁶ However, it has not been possible to establish the absolute configurations of these isomers at C24 to determine which is 20*S*,24*S*-dihydroxyvitamin D3 [20*S*,24*S*(OH)₂D3] and which is 20*S*,24*R*-dihydroxyvitamin D3 [20*S*,24*R*(OH)₂D3], due to the limited amount of these enzymatic products.

Thus, the aim of this study was to unambiguously assign the C24 configurations to the two 20*S*,24(OH)₂D3 isomers as well as their 1α-hydroxy derivatives and to more comprehensively evaluate their biological activities. To this end, we made both isomers by chemical synthesis, assigned their structures by NMR and molecular modeling, determined their differential kinetics of 1α-hydroxylation by CYP27B1, and tested their biological activities. In addition, their 1α-hydroxy derivatives were enzymatically synthesized and tested for comparison of their activities with their parent 20,24(OH)₂D3 isomers.

RESULTS AND DISCUSSION

Chemistry

The detailed syntheses and spectral characterization of 20*S*,24*S*(OH)₂D3 and 20*S*,24*R*(OH)₂D3 are described in the Supporting Information. Briefly, as shown in Scheme 1, we started from commercially available pregnenolone acetate (**1**). We chose to avoid the formation of the C7 double bond in the initial steps, due to the instability of the 5,7-diene under light and acidic conditions. The 3-acetyl of **1** was removed under KOH/MeOH condition and replaced by TBS. The TBS protected silyl ether **3** was treated with allylmagnesium bromide to get the desired 20*S*-homoallylic alcohol **4** as a major product,²⁵ in which the 20*S*-OH was protected with chloromethyl ethyl ether (EOMCl) using diisopropylethylamine in DCM at rt. Hydroboration of the protected alkene **5** using 9-BBN followed by NaOH/H₂O₂ oxidation selectively gave the primary alcohol **6**, which was then oxidized by pyridinium dichromate (PDC) in dichloromethane (DCM) into the aldehyde **7**. This aldehyde was treated with isopropylmagnesium bromide in THF to produce the diastereomeric mixture **8**, which showed two separable spots on TLC (ethyl acetate/hexane) and gave two pure alcohols (**8a**, **8b**) after separation by flash chromatography. The separated alcohols were used for subsequent reactions separately to get both 7DHC diastereomers. Removal of C3 TBS by TBAF followed by reaction with acetic anhydride in pyridine gave the acetylated **10**. To produce the 5,7-diene, we used 1,3-dibromo-5,5-dimethylhydantoin (dibromantin), AIBN, TBAB, TBAF conditions as reported previously.²⁵ To ensure the stereochemistry of C3 and C24 stayed intact, EOM deprotection under acidic condition was carried out before deacetylation. EOM deprotection of **11** by strong acids such as HCl destroys the 5,7-diene structure; thus, the weak acid CSA in methanol was chosen to deprotect EOM slowly with 30% yield (61% recovered). Further treatment of aqueous KOH in MeOH gave 20,24-dihydroxyl-7DHC **13**. Irradiation of 7-DHC in ether by UVB light for 15 min followed by reflux in ethanol produced vitamin D3 compounds **17a** and **17b** which were further separated by preparative HPLC using acetonitrile and water as mobile phases.

HPLC Showed Matched Retention Times between Chemically Synthesized and Enzymatically Generated Counterparts

To confirm the chemically synthesized 20,24-(OH)₂D₃ isomers were identical with enzymatically generated isomers, HPLC was used to compare their retention times. As shown in Figure 2 and Figure S15, HPLC separation was carried out using two different solvent systems, either an acetonitrile in water gradient or a methanol in water gradient. Under both conditions retention times of each chemically synthesized isomer exactly matched that of the corresponding enzymatic product (see chromatograms of spiked samples in Figure S15). Thus, the enzymatically generated isomer originally designated as product B in the original reports of its production by CYP24A1^{6,23} is isomer I and that designated as product C is isomer II.

Structure Assignments of Isomer I as 17a and Isomer II as 17b

Further confirmation for the same structures between the synthesized and the original enzymatically generated isomers is provided by NMR. The NMR spectra of the chemically synthesized 20*S*,24*S*/*R*(OH)₂D₃ isomers are identical to those of the enzymatically generated products (Figure 3A,C for isomer I and Figure 3B,D for isomer II).^{12,26} The distinct splitting patterns of the 26/27 methyl groups served as a fingerprint between isomers I (doublet) and II (pseudo triplet) as clearly indicated in Figure 3A–D. Full assignments for the ¹H and ¹³C chemical shifts using 2D NMR for both chemically synthesized isomers based on data of several hydroxy derivatives of vitamin D₃ published previously were made.^{12,26} These assignments are summarized in Table S2, along with previously reported assignments for the enzymatic products for comparison.²³

To determine the absolute configuration at C24, we analyzed the NOESY spectra. In the NOESY spectra the relative integrals are 0.324 (isomer I) and 0.285 (isomer II) for the crosspeaks from 24H to 26/27-Me, using the NOE integral of the geminal 19-CH₂ as the internal reference (Figure 2E). The average distances from 24H to 26/27-Me are 3.07 Å (average of 3.04 and 3.10 Å), 3.44 Å (average of 2.99 and 3.88 Å), in 24*R* and 24*S*-configurations, respectively, based on the model structures (Figure 2F). Therefore, isomer I is tentatively assigned the 24*R*-configuration (**17a**), and isomer II to the 24*S*-configuration (**17b**).

In addition, the ¹H chemical shift patterns of the two geminal protons at C23 are distinct between isomer I (1.75 and 1.39 ppm, a difference of 0.36 ppm) and isomer II (1.63 and 1.44 ppm, a difference of 0.19 ppm) as shown in Figure 3E and HSQC spectra in Supporting Information (Figure S16). Due to the severe overlap with other protons, reliable NOE integrals cannot be obtained between C24 proton and C23 protons. However, the distances from the oxygen atom of the C24-OH group to the two proton atoms at C23 were 2.62 and 3.32 Å in 20*S*,24*R*(OH)₂D₃ and 2.57 and 2.62 Å in the 20*S*,24*S*(OH)₂D₃ model structures (Figure 2F). Since the ¹H chemical shift is sensitive to the chemical environment, it is expected that difference between the ¹H chemical shifts of the two protons at C23 is larger in the 24*R*-configuration than that in the 24*S*-configuration, consistent with our earlier tentative assignments. Collectively, these NMR results indicate that isomers I and II, regardless of whether they were chemically synthesized as performed in this report or were

enzymatically generated as we reported previously,^{6,23} are 20*S*,24*R*(OH)₂D3 (**17a**) and 20*S*,24*S*(OH)₂D3 (**17b**), respectively.

To further confirm the absolute configurations of C24, **8a** and **8b** were chosen to be esterified to *S*- and *R*-Mosher esters, separately. Analysis of the ¹H NMR spectra for **8a**, **8b**, and their Mosher esters unambiguously supported that isomers I and II had 24*R* and 24*S* configurations, respectively (Figure S1–S6 and Table S1).

Metabolism by Mouse CYP27B1

Previously we have shown that CYP27B1 can hydroxylate both 20*S*(OH)D3 and 20*S*,24*R*(OH)₂D3 in the 1α-position^{24,27} and that this modifies their biological activity in a cell-type-dependent manner.^{6,28,29} We therefore compared the kinetics of the metabolism of 20*S*,24*R*(OH)₂D3 and 20*S*,24*S*(OH)₂D3 by mouse CYP27B1 (Table 1). Each isomer was able to be 1α-hydroxylated to produce a single product, previously identified as 1α,20*S*,24*R*-trihydroxyvitamin D3 in the case of 20*S*,24*R*(OH)₂D3²⁷ and presumed to be the 1α-hydroxy derivative in the case of 20*S*,24*S*-dihydroxyvitamin D3 based on the high specificity for 1α-hydroxylation by CYP27B1.^{27,30} 20*S*,24*R*(OH)₂D3 was hydroxylated with a *k*_{cat} 18-fold higher than that for the 24*S* isomer. Thus, despite its 3.2-fold higher *K*_m, 20*S*,24*R*(OH)₂D3 is metabolized with a 5-fold higher catalytic efficiency (*k*_{cat}/*K*_m) than 20*S*,24*S*(OH)₂D3. The efficiency of 1α-hydroxylation of 20*S*,24*R*(OH)₂D3 by CYP27B1 is comparable to that of 25(OH)D3,²⁷ so we predict that this hydroxylation does occur in vivo.

VDR-Induced Transcriptional Activity

To investigate the activation of the vitamin D receptor (VDR) by 20*S*,24*R*(OH)₂D3 (**17a**) and 20*S*,24*S*(OH)₂D3 (**17b**) together with their 1α-OH derivatives (**18a** and **18b**), two cell lines (HaCaT and Caco-2) were transduced by a lentiviral VDRE luciferase reporter vector. 1,25-(OH)₂D3 and 22-oxa were used as positive controls.³¹ As seen from Table 2, both 1,25-(OH)₂D3 and 22-oxa-1,25(OH)₂D3 (22-oxa) showed the expected strong activations of VDRE in both cell lines, with the EC₅₀ values showing that 22-oxa is more potent than 1,25(OH)₂D3. Interestingly, **17a** and **17b** were incapable of activating VDRE significantly up to 1000 nM, but their 1α-OH derivatives **18a** and **18b** activated VDR. Both **18a** and **18b** showed higher potency than 1,25(OH)₂D3 in promoting VDRE-LUC activity, at least with respect to the synthetic promoter construct utilized in these assays. Compound **18a** is about 4- to 5-fold more potent than **18b**, and its potency is comparable with that of 22-oxa in both of the two cell lines tested. Interestingly, 20*S*,24*R*(OH)₂D3 (**17a**), which did not cause VDRE activation in these assays, proved to be more potent than 1,25(OH)₂D3 with respect to inhibiting colony formation by SKMEL 188 melanoma cells,⁶ suggesting that there are varied (biased) effects of 20(OH)D3 metabolites toward different promoters containing vitamin D responsive elements, which should be dependent on cofactors activities.¹¹

Inhibition of IFNγ Production

IFNγ, the only member of type II interferons, is a critical cytokine for innate and adaptive immunity, and it is also a commonly used inflammation marker. We have previously shown that 1,25(OH)₂D3 and 20(OH)D3 downregulated IFNγ production by mouse spleen cells.¹¹ To evaluate the activities of the two synthesized 20,24(OH)₂D3 isomers **17a,b** and their 1α-

OH derivatives, their inhibitory effects on IFN γ production by mouse splenocytes were evaluated. As shown in Table 2, all secosteroids tested (100 nM) except **17b** significantly reduced production of IFN γ by splenocytes, while **17b** slightly increased production. Interestingly, **17a** decreased IFN γ levels more than 22-oxa. With respect to the **17a,b** isomers and their 1 α -hydroxyderivatives, isomers with the 24*R* configuration (**17a** and **18a**) caused a greater reduction of IFN γ production than the 24*S* isomers (**17b** and **18b**), suggesting that the 24*R* configuration is beneficial for anti-inflammatory activity. In addition, the 1 α -hydroxy derivatives caused greater reduction of IFN γ production than their corresponding parent compounds, which may be due to their greater potency for activation of the VDR, as shown in Table 2.

Upregulation of LAIR-1

Leukocyte associated immunoglobulin- like receptors (LAIR-1) are expressed on T cells and other immune cells and are thought to modulate T cell receptor signaling to downregulate immune responses and autoimmunity.³² We have recently found that 1,25(OH) $_2$ D3 and 20*S*(OH)D3 can upregulate LAIR-1 expression on T cells which may represent a previously unrecognized mechanism by which vitamin D downregulates autoimmunity (Myers et al., in preparation). To determine whether the anti-inflammatory effects of 20*S*,24*R/S*(OH) $_2$ D3 and their 1 α -hydroxylation products are mediated, at least partially, through LAIR-1 upregulation, we performed a flow cytometry study on LAIR-1. As shown in Table 2, all secosteroids tested significantly increased the expression of LAIR-1 compared to the control. The 1 α -hydroxyl derivatives of the C24 isomers showed similar stimulation as their parent compounds, while the isomers with the C24*R* configuration exhibited greater stimulation of LAIR-1 levels than their 24*S* counterparts.

CONCLUSION

20*S*,24*S*(OH) $_2$ D3 and 20*S*,24*R*(OH) $_2$ D3 were chemically synthesized for the first time. The C24 stereochemistry of the two isomers was unambiguously assigned by NMR analysis. HPLC retention times of chemically synthesized 20*S*,24*S*(OH) $_2$ D3 and 20*S*,24*R*(OH) $_2$ D3 enabled the identification of the major isomer (I) produced from 20*S*(OH)D3 by CYP24A1 as 20*S*,24*R*(OH) $_2$ D3 and the minor isomer (II) as 20*S*,24*S*(OH) $_2$ D3.^{6,23} 20*S*,24*R*(OH) $_2$ D3 is also the isomer produced from 20(OH)D3 by an unidentified P450, distinct from CYP24A1 (which is not expressed in liver) in mouse liver microsomes.²⁴ Biological studies showed that the 24*R*-epimer had stronger or more potent biological activity, regardless of whether the compound was 1 α -hydroxylated or not. The 20,24(OH) $_2$ D3 isomers lacked the ability to activate VDRE using a synthetic promotor construct; however, their 1 α -OH products showed potent VDRE-LUC activation, significantly more potent than that of 1,25(OH) $_2$ D3 and comparable with that of 22-oxa. In addition, inhibition of INF γ production by splenocytes and stimulation of LAIR-1 production indicate that the 24*R*-epimer is also more active than 24*S*-epimer with respect to anti-inflammatory activities. The different properties of 20*S*,24*S*(OH) $_2$ D3 and 20*S*,24*R*(OH) $_2$ D3 are further demonstrated by the ability of CYP27B1 to metabolize 20*S*,24*R*(OH) $_2$ D3 with a catalytic efficiency 5.5-fold higher than that for 20*S*,24*S*(OH) $_2$ D3 but comparable to 1,25- (OH) $_2$ D3. In summary, 20*S*,24*R*(OH) $_2$ D3 displays greater biological activity than 20*S*,24*S*(OH) $_2$ D3, with 1 α -hydroxylation enhancing

the activities of both epimers. Further investigation on the interaction with the vitamin D receptor and subsequent signal transduction pathways would likely explain their differential biological activities.

EXPERIMENTAL SECTION

General Methods

All reagents and solvents for the synthesis and separation were purchased from commercial sources and were used as received. Reactions of 5,7-diene structures were all carried out in the dark by wrapping the flasks with aluminum foil. Moisture- or air-sensitive reactions were performed under an argon atmosphere. All reactions were routinely monitored by TLC on silica gel using ethyl acetate and hexane as mobile phases and visualized by 5% phosphomolybdic acid in ethanol or UV light. Mass spectra of all compounds were obtained by a Bruker ESQUIRE-LC/MS system equipped with an ESI source. The purities of final D3 compounds, as analyzed by an Agilent 1100 HPLC system (Santa Clara, CA), were above 98%. Ethyl acetate was used for extraction of reaction mixtures and then dried over anhydrous Na_2SO_4 , filtered, and removed by rotary evaporator under reduced pressure. For comparison of retention times of enzymatically^{6,23} and chemically generated 20S, 24(OH)₂D3 isomers, HPLC was carried out with an acetonitrile in water gradient comprising 45–100% acetonitrile for 25 min, then 100% acetonitrile for 15 min or a methanol in water gradient comprising 64–100% methanol for 20 min, then 100% methanol for 25 min, using a flow rate of 0.5 mL/min and a C18 column (Grace Alltima, 25 cm $\times$ 4.6 mm, particle size 5 μm).

NMR

A series of 1D and 2D NMR measurements were performed on the two chemically synthesized isomers, 20S,24R(OH)₂D3 (isomer I) and 20S,24S(OH)₂D3 (isomer II), using either a Bruker Avance III 400 MHz, with a BBO 5 mm probe with Z-gradient (Bruker BioSpin, Billerica, MA), or a Varian Unity Inova 500 MHz spectrometer using a 5 mm TXI probe (Agilent Technologies Inc., Santa Clara, CA, USA). Samples (~1.5 mg) were dissolved in 0.3 mL of CD_3OD using 5 mm Shigemi NMR tubes (Shigemi Inc., Allison Park, PA, USA). NMR data were collected at 25 °C. Chemical shifts were referenced to residual solvent peaks for CD_3OD (3.31 ppm for proton and 49.15 ppm for carbon).

Kinetics of Metabolism by Mouse CYP27B1

To measure the kinetics of 1 α -hydroxylation by CYP27B1, substrates [20S,24R(OH)₂D3 and 20S,24S(OH)₂D3] were incorporated into phospholipid vesicles prepared from dioleoylphosphatidylcholine and cardiolipin, as before.¹³ Mouse CYP27B1 (0.01 μM for isomer I and 0.125 μM for isomer II) was incubated for 2 min with a range of substrate concentrations. Products were extracted with dichloromethane and analyzed by reverse-phase HPLC on a C18 column (Grace Smart, 15 cm $\times$ 4.6 mm, particle size 5 μm) with an acetonitrile gradient comprising 45–100% acetonitrile for 10 min, then 100% acetonitrile for 10 min, at a flow rate of 0.5 mL/min. The Michaelis–Menten equation was fitted to the experimental data with correlation coefficients of 0.991 and 0.993 for isomers I and II, respectively. Data for K_m and k_{cat} are presented as the mean $\pm$ standard error of the curve fit.

Enzymatic Synthesis of 1 α -Hydroxy Derivatives Using CYP27B1

These reactions were carried out with the 20S,24R(OH)₂D3 and 20S,24S(OH)₂D3 substrates incorporated into phospholipid vesicles using purified mouse CYP27B1, as reported previously,²⁷ in a scaled up version of the incubations described above. 1 α ,20S,24R(OH)₂D3 and 1 α ,20S,24S(OH)₂D3 were purified by HPLC on a C18 column (Grace Alltima, 25 cm $\times$ 4.6 mm, particle size 5 μ m) using an acetonitrile gradient comprising 45–100% acetonitrile for 25 min, then 100% acetonitrile for 15 min, at a flow rate of 0.5 mL/min.

Cell Culture

Dulbecco's modified Eagle medium (DMEM) supplemented with glucose, L-glutamine, pyridoxine hydrochloride, 5% fetal bovine serum (FBS), and 1% penicillin–streptomycin–amphotericin antibiotic solution (Ab) (Sigma-Aldrich, St. Louis, MO) was used to culture immortalized human keratinocytes (HaCaT). The same medium using 10% FBS instead of 5% FBS was used to culture colonic Caco-2 cells. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

VDRE Reporter Assays

HaCaT and Caco-2 cells were transduced with lentiviral VDRE luciferase using a Cignal Lenti VDRE reporter (luc) kit according to the manufacturer's protocol (QIAGEN, Valencia, CA). After 1 week selection by puromycin (1 μ g/mL), transduced HaCaT and Caco-2 cells were seeded in a 96-well plate (1000 cells/well) using partial media (without FBS) and incubated for 24 h. DMSO solutions of secosteroids to be tested were added to cells, which were then incubated for another 24 h, and the luciferase signal was measured according to the manufacturer's procedure for the ONE-Glo luciferase assay system (Promega, Madison, WI). The final concentration of DMSO was 0.1%, and 0.1% DMSO was used as the vehicle control. All concentrations were tested in triplicate.

Flow Cytometry

Splenocytes from DBA/1 mice were isolated,³³ and the expression of LAIR-1 was determined following overnight culture with each secosteroid at 10^{−7} M (or vehicle control) by multiparameter flow cytometry using an LSRII flow cytometer (BD Biosciences, San Jose, CA). Cells were labeled with fluorochrome antibodies specific for CD4 (BD Biosciences, San Jose, CA) and for LAIR-1 (eBioscience, San Diego, CA). Gating was performed on CD4 cells, and the data were expressed as mean fluorescence $\pm$ SD. A minimum of 10 000 cells were analyzed from each sample, and the final analysis was performed using Flow software (Tree Star, Ashland, OR). Values represent the mean fluorescence from two separate analyses.

IFN γ Inhibition Assay

Secosteroids were solubilized in absolute EtOH at 10^{−4} M and diluted to 10^{−6} M by adding Eagle's minimal essential medium (EMEM) containing 9% charcoal-stripped fetal calf serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, nonessential amino acids, 2.5 mM 2-mercaptoethanol, 2.5 mM L-glutamine.³³ Splenocytes from mice were isolated,

erythrocytes lysed by hypotonic shock, washed twice with EMEM, and suspended at a concentration of $(2 \times 10^6)/\text{mL}$ in EMEM described above. To each well in a 48-well tissue culture plate, an amount of 450 μL of the splenocytes was added. Secosteroids (50 μL of the 10^{-6} M stock) or EtOH diluted 1:100 with the above culture medium were added to triplicate wells and then incubated at 37 °C in 5% CO_2 in a humidified tissue culture incubator for 2 h, after which 1 $\mu\text{g}/\text{well}$ of rat anti-mouse CD3 MOAB was added. After 72 h culture, supernatants from each well were harvested and analyzed by ELISA for levels of α -murine $\text{IFN}\gamma$ (RAD Systems, Minneapolis, MN), according to the manufacturer's instructions. The concentration of $\text{IFN}\gamma$ in supernatants from cultures containing secosteroids was compared to the concentration of $\text{IFN}\gamma$ in the supernatants of EtOH-treated control cultures, by ANOVA. Results are expressed as the mean of triplicate determinations $\pm$ SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

1,24,25(OH)₃D₃	1 α ,24,25-trihydroxyvitamin D ₃
1,25-(OH)₂D₃	1 α ,25-dihydroxyvitamin D ₃
22-oxa	22-oxa-1 α -25-dihydroxyvitamin D ₃
25(OH)D₃	25-hydroxyvitamin D ₃
20S(OH)D₃	20S-hydroxyvitamin D ₃
20,24(OH)₂D₃	20S,24-dihydroxyvitamin D ₃
20S,24R(OH)₂D₃	20S,24R-dihydroxyvitamin D ₃
20S,24S(OH)₂D₃	20S,24S-dihydroxyvitamin D ₃
24,25(OH)₂D₃	24,25-dihydroxyvitamin D ₃
7DHC	7-dehydrocholesterol
9-BBN	9-borabicyclo[3.3.1]nonane
COSY	¹ H– ¹ H correlation spectroscopy
CSA	camphorsulfonic acid
dibromantin	1,3-dibromo-5,5-dimethylhydantoin
HSQC	¹ H– ¹³ C heteronuclear single quantum correlation spectroscopy

HMBC	^1H – ^{13}C heteronuclear multiple bond correlation spectroscopy
IFNγ	interferon γ
NOESY	nuclear Overhauser effect spectroscopy
TBAB	tetra- <i>n</i> -butylammonium bromide
TBAF	tetra- <i>n</i> -butylammonium fluoride
TOCSY	^1H – ^1H total correlation spectroscopy
D3	vitamin D3
VDR	vitamin D receptor
VDRE	vitamin D response element

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Figure 1. D₃ conversion to 25(OH)D₃, 1,25(OH)₂D₃, 20S(OH)D₃, 20S,24R(OH)₂D₃, and 20S,24S(OH)₂D₃.

Figure 2.

Comparison of HPLC retention times of 20,24(OH)₂D₃ isomers produced enzymatically and chemically: (A, C) isomer I chemical; (B, D) isomer I enzymatic; (E, G) isomer II chemical; (F, H) isomer II enzymatic. Samples were analyzed by reverse phase HPLC utilizing acetonitrile in water (A, B, E, F) or methanol in water (C, D, G, H) gradients, as described in the Experimental Section.

Figure 3.

^1H NMR spectral comparison between biologically generated (A, B) and chemically synthesized (C, D) **17a,b**. ^1H - ^1H NOESY spectra (E) of isomer I (identified as **17a**) and isomer II (**17b**) together with their structural models (F).

Scheme 1.**Synthesis of Compounds 17a and 17b^a**

^aReagents and conditions: (a) aq KOH, MeOH, rt, 2 h, 95%; (b) TBSCl, imidazole, DMF, rt, overnight, 90%; (c) allylmagnesium bromide, THF, 0 °C to rt, overnight, 82%; (d) EOMCl, DIPEA, CH₂Cl₂, rt, overnight, 89%; (e) 9-BBN, THF, 0 °C to rt, 24 h; H₂O, rt, 0.5 h; NaOH, H₂O₂, -20 °C to rt, overnight, 76%; (f) PDC, CH₂Cl₂, rt, 24 h, 94%; (g) isopropylmagnesium bromide, THF, 0 °C to rt, 6 h, 85%; (h) TBAF, THF, rt, 12 h, 100%; (i) Ac₂O, pyridine, DMAP, 6 h, 91%; (j) dibromantoin, AIBN, benzene/hexane (1:1), reflux 20 min; TBAB, THF, rt, 75 min, then TBAF, rt, 50 min, 44%; (k) CSA, MeOH/DCM (1:1), 0 °C to rt, 12 h, 30% (61% recovered); (l) aq KOH, MeOH, 2 h, 90%; (m) UVB, Et₂O, 15 min; (n) ethanol, reflux, 3 h; (o) HPLC, ACN/H₂O, 13% (steps m–o), overall yield 1.6%. TBSCl, *tert*-butyldimethylsilyl chloride; DMF, dimethylformamide; PDC, pyridinium dichromate; AIBN, azobis(isobutyronitrile); ACN, acetonitrile.

Table 1Kinetics of the Metabolism of the 20,24(OH)₂D₃ Isomers by Mouse CYP27B1^a

substrate	K_m	k_{cat}	k_{cat}/K_m
20S,24R(OH) ₂ D ₃ (17a)	7.99 ± 4.01	31.2 ± 10.7	3.90
20S,24S(OH) ₂ D ₃ (17b)	2.49 ± 0.59	1.76 ± 0.15	0.71

^a K_m , (mmol of substrate)/(mol of phospholipid); k_{cat} , (nmol/min)/nmol P450; k_{cat}/K_m , min⁻¹ (mmol/mol phospholipid)⁻¹. Data for k_{cat} and K_m are ±SE of the curve fit.

Table 2

Biological Activities of 20*S*,24*R*/*S*(OH)₂D3 and Their 1α-Hydroxylated Derivatives Using 1,25(OH)₂D3 and 22-Oxa-1,25(OH)₂D3 as Positive Controls^a

compd	LAIR (MF)	IFNγ (pg/mL)	VDRE activation (EC ₅₀ , nM)	
			HaCaT	Caco-2
vehicle control	973 ± 95	731 ± 15	N/A	N/A
20 <i>S</i> ,24 <i>R</i> (OH) ₂ D3 (17a)	1907 ± 198	465 ± 5	NS	NS
1α,20 <i>S</i> ,24 <i>R</i> (OH) ₂ D3 (18a)	2100 ± 583	291 ± 5	64.9 ± 1.6	66.9 ± 3.6
20 <i>S</i> ,24 <i>S</i> (OH) ₂ D3 (17b)	1537 ± 74	923 ± 10	NS	NS
1α,20 <i>S</i> ,24 <i>S</i> (OH) ₂ D3 (18b)	1819 ± 612	589 ± 6	270.9 ± 2.6	207.9 ± 2.1
1,25(OH) ₂ D3	1642 ± 220	562 ± 5	321.5 ± 14.2	515.2 ± 22.4
22-oxa-1,25(OH) ₂ D3	1511 ± 366	356 ± 6	37.9 ± 1.2	40.8 ± 1.7

^aN/A: not applicable. NS: no significant activation. MF: mean fluorescence.