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Photo-conversion of two epimers (20*R* and 20*S*) of pregna-5,7-diene-3 β ,17 α ,20-triol and their bioactivity in melanoma cells

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Abstract

Pregna-5,7-dienes and their hydroxylated derivatives can be formed *in vivo* when there is a deficiency in 7-dehydrocholesterol (7-DHC) Δ -reductase function, e.g., Smith-Lemli-Opitz syndrome (SLOS). Ultraviolet B (UVB) radiation induces photoconversion of 7-DHC to vitamin D₃, lumisterol₃ and tachysterol₃. Two epimers (20*R* and 20*S*) of pregna-5,7-diene-3 β ,17 α ,20-triol (**4*R*** and **4*S***, respectively) were synthesized and their UVB photo-conversion products identified as corresponding 9,10-secosteroids with vitamin D-like and tachysterol-like structures, and 5,7-dienes with inverted configuration at C-9 and C-10 (lumisterol-like). The number and character of the products and the dynamics of the process were dependent on the UVB dose. At high UVB doses, the formation of multiple oxidized derivatives of the primary products, and the formation of 5,7,9(11)-triene, were observed. The production of vitamin D-like, tachysterol-like and luminosterol-like derivatives was also observed in human skin treated with **4*R*** and **4*S***, and subjected to UV irradiation, as shown by RP-HPLC. Newly synthesized compounds inhibited melanoma growth in dose dependent manner, and some of them showed equal or higher potency than 1,25(OH)₂D₃. In summary, we have characterized for the first time the products of UV induced conversion of pregna-5,7-diene-3 β ,17 α ,20-triols and documented that the newly synthesized compounds have antiproliferative properties against melanoma cells.

Keywords

secosteroids; UV radiation; lumisterol; tachysterol; SLOS; melanoma

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Introduction

The UVB-driven isomerization of cholesta-5,7-diene-3 β -ol (7-dehydrocholesterol, 7DHC) to (3 β ,5Z,7E)-9,10-secocholesta-5,7,10(19)-trien-3-ol (cholecalciferol, vitamin D₃) is one of the most fundamental reactions in the photobiology of the skin.[1-3] This conversion is initiated by photolysis of the unsaturated B ring, with production of pre-D₃ intermediate, and is followed by its slow isomerization to three main products: vitamin D₃, tachysterol₃ (6E-9,10-secocholesta-5(10),6,8-trien-3 β -ol, T₃) and lumisterol₃ (9 β ,10 α -cholesta-5,7-diene-3 β -ol, L₃) (Scheme 1). The relative ratio and the structure of products depend on a number of variables, including the ultraviolet B (UVB) dose (absorbed energy), temperature, wavelength, the presence of biological membranes or when the reaction is carried out in the skin.[3-5] Higher doses of UV or the presence of trace amounts of acid trigger further isomerization of T₃ to Isotachysterol₃ (isoT₃) products, and is followed by autooxidation.[4,6] Another pathway of degradation/isomerization of vitamin D₃ with production of 5,6-transvitamin D₃, suprasterols I and II in response to high doses of UVB was also demonstrated in the skin.[4] It was shown that 7DHC in biological systems can also produce corresponding 5,7,9(11)-trienes. Although the detailed mechanism is not fully understood; it has been suggested that singlet oxygen and photosensitizers may play a role in this reaction.[7-11]

Accumulation of 5,7-dienes and 5,7,9(11)-trienes is important in the etiology of a 7 Δ -reductase deficiency syndrome (Smith-Lemli-Opitz syndrome – SLOS). [9,10,12-15] The lack of the key enzyme converting 7DHC to cholesterol results in multiple metabolic defects with accumulation of 7DHC and its 5,7-diene steroidal derivatives, including cholesta-5,7,9(11)-trien-3 β -ol (9DDHC)[10] and pregna-5,7-diene-3 β ,17 α ,20-triol.[12-14] Recently, it was proposed that oxidation of 7DHC and 9DDHC leads to severe photosensitivity in SLOS patients.[7,9]

Vitamin D₃ is not only a master regulator of calcium metabolism but also a powerful oncostatic agent affecting proliferation, differentiation and apoptosis of normal and malignant cells.[2, 16] Although its potential therapeutic use against cancer or hyperproliferative disorders is largely limited due to toxicity (hypercalcemia) at pharmacological concentrations, this adversity can be reduced or attenuated by shortening or removal of the side chain.[17,18] Thus, the generation of vitamin D-like compounds with pregnane side chains may have a potential utility in treatment of cancers or other pathological disorders.

Here we report on the UVB photolysis of two epimers (20*R* and 20*S*) of pregna-5,7-diene-3 β ,17 α ,20-triols synthesized from series of 3-acetylated 5-diene precursors.[13,19] The dynamics of the photolytic reactions and identification of novel 9,10-secosteroids with D- T- and L-like structures, and corresponding 5,7,9(11)-trienes. In addition, we studied the formation of secosteroidal derivatives in human skin exposed to UVB and tested their biological activity on the human melanoma SKMEL-188 cell line.

Experimental

Chemical synthesis

The synthesis of compounds **4R** and **4S** is shown in Scheme 1.

Synthesis of 2

The acetylation of steroid **1** was carried out following a known procedure [20]. Yield: 95%. ¹H NMR (500MHz, CDCl₃) for compound **2a**: δ 5.39 (d, *J* = 5 Hz, 1H), 4.58-4.64 (m, 1H), 2.92-2.96 (m, 1H), 2.30-2.36 (m, 2H), 2.12 (s, 3H), 2.04 (s, 3H), 2.05 (s, 3H), 1.98-2.02 (m, 2H), 1.86-1.90 (m, 2H), 1.46-1.80 (m, 9H), 1.26-1.32 (m, 1H), 1.14-1.18 (m, 1H), 1.06-1.08

(m, 1H), 1.03 (s, 3H), 0.64 (s, 3H). ESI-MS: calculated for $C_{25}H_{36}O_5$, 416.26, found 439.3 [M+Na]⁺.

General procedure for the synthesis 3

Compounds **3** were synthesized according to a known procedure [13]. Yield: 40-50%. ¹H NMR (500MHz, CDCl₃) for compound **3**: δ 5.57-5.59 (m, 1H), 5.44-5.46 (m, 1H), 4.68-4.74 (m, 1H), 2.96-2.90 (m, 1H), 2.59-2.63 (m, 1H), 2.49-2.54 (m, 1H), 2.36 (t, *J* = 15 Hz, 1H), 2.11 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 2.02-2.04 (m, 1H), 1.82-1.94 (m, 4H), 1.56-1.73 (m, 6H), 1.38 (dt, *J* = 15 Hz, 5 Hz, 1H), 0.95 (s, 3H), 0.57 (s, 3H). ESI-MS: calculated for $C_{25}H_{34}O_5$, 414.24, found 437.3 [M+Na]⁺.

General procedure for the synthesis of 4 (4R and 4S)

Compounds **4** (**4R** and **4S**) were synthesized according to a known procedure [13]. Reaction resulted in a crude, white, solid mixture of compounds **4R** and **4S**: 40-50% yield. The mixture was subjected to flash chromatography (column eluted with hexane–ethyl acetate 20:1, 10:1, 5:1, 1:1 in order), but only the isomer with a faster retention time (Peak 1, **4R**), was purified in this manner. The separation of the second isomer (Peak 2, **4S**) was accomplished using RP-HPLC.

¹H NMR (500MHz, CD₃OD) for compound **4S**: δ 5.54 (dd, *J* = 7.5Hz, 3.2Hz, 1H), 5.38-5.40 (m, 1H), 3.94 (q, *J* = 18Hz, 1H), 3.47-3.55 (m, 1H), 2.57 (t, *J* = 10Hz, 1H), 2.38-2.43 (m, 1H), 2.2-2.28 (m, 1H), 1.88–1.98 (m, 3H), 1.62–1.88 (m, 8H), 1.42-1.58 (m, 3H), 1.26-1.34 (m, 2H), 1.15 (d, *J* = 6Hz, 3H), 0.96 (s, 3H), 0.77 (s, 3H). ESI-MS: calculated for $C_{21}H_{32}O_3$, 332.24, found 355.3 [M+Na]⁺; **4R**: δ 5.54 (dd, *J* = 8.3Hz, 2.5Hz, 1H), 5.41-5.44 (m, 1H), 3.79 (q, *J* = 18Hz, 1H), 3.47-3.56 (m, 1H), 2.60 (t, *J* = 10Hz, 1H), 2.38-2.43 (m, 1H), 2.20-2.28 (m, 1H), 2.07-2.14 (m, 1H), 1.89–1.97 (m, 2H), 1.62–1.89 (m, 8H), 1.42-1.53 (m, 2H), 1.26-1.34 (m, 2H), 1.12 (d, *J* = 6Hz, 3H), 0.95 (s, 3H), 0.7 (s, 3H). ESI-MS: calculated for $C_{21}H_{32}O_3$, 332.24, found 355.3 [M+Na]⁺.

General procedure for UVB irradiation of 4R and 4S

The UVB irradiation was performed as described previously[21] with some modifications. Briefly, a methanol solution of **4R** or **4S** at 1 mg/mL was subjected to UV irradiation for various times in a quartz cuvette (or glass HPLC insert) using Biorad UV Transilluminator 2000 (Biorad, Hercules, CA). Spectral characteristics of the UVB (280-320 nm) source were published previously [22] and its strength (4.8 ± 0.2 mW cm⁻²) was routinely measured with digital UVB Meter Model 6.0 (Solartech Inc., Harrison Twp, MI). Irradiation was followed by 14 hours incubation at room temperature and selected products were purified by RP-HPLC chromatography as described.[21] The major products were identified on the basis of their retention time and characteristic UV absorption. Initial identification was confirmed by means of MS and NMR measurements. The quantities of products varied and were predominantly dependent on the UVB radiation dose. Fifteen minutes reaction resulted in 30-35% of pre-D-like, 20% T-like, 10% of substrate and lower quantity of other products.

UVB irradiation of 4R and 4S incorporated into human skin

Human neonatal foreskin from African-American donors was collected after routine circumcision, fat was removed, and the skin was washed with DMEM medium and cut into approximately 5×5 mm pieces. Skin fragments (epidermis facing up), were placed into 24 well plates containing 100 μL of DMEM medium, leaving the epidermis above the surface of the medium. The compounds **4R** or **4S** (2 μL of 10 mM solution) were dropped on the epidermis and the samples were incubated for 1-2 hours in DMEM with dermis down (liquid phase) and epidermis exposed to air a room temperature. Next, the samples were washed two times for 5

minutes with PBS to remove not absorbed steroids. Plates containing skin fragments were placed upside down on Biorad UV Transilluminator 2000 (Biorad, Hercules, CA) and irradiated with 100, 500, 1000 and 2500 mJ/cm². After irradiation tissue was shredded and steroids were extracted with excess of methylene chloride (twice). The samples were dried under nitrogen and analyzed by RP-HPLC. Untreated skin (irradiated and non-irradiated), skin treated with **4R** or **4S** without irradiation and compounds **4R** or **4S** irradiated in ethanol served as controls.

Colony forming assay

To measure colony formation and growth of melanoma SKMEL-188, cells were plated at a colony-forming density (200 cells per well on six well plates). Cells were grown in F10 medium (GIBCO, Invitrogen Corp., Carlsbad, CA) supplemented with charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and an antibiotic-antimycotic solution (Sigma, St. Louis, MO) at 37°C in an atmosphere of 95% air and 5% CO₂. [23] Cells were treated with compounds: **4R**, **4R-pD**, **4R-pL**, **4S**, **4S-pD**, **4S-pL** and 1,25-dihydroxyvitamin D₃ as a positive control at different concentrations (0.1, 10 and 100 nM). Compounds were added to the media from 1 mM ethanol stock solutions, and corresponding dilution of methanol was used as negative (vehicle) control. Cells were grown for 6-10 days and colonies were fixed with 4% paraformaldehyde in PBS at 4°C. The fixed cells were washed with distilled water, stained with 0.1% crystalline blue for 30 min and rinsed with distilled water to remove excess of the dye. Plates were photographed and the number and the size of colonies were measured by ImageJ software (NIH, Bethesda, MD). Colony forming efficiency (CFU) was determined by dividing the number of colonies formed by the number of cells plated, and multiplied by 100.

Statistical analyses

Data were tested for distribution and homogeneity of variances, and for statistical significance with one-way analysis of variance (Anova) with Tuckey post-hoc test (for more than two groups) using Prism 4.00 (GraphPad Software, San Diego, CA). Data is presented as the mean $\pm$ SEM; for n=4-6. *P<0.05, **P<0.01 and ***P<0.001.

Other procedures

All RP-HPLC analyses were performed using a Waters' HPLC-system equipped with a diode-array detector, auto-sampler and fraction collector (Waters Associates, Milford, MA) as was described elsewhere. [21] Samples were injected into an Atlantis C18 column running on 70/30 methanol/water at a flow rate of 1.5 mL/min. Collected fractions (15 sec) containing above 95% of pure compound (for 240 nm and 280 nm spectra) were pooled together and used for further characterization. The procedure was optimized to achieve the best separation of each selected product.

Mass spectra were recorded using a Bruker Esquire-LC/MS Spectrometer equipped with an electrospray ionization (ESI) source, as described previously [21].

NMR measurements were performed on a Varian Unity Inova-500 MHz spectrometer (Varian Inc., Palo Alto, CA) using either a 3 mm probe or a 5 mm probe. Deuterated methanol was used instead of CDCl₃ to avoid observed decomposition due to traces of acid that are present in commercial chloroform.

Results

Synthesis of pregna-5,7-diene-3 β ,17 α ,20-triols

The synthesis was performed essentially as described previously [21] (See Scheme 2 and Material and Methods for details).

The synthesis of pregna-5,7-diene-3 β ,17 α ,20-triols (**4**) was carried out from 17 α -acetylated 5-en precursor (**1**) by acetylation, bromination-dehydrobromination and reduction, following the known procedure.[13] Deprotection was performed simultaneously with reduction of the carbonyl group. The procedure resulted in formation of two diastereomers: **4R** and **4S** (20R and 20S) with a ratio of 50:50. The mixture was effectively separated by reverse phase HPLC (RP-HPLC). The presence of 4,6-dienes was not detected, as was reported during the synthesis of 3 β -hydroxypregna-5,7-dien-20-one and androsta-5,7-diene-3 β ,17 β -diol [21]. Physico-chemical properties of pregna-5,7-diene-3 β ,17 α ,20-triols are summarized in Table 1. The detailed NMR data are presented on Table 2. NMR chemical shifts for **4R** and **4S** are in agreement with those presented previously,[13] with small differences related to a solvent effect due to the use of CD₃OD instead of CDCl₃ for NMR experiments.

UVB irradiation of pregna-5,7-diene-3 β ,17 α ,20-triols (**4**) and identification of products by combination of RP-HPLC, UV and mass spectrometry

The UV conversion of **4R** and **4S** were performed using a UVB light source (4.8 ± 0.2 mW cm⁻²) with maximum emission spectrum in a range of 290–320 nm[22] as described previously. [21] The photo-conversion and subsequent time dependent structural rearrangements were monitored by an HPLC equipped with a diode array that enabled fast detection of products by characteristic UV spectra.[24] Similarly to cholesta-5,7-dien-3 β -ol (7DHC)[24], four main products (Scheme 3) were formed in time-dependent fashion, namely pro-D-like, T-like, L-like and D-like. All products were separated by HPLC and identified based on their unique UV absorption spectra (Figure 1).

The irradiation of **4R** and **4S** with increasing UV doses resulted in the formation of diverse products, as shown in Figure 2. Short irradiation (5 min.; Figure 2A, B) of both **4R** and **4S** resulted in the formation of pre-D-like (λ_{max} at 260 nm; **4R-pD** and **4S-pD**), T-like (λ_{max} at 274, 281, 290 nm **4R-T** and **4S-T**) and L-like (262, 271, 281; **4R-L** and **4S-L**) products. The products **4R-pD** and **4S-pD** underwent subsequent slow isomerization into vitamin D-like compounds (**4R-D** and **4S-D**) with maximum UV absorption at 265 nm. Longer UVB irradiation (15 minutes; Figure 2C, D) resulted in conversion of T-like products to isoT-like compounds with characteristic UV absorption for a conjugated double bond (λ_{max} at 248 nm). Irradiation for 30 and 60 minutes (Figure 2E-H) resulted in further conversion into numerous iso-T-like products with a corresponding decrease in pre-D-like and T-like products, indicating further metabolism (Scheme 2) with the potential formation of suprasterols [4] with a λ_{max} below 250 nm.

Further identification was performed after purification by RP-HPLC (see Materials and Methods for details) and the corresponding fractions were analyzed by MS. As expected, all D-like, L-like and T-like products had identical mass ($m/z = 355.25$ [M+Na]⁺) with the parental compounds (Table 1). In addition to a molecular ion at $m/z = 355.25$ [M+Na]⁺ the majority of iso-T-like products of irradiation had an additional ion at $m/z = 387.15$ (Figure 3). This indicates either the addition of O₂ with formation of peroxide or hydroperoxide derivatives similar to those shown for iso-T3 (6E-9,10-secocholesta-5(10),6,8(14)-trien-3 β -ol)_[6], or oxidation of **4S** and **4R** without photolysis of the B ring, with production of endoperoxide and hydroperoxide, as was shown for 7-DHC.[8,10] The small scale of reaction and instability of isoT-like compounds prevented their more complete characterization.

Identification of novel L-like, D-like, and T-like compounds by NMR

The D-, L- or T-like irradiation products of **4R** and **4S** of defined UV and mass spectra were subjected to NMR analysis. Elucidation of the structures was based on ¹H-NMR data and selected 2D experiments (COSY, TOCSY). The detailed list of chemical shifts with an assignment of signals is shown in Table 2. The D-like (**4R-D** and **4S-D**), and L-like (**4R-L** and

4S-L) compounds were assigned based on expected chemical shifts for vinylic and methyl protons with the characteristic pattern. The main difference between NMR data for L-like compounds (**4R-L** and **4S-L**) and their respective parental compounds is a downfield shift of the methyl group at C19 (~0.20 ppm).

Although we were able to detect and initially characterize T-like and isoT-like compounds derived from **4R** and **4S**, these were not stable, which prevented their in-depth characterization.

Detection and characterization of novel triene-like products of UVB irradiation of **4R** and **4S**

In addition to well-characterized products of 5,7-diene irradiation (D-like, L-like, T-like and isoT-like compounds), we detected other products with UV absorption (λ_{max}) UV absorption suggests the presence of a triene system, presumable similar to cholesta-5,7,9(11)-trien-3 β -ol (9-DDHC), with reported λ_{max} at 324 nm.[9,10] Although irradiation of both **4R** and **4S** resulted in the formation of compounds with λ_{max} max above 300 nm, the process was more efficient from **4S** precursor (Figure 2). NMR analysis of this product confirmed the presence of 5,7,9(11)-triene system, and the product was identified as pregna-5,7,9(11)-triene-3 β ,17 α ,20S-triol (Figure 4), a compound that was previously reported.¹³ The chemical shifts we observe for **5S** (Table 2) are essentially identical (with small changes due to different solvent) to these earlier data.

Production of pregna-5,7-diene-3 β ,17 α ,20-triols (**4**) derivatives in human skin subjected to UV radiation (UVR)

Fragments of neonatal human skin (approximately, 5×5 mm) were treated with **4R** and **4S**, incubated in culture medium for 1-2 hours, washed and subjected to UV-irradiation as described in Experimental section. The products of irradiation were separated using RP-HPLC (Figure 5). Previously identified products of UV radiation of **4R** and **4S** in methanol were used as standards. Based on identical retention times we detected UVR induced production of L-like, D-like, T-like and preD-like compounds in human skin from **4R** (Figure 5A) and **4S** (Figure 5B). The formation of **4R** and **4S** secoderivatives was UVR dose dependent and the maximum production was observed at 2500 mJ/cm² (Figure 5; chromatograms 7 on panels A and B). These secosteroids were absent in the untreated human skin (Figure 5, chromatograms 2) or human skin exposed only to pregna-5,7-diene-3 β ,17 α ,20-triols without following UVR (Figure 5, chromatograms 3 on panels A and B). We also noted appearance of products corresponding to isoT-like derivatives with characteristic UV absorption (λ_{max} at 250 nm) and retention time around 2 minutes (not shown). Further identification of these products was not possible due to limited absorption of steroidal precursors by skin and subsequently relatively low production of secosteroids.

Inhibition of melanoma SKMEL-188 colony formation by pregna-5,7-diene-3 β ,17 α ,20-triols (**4R** and **4S**) and their D-like and L-like derivatives

The inhibition of melanoma SKMEL-188 colony formation by **4R** or **4S** and their D-like and L-like derivatives was used to assay their biological activity as described in the Experimental section. All studied compounds had antiproliferative properties as showed by inhibition of colony formation (Figure 6). When all colonies were counted, the average inhibition for the highest concentrations of tested compounds (100 nM) was 50-70%. The inhibitory effect was relatively lower ($\leq 50\%$) for small colonies with diameter below 20 nm and higher for colonies above 20 nm (50-80%) at 100 nM concentrations. Interestingly, the pregna-5,7-diene-3 β ,17 α ,20-triols precursors (**4R** and **4S**) were more efficient in growth inhibition than their photoderivative products (Figure 6 B and E). When comparing to 1,25(OH)₂D₃, (Fig. 6 A) we found that the precursor **4S** (Fig. E) was the most potent compound, while L-like compound **4R-L** showed the lowest activity (Fig. 6 D). Furthermore other compounds including **4S-D**, **4S-L** and **4R-D** had similar effects as 1,25-(OH)₂D₃.

Discussion

We are reporting for the first time an efficient, reproducible and detailed method for the UV-driven derivatization of two epimers (20R and 20S) of pregna-5,7-diene-3 β ,17 α ,20-triol (**4R** and **4S**). To our knowledge, this is the first description of vitamin D-like (**4S-D**, **4R-D**), L-like (**4S-L** and **4R-L**), T-like (**4S-T** and **4R-T**) derivatives of **4R** and **4S**. Careful observation of the dynamics of conversion reveal a rapid, UVB dose-dependent conversion of T-like compounds to iso-T, and their further oxidation. Longer UVB irradiation results in the synthesis of pregna-5,7,9(11)-triene-3 β ,17 α ,20S-triol (**5S**), its structure being confirmed by NMR. Although, chemical synthesis of **5S** was previously reported,[13] the role of UV in its formation has not been described. Interestingly, it was postulated that pregna-5,7,9(11)-triene-3 β ,17 α ,20S-triol is produced in humans because 21-deoxypregnane is almost exclusively reduced to 20S-hydroxyls.[14] In our hands, compound **4S** was more efficiently converted to triene by UVB irradiation, despite the reduction of compound **3** resulting in an almost 50-50 mixture of **4R** and **4S**. It was also shown that the synthesis of steroidal trienes has two potential intermediates of endoperoxide and hydroperoxide nature, and could be triggered by singlet oxygen in the presence of photosensitizing agents.[8,9,11] The 9-DDHC can also act as chromophore, stimulating the production of singlet oxygen and subsequent oxidation of 7DHC to 7-hydroxy-5,8(9)-cholestatriene, which undergoes decomposition to give 9-DDHC and hydrogen peroxide.[9] Such a self regenerating system producing singlet oxygen was also shown for ergosterol and its triene derivative.[25] Our data not only support such a model but also indicate for the first time that UV irradiation is sufficient for the auto-catalytic conversion of pregna-5,7-diene-3 β ,17 α ,20S-triol (**4S**) to pregna-5,7,9(11)-triene-3 β ,17 α ,20S-triol (**5S**), as is presented in Scheme 3; the presence of oxidized derivatives was confirmed by MS (Figure 3). Interestingly, formation of triene for 7DHC was observed only in the presence of photosensitizers [8-11,25] and extensive irradiation of 3 β -hydroxypregna-5,7-dien-20-one resulted in the production of oxidized iT-like compounds.[21] This suggests a correlation between the presence of two hydroxyls at C17 and C20 and UVR induced formation of the triene.

Since **4S** and **4R** and other androsta- and pregna-5,7-dienes derivatives were detected in living organisms under physiological and pathological conditions [10,13,14,26-30] our current data suggest (by inference) that they may be converted into corresponding secosteroids in the skin if exposed to solar radiation [31]. To test this hypothesis we performed *ex vivo* experiments with photolytic reactions conducted in human skin. Thus, using a physiologically relevant topical application of pregna-5,7-diene-3 β ,17 α ,20-triols with following *ex-vivo* incubation, washing of unincorporated products and UVR exposure, we were able to detect peaks with retention times corresponding to L-like, D-like, T-like and preD-like standards that were absent in negative controls. This indicates that under physiological conditions (skin) steroidal dienes without the side chain (pregna-5,7-diene-3 β ,17 α ,20-triols) undergo similar pathway of photochemical transformations to their corresponding secosteroidal products as in the model UVR induced transformation of 7-dehydrocholesterol to secosteroids containing the full side chain (vitamin D₃, tachysterol₃ and luminosterol₃) described by the Holick group [1,3]. These findings also substantiate future effort to study photolytic transformation of pregna-5,7-diene-3 β ,17 α ,20-triols in SLOS patients, since these were detected in body fluids of those patients [13,14].

Analog of vitamin D₃ without cholesterol-type side-chain have low calcemic activity [18, 32] and vitamin D₃ and its derivatives have broad spectrum of biological functions [2,16,33], including anti-carcinogenic properties in the skin (reviewed in [34,35]) and melanomas (reviewed in [36]). In the present studies we have shown that pregna-5,7-diene-3 β ,17 α ,20-triols and its D-like and L-like derivatives have clear tumorostatic activity as defined by their inhibition of colonies formation by human melanoma cells. Interestingly, 5,7-diene-3 β ,17 α ,

20-triols precursors showed the highest activity with **4S** being the most active, even more active than 1,25(OH)₂D₃, while L-like compound **4R-L** was the least potent. The other compounds showed similar activity to 1,25(OH)₂D₃. Thus, these studies may help to identify efficient drugs for adjuvant melanoma therapy including the described secosteroids and, interestingly, their precursor 5,7-diene-3 β ,17 α ,20-triols. Thus, newly generated dihydroxy derivatives, as described in this paper, may serve as promising candidates for the treatment of various pathological processes, including malignant melanoma, which is resistant to any type of therapy once metastatic process has started.[37]

In conclusion, we have characterized in details an *in vitro* UVB induced conversion of two epimers (20R and 20S) of pregna-5,7-diene-3 β ,17 α ,20-triol, identified and defined the structures of new products of this process, demonstrated that similar transformation can occur in the skin and demonstrated anti-melanoma activity of the substrates and secosteroidal products of the photoconversion.

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Figure 1. UVB- driven photolysis of 4S (as an example)

(A) RP-HPLC separation of 4S irradiation products after treatment with UVB for 15 minutes.

(B) Representative UV spectra of irradiated samples. Peak number (Panel A) corresponds to predicted structure based on UV spectra (Panel B). Peaks were assigned as follows: 1-4, 10, 11 – isoT-like and oxidized isoT-like; 5 – 5S; 6 – 4S-L; 7 – 4S-D; 8 – 4S; 9 – 4S-T; 12 – 4S-pD. The UV spectra for RP-HPLC (Panel A) were measured at 280 nm.

Representative UV spectra of irradiated samples of 4R (c) and 4S (d). (1 – 5c, 2-5cD, 3 – unknown product, 4 – 5cT, 5 – 5c1, 6 – unknown product).

Figure 2.

Dose (time of irradiation) dependence of prenat-5,7-diene-3 β ,17 α ,20-triols (**4R** and **4S**) photo-conversion. The compound **4R** (Panels A, C, E and G) or **4S** (Panels B, D, F and H) was irradiated for 5 (A,B), 15 (C,D), 30 (E,F) or 60 (G,H) minutes and products were separated by using RP-HPLC. The absorption profiles of products were recorded by using diode-array detector. The samples were separated after incubation for 2 hours at room temperature.

Figure 3. Identity of oxidized derivatives of **4R** (A) and **4S** by mass spectrometry. Sample were purified after UV treatment using RP-HPLC and analyzed with LC-MS.

Figure 4. Identification of pregna-5,7,9(11)-triene-3β,17α,20S-triol (A) 500 MHz proton NMR (B) COSY showing correlation from 11-CH to 12a-CH₂, 20-H to 21-CH₃ and 3-CH to 4-CH₂ and 2CH₂. Additionally, correlation from 7-CH to C-14 and from 14-CH to 15CH₂. Impurities are marked with asterisk.

Figure 5. *Ex vivo* production of pregnane-5,7-diene-3 β ,17 α ,20-triols (4R** and **4S**) derivatives in human neonatal skin**

Skin fragments were treated with 2 μ L of 10 mM of **4R** (Panel A) or **4S** (Panel B) and subjected to UV irradiation. The products of photolysis in skin were subjected to RP-HPLC chromatography (chromatograms 2-7) and their retention times were compared with previously identified products of UV irradiation of **4R** and **4S** in methanol (chromatogram 1). Different UV doses were used to show dynamics of the process. The UVB doses per cm² were as follows: chromatograms 1 and 7 – 2500 mJ/cm², 3 – 0 mJ/cm², 4 – 100 mJ/cm², 5 – 500 mJ/cm², 6 – 1000 mJ/cm². Chromatograms 2 show normal profile of non-treated and non-irradiated skin.

Figure 6.

Inhibition of colony formation of melanoma SKMEL-188 treated with pregna-5,7-diene-3 β , 17 α ,20-triols **4R** (B) and **4S** (E) and their D-like and L-like derivatives: **4R-D** (C), **4R-L** (D), **4S-D** (F) and **4S-L** (G).

An active form of vitamin D₃ 1,25(OH)₂D₃ was used as positive control (A). The inhibition of melanoma SKMEL-188 colony formation was used to assay biological activity of newly synthesized compounds at 0.1, 10 and 100 nM concentrations. The average value (n=6) for ethanol treated cells was used as a 0 nM on all graphs. The total number of colonies and their diameters with cutoff below and above 20 nm was measured and expressed in colony forming units (CFU). Data are presented as mean $\pm$ SEM for colony-forming assay (n=4 to 6); *P<0.05,

** $P < 0.005$, and *** $P < 0.001$. Representative pictures of SKMEL treated with studied compounds and the vehicle control are shown (H).

Scheme 1.
Photolysis of cholesta-5,7-diene-3 β -ol.

Scheme 2. Synthesis of 4R and 4S.^a

^a Reagents and conditions: (a) Ac_2O , microwave, p-toluenesulfonic acid monohydrate; (b) dibromantin, 2,2'-azobisisobutyronitrile, benzene/hexane (1:1), 100°C, reflux; (c) Bu_4NBr , Bu_4NF , THF, room temperature; (d) LiAlH_4 , THF, 0°C.

Scheme 3.
Photolysis of pregna-5,7-diene-3 β ,17 α ,20-triols.

Scheme 4. Putative model of UVR induced generation of pregnane-5,7,9(11)-triene-3β,17α,20S-triol
 (1) Singlet oxygen initiates photooxidation of **4S** or **4R**, with formation of endoperoxide and hydroperoxide.
 (2) UV stimulates H_2O_2 and triene formation.
 (3) Triene acts as a photosensitizer for singlet oxygen generation.
 (4) The source of initial singlet oxygen is uncertain, but possibly it is formed from O_2 during irradiation.

Table 1
UV and MS spectra data for pregna-5,7-diene-3 β ,17 α ,20-triols and its derivatives

No	Parental compound	Structure type	UV max (nm)	Predicted MW	MS+
4R	1	5,7-diene	261,270,281,290	332.25	355.25 [M+Na]⁺
<i>4R-I</i>	<i>4R</i>	<i>preD-like</i>	<i>260</i>	<i>332.25</i>	<i>ND</i>
4R-D	4R	D-like	265	332.25	355.25 [M+Na]⁺
4R-L	4R	L-like	262,271,281	332.25	355.25 [M+Na]⁺
<i>4R-T</i>	<i>4R</i>	<i>T-like</i>	<i>270,279,291</i>	<i>332.25</i>	<i>355.25 [M+Na]⁺</i>
<i>4R-IT</i>	<i>4R</i>	<i>isoT-like (oxide)</i>	<i>240,248, 257</i>	<i>364.25</i>	<i>387.15 [M+Na]⁺</i>
<i>4R7</i>	<i>4R</i>	<i>5,7,9(11) triene</i>	<i>312,323,340</i>	<i>330.22</i>	<i>ND</i>
4S	1	5,7-diene	263,271,282,292	332.25	355.25 [M+Na]⁺
<i>4S-I</i>	<i>4S</i>	<i>preD-like</i>	<i>260</i>	<i>332.25</i>	<i>ND</i>
4S-D	4S	D-like	265	332.25	355.25 [M+Na]⁺
4S-L	4S	L-like	262,271,282	332.25	355.25 [M+Na]⁺
<i>4S-T</i>	<i>4S</i>	<i>T-like</i>	<i>272,282,291</i>	<i>332.25</i>	<i>355.25 [M+Na]⁺ND</i>
<i>4S-IT1</i>	<i>4S</i>	<i>isoT-like</i>	<i>241,248, 257</i>	<i>332.25</i>	<i>355.25 [M+Na]⁺</i>
<i>4S-IT2</i>	<i>4S</i>	<i>isoT-like (oxide)</i>	<i>241,248, 257</i>	<i>332.25</i>	<i>387.15 [M+Na]⁺</i>
<i>4S7</i>	<i>4S</i>	<i>5,7,9(11) triene</i>	<i>312,323,340</i>	<i>330.22</i>	<i>ND</i>

Bold – purified and characterized (NMR), *italics* – characterized by UV spectra, ND – not determined

Table 2
¹H NMR chemical shifts of pregna-5,7-diene-3 β ,17 α ,20-triols, D-like, L-like and 5,7,9(11)-triene

	4R	4S	4R-D	4S-D	4R-L	4S-L	5S
1 CH ₂	α 1.31 β 1.90	α 1.29 β 1.90	α 2.12 β 2.42	α 2.12 β 2.41	α 1.31 β 1.90	α 1.31 β 1.90	1.48 1.70
2 CH ₂	α 1.92 β 1.45	α 1.93 β 1.54	α 1.97 β 1.68	α 1.97 β 1.68	α 1.92 β 1.54	α 1.92 β 1.54	1.89 1.68
3 CH	3.51	3.51	3.76	3.76	4.03	4.03	3.47
4 CH ₂	α 2.41 β 2.23	α 2.40 β 2.23	α 2.55 β 2.19	α 2.54 β 2.19	α 2.41 β 2.29	α 2.44 β 2.35	2.34 2.42
6 CH	5.54	5.54	6.23	6.24	5.59	5.54	5.65
7 CH	5.42	5.39	6.09	6.09	5.47	5.39	5.40
9 CH	2.11	2.02	2.85	2.85	2.02	2.02	
11 CH ₂	α 1.67 β 1.75	α 1.6-1.8 β 1.6-1.8	α 1.77 β 1.77	α 1.77 β 1.77	α 1.7-1.8 β 1.7-1.8	α 1.7-1.8 β 1.7-1.8	5.61
12 CH ₂	α 1.49 β 1.80	α 1.49 β 2.12	α 1.54 β 2.05	α 1.56 β 2.04	α 1.49 β 2.18	α 1.49 β 2.18	2.61 2.14
14 CH	2.62	2.57	2.12	2.12	2.05	2.05	2.78
15 CH ₂	α 1.83 β 1.63	α 1.84 β 1.56	α 1.54 β 2.53	α 1.61 β 1.61	α 1.82 β 1.51	α 1.82 β 1.51	1.85 1.50
16 CH ₂	α 1.56 β 1.74	α 1.7-1.8? β 1.9?	α 1.87 β 2.7	α 1.7 β 2.66	α 1.76 β 2.22	α 1.76 β 2.22	1.60 1.75
18 CH ₃	0.7	0.77	0.6	0.67	0.76	0.76	0.67
19 CH ₃	0.94	0.95	α 4.76 β 5.05	α 4.74 β 5.05	0.67	0.75	1.24
20 C	3.78 CH	3.94 CH	3.76	3.61	3.72 CH	3.65 CH	3.9
21 CH ₃	1.19	1.15	1.18	1.14	1.14	1.15	1.16

Italics - not fully determined presumably similar to parental compound