

# Novel vitamin D photoproducts and their precursors in the skin

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Novel metabolic pathways initiated by the enzymatic action of CYP11A1 on 7DHC (7-dehydrocholesterol), ergosterol, vitamins D<sub>3</sub> and D<sub>2</sub> were characterized with help of chemical synthesis, UV and mass spectrometry and NMR analyses. The first pathway follows the sequence 7DHC → 22(OH)7DHC → 20,22(OH)<sub>2</sub>7DHC → 7DHP (7-dehydropregnolone), which can further be metabolized by steroidogenic enzymes. The resulting 5,7-dienes can be transformed by UVB to corresponding, biologically active, secosteroids. Action of CYP11A1 on vitamin D<sub>3</sub> and D<sub>2</sub> produces novel hydroxyderivatives with OH added at positions C17, C20, C22, C23 and C24, some of which can be hydroxylated by CYP27B1 and/or by CYP27A1 and/or by CYP24A1. The main products of these pathways are biologically active with a potency related to their chemical structure and the target cell type. Main products of CYP11A1-mediated metabolism on vitamin D are non-calcemic and non-toxic at relatively high doses and serve as partial agonists on the vitamin D receptor. New secosteroids are excellent candidates for therapy of fibrosing, inflammatory or hyperproliferative disorders including cancers and psoriasis.

## New Secosteroidal Systems

**An overview.** While defining neuroendocrine activities of the skin,<sup>1–3</sup> we discovered novel metabolic pathways initiated by the enzymatic action of cytochrome P450ccc (CYP11A1) on 7-dehydrocholesterol (7DHC; pro-vitamin D<sub>3</sub>),<sup>4–6</sup> ergosterol,<sup>7,8</sup> vitamin D<sub>3</sub><sup>9–16</sup> and vitamin D<sub>2</sub>.<sup>17,18</sup> These substrates have structural similarity to the well characterized substrate for CYP11A1, cholesterol, where cleavage of its side chain between C20 and C22 producing pregnenolone represents the initial reaction in the synthesis of steroid hormones.<sup>19,20</sup> We have now established that P450ccc can hydroxylate vitamin D<sub>3</sub> in a sequential manner at positions C17, C20, C22 and C23 to produce 20-hydroxyvitamin D<sub>3</sub> (20(OH)D<sub>3</sub>), 20,23(OH)<sub>2</sub>D<sub>3</sub>, 20,22(OH)<sub>2</sub>D<sub>3</sub> and 17,20,23(OH)<sub>3</sub>D<sub>3</sub> as the main products, with additional production of 17(OH)

D<sub>3</sub>, 22(OH)D<sub>3</sub>, 23(OH)D<sub>3</sub> and 17,20(OH)<sub>2</sub>D<sub>3</sub>. Some of these products can be hydroxylated, by CYP27B1<sup>15,16</sup> to produce 1,20(OH)D<sub>3</sub> and 1,20,23(OH)<sub>3</sub>D<sub>3</sub>, by CYP27A1<sup>14</sup> to produce 20,25(OH)<sub>2</sub>D<sub>3</sub> or 20,26(OH)<sub>2</sub>D<sub>3</sub> or by CYP24A1<sup>21</sup> to produce 20,24(OH)<sub>2</sub>D<sub>3</sub> or 20,25(OH)<sub>2</sub>D<sub>3</sub>. In addition, products of P450ccc activity on vitamin D<sub>2</sub> include 20(OH)D<sub>2</sub>, 17,20(OH)<sub>2</sub>D<sub>2</sub> and 17,20,24(OH)<sub>3</sub>D<sub>2</sub><sup>17,18</sup> with 20(OH)D<sub>2</sub> also being hydroxylated by CYP27B1.<sup>22</sup> The main products of these pathways, are biologically active with a potency defined by their chemical structure and the cell lineage.<sup>21–31</sup>

Cleavage of the side chain of 7DHC generates 7-dehydropregnolone (7DHP), which can be further metabolized by classical steroidogenic enzymes to produce 5,7-diene steroids that are identifiable in pathological conditions [Smith Lemli Opitz syndrome (SLOS)<sup>32,33</sup>], or ex vivo in adrenal glands,<sup>5</sup> placenta or keratinocytes<sup>6</sup> incubated with 7DHC. These 5,7-diene steroids can be converted by UV B radiation (UVB) to androsta-calciferols (aD) and pregra-calciferols (pD), i.e., vitamin D compounds with a short or absent side chain, which are indeed biologically active in skin cells.<sup>34–38</sup>

In this review we will focus on Δ7 steroids and photolytically generated secosteroids, and describe their biological activity and methods for their synthesis. The review will be complemented by a description of Δ7 steroidogenic pathways.

**General overview of Δ7 steroidogenic pathways.** *Studies on the metabolism of Δ7-steroids by purified enzymes, organelles and tissue fragments.* 7DHC can serve as the substrate for the synthesis of a range of Δ7 steroids including Δ7 pregnenes, androgens and estrogens. Pathways leading to Δ7-steroids have been elucidated in part by studies with purified enzymes, subcellular organelles and tissue fractions, and in part from the analysis of steroids present in patients with Smith-Lemli-Opitz Syndrome (SLOS).

Steroidogenesis in tissues such as the adrenal cortex and gonads starts with the transport of cholesterol from the outer to the inner mitochondrial membrane mediated by the Steroidogenic Acute Regulatory (StAR) protein.<sup>20</sup> This transfer represents the rate-limiting step in the synthesis of progesterone, androgens or corticosteroids in these tissues and is regulated by the tropic hormones.<sup>20</sup> Purified StAR protein can mediate the transfer of 7DHC between phospholipid vesicle membranes with

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comparable efficiency to that for cholesterol.<sup>5</sup> The addition of purified StAR protein to placental mitochondria resulted in a 7.5-fold stimulation of 7DHC metabolism by the mitochondria with the rate being higher than that for cholesterol metabolism under similar conditions.<sup>6</sup> It is therefore clear that the StAR protein can efficiently transport 7DHC to the inner mitochondrial membrane for steroid synthesis.

The ability of CYP11A1 to catalyze the removal of the side chain of 7DHC and thus initiate  $\Delta 7$ -steroidogenic pathways starting from 7DHC is now well established.<sup>4-6,39</sup> Studies with purified bovine and human CYP11A1 have shown that both can catalyze the side chain cleavage of 7DHC producing 7-dehydro-pregnenolone (7DHP), with a catalytic efficiency slightly higher than that for cholesterol conversion to pregnenolone.<sup>4</sup> Conversion of 7DHC to 7DHP by human placental mitochondria has also been demonstrated, with inhibition by 22R-hydroxycholesterol, a tight-binding CYP11A1 intermediate,<sup>19</sup> clearly demonstrating that the reaction is mediated by CYP11A1.<sup>6</sup> Fragments of rat, dog, pig and rabbit adrenal glands incubated with 7DHC ex vivo also converted 7DHC to 7DHP as did mitochondria from rat skin.<sup>5</sup>

Conversion of 7DHC to 7DHP by human CYP11A1 is accompanied by accumulation of the two reaction intermediates, 22-hydroxy-7DHC [22(OH)7DHC] and 20,22-dihydroxy-7DHC [20,22(OH)<sub>2</sub>7DHC]. These intermediates accumulate well above the concentration of CYP11A during incubations with 7DHC and therefore should be considered as reaction products, not enzyme-bound reaction intermediates.<sup>6</sup> Other minor, monohydroxylated 7DHC derivatives that remain to be identified are also produced in this reaction.<sup>6</sup> Production of (20,22(OH)<sub>2</sub>7DHC) and other mono-hydroxylated products was also observed in incubations of human placental mitochondria with 7DHC,<sup>6</sup> as well as for adrenal fragments from rat, dog, rabbit and pig incubated with 7DHC ex vivo.<sup>5</sup>

Another  $\Delta 7$  steroid found in mammals which is of dietary origin is ergosterol, a fungal sterol which is the precursor of vitamin D.<sup>40,41</sup> This sterol can be metabolised by CYP11A1 but unlike cholesterol and 7DHC, no cleavage of the side chain occurs.<sup>7,8</sup> While the products cannot enter the classical steroidogenic pathways, some of the products do display antiproliferative activity on skin cells.<sup>8</sup> Human CYP11A1 catalyzes both epoxidation and hydroxylation of the ergosterol side chain producing 20-hydroxy-22,23-epoxy-22,23-dihydroergosterol as a major metabolite.<sup>7</sup>

Studies with partially purified 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta 5\text{-}4$  isomerase (3 $\beta$ HSD) type 1 from the human placenta show that this enzyme can act on 7DHP with a catalytic efficiency 40% of that measured for pregnenolone. The lower efficiency was largely due to an increase in the  $K_m$  for substrate.<sup>5</sup> The product of 3 $\beta$ HSD action on 7DHP was identified as 7-dehydroprogesterone (4,7-pregnadien-3-20-dione) by collecting the product from incubations of placental microsomes with 7DHP and subjecting it to structural analysis by mass spectrometry and NMR.<sup>6</sup> 7-Dehydroprogesterone proved to be unstable, with considerable conversion to several unidentified products on storage at room temperature for a few hours. 7-Dehydroprogesterone was also produced by adrenal fragments from several mammalian

species, including the pig, incubated with 7DHC or 7DHP.<sup>5,6</sup> Pig adrenal fragments also produced a metabolite tentatively identified as 17-hydroxy-7DHP, suggestive of CYP17A1 action on 7DHP.

**$\Delta 7$  Estrogen synthesis and metabolism in the pregnant mare.** The urine and blood of the pregnant mare contain a high concentration of the B-ring unsaturated estrogen, equilin [3-hydroxy-1,3,5(10),7-estratetraen-17-one] and 17-dihydroequilin, both of which contain a  $\Delta 7$  double bond, and 17-dihydroequilinin [3 $\beta$ ,17-dihydroxy-1,3,5(10),6,8-estrapentaen], which contains double bonds at carbons 6 and 8 of the B-ring.<sup>42,43</sup> The former two can be made by the equine placenta incubated with 5,7-androstadiene-3 $\beta$ ,17 $\beta$ -diol, in a pathway proposed to involve 17 $\beta$ HSD, 3 $\beta$ HSD and CYP19A1 (aromatase). Dihydroequilinin appears to arise from dehydrogenation of dihydroequilin.<sup>44</sup> In parallel studies on microsomes from the horse and human placenta, both were able to convert the  $\Delta 7$  androgen, 3-hydroxy-3,5,7-androstanetri-en-17-one to equilin,<sup>45</sup> illustrating the ability of both human and equine CYP19A1 to act on  $\Delta 7$  substrates. The perfused human placenta also made equilin and 17 $\beta$ -dihydroequilin from 3 $\beta$ -hydroxy-5,7-androstadien-17-one (7-dehydro-DHEA).<sup>46</sup>

The androgen precursors for the synthesis of B-ring unsaturated estrogens in the pregnant mare are from the fetal gonads. 7-Dehydro-DHEA has been identified in the gonadal veins of fetal ovaries and testes *in situ*.<sup>47</sup> The horse fetal gonad has been shown to convert 7DHC to 7DHP and then to 7-dehydro-DHEA. Thus, the equine feto-placental unit displays a complete  $\Delta 7$  steroidogenic pathway from 7DHC right through to estrogens.<sup>42,44,47</sup> The high concentration of B-ring unsaturated estrogens in the pregnant mare compared with other mammalian species appears to arise from the ability of the fetal gonads to produce a high proportion of  $\Delta 7$  androgens, perhaps reflecting limited 7DHC reductase activity. The pathways in the pregnant mare provide strong evidence for the ability of all the enzymes in the estrogen biosynthetic pathway, including CYP17A1 and aromatase, to work on the  $\Delta 7$  isoforms of their substrates.

***Pathways of 7DHC metabolism indicated by studies on Smith-Lemli-Opitz Syndrome patients.*** Smith-Lemli-Opitz Syndrome (SLOS) is caused by a deficiency in the enzyme 7-dehydrocholesterol reductase (DHCR7) which catalyzes the last step in cholesterol synthesis, the conversion of 7DHC to cholesterol.<sup>48</sup> This syndrome is associated with decreased cholesterol levels and markedly elevated 7DHC levels. The  $\Delta 7$  steroids identified in the urine of these patients provide a good indicator of the ability of various steroidogenic enzymes to act on  $\Delta 7$  steroids.<sup>33,49</sup> Interestingly, corresponding  $\Delta 8$  steroids are also observed in these samples indicating conversion of  $\Delta 7$  steroids to  $\Delta 8$  steroids occurs in these patients, either enzymatically or non-enzymatically. The identification of 5,7-pregnadiene-3 $\beta$ ,17 $\alpha$ ,20 $\alpha$ -triol and other 17 $\alpha$ -hydroxy-7DHP derivatives in SLOS patients clearly supports the above studies with purified enzymes, organelles and tissue fractions demonstrating the sequential involvement of the StAR protein and CYP11A1 in  $\Delta 7$  steroid synthesis.

The finding that many of the  $\Delta 7$  steroids identified in SLOS patients possess a 17 $\alpha$ -hydroxyl group indicates that CYP17A1 displays 17 $\alpha$ -hydroxylase activity on 5,7-pregnadien. Many of

the products including  $5\beta$ -pregn-7-ene- $3\alpha,17\alpha,20\alpha$ -triol and  $5,7$ -pregnadiene- $3\beta,17\alpha,20\alpha$ -triol and other  $17\alpha$ -hydroxy 7DHP derivatives possess a  $20\alpha$ -hydroxy group. Thus the involvement of  $20\alpha$ -hydroxysteroid dehydrogenase in converting the ketone group at C20 of 7DHP and/or 17-hydroxy7DHP is indicated.  $\Delta 7$ -Androgens (and  $\Delta 8$  analogs) such as 7-dehydro-DHEA and  $5,7$ -androstene- $3\beta,17\beta$ -diol, have also been identified in the urine of SLOS patient indicating that CYP17A1 can catalyze the C17-C20 lyase reaction on 17-hydroxy  $\Delta 7$  steroids. The  $\Delta 7$  17-hydroxy products accumulate relative to the usual steroids (lacking  $\Delta 7$  unsaturation) more than the  $\Delta 7$  androgens accumulate, suggesting that CYP17A1 displays higher hydroxylase activity than lyase activity on  $\Delta 7$  C21 steroids.<sup>33</sup>

No  $\Delta 7$  steroids with a 21-hydroxyl group or an  $11\beta$ -hydroxy group have been identified in the urine of SLOS patients.<sup>33</sup> Thus it would appear that neither CYP21A2, CYP11B1 nor CYP11B2 can act on  $\Delta 7$  steroids. Interestingly, no steroids with a 4,7-diene structure as in 7-dehydroprogesterone have been identified in SLOS patients. As mentioned above, we have found that this  $\Delta 7$  steroid is unstable,<sup>6</sup> which could potentially prevent the isolation and identification of it or its hydroxy derivatives in the urine of SLOS patients. It is likely to be unstable in vivo as well as in vitro, with the possibility that the resulting non-enzymatic products have adverse physiological effects in SLOS. C21 steroids with only a double bond in the  $\Delta 7$  position such as  $5\beta$ -pregn-7-ene- $3\alpha,17\alpha,20\alpha$ -triol have been isolated from SLOS patients. These are believed to have arisen from 7-dehydroprogesterone (or its derivatives that retain the 3-oxo-4,7-diene structure) since the action of  $5\beta$ -reductase and  $3\alpha$ -hydroxysteroid dehydrogenase requires the 3-oxo- $\Delta 4$  structure.<sup>50</sup>

Estriol levels are low in women pregnant with SLOS-affected fetuses, presumably due to decreased cholesterol synthesis. 7-Dehydroestrogens, especially  $16\alpha$ -hydroxy- $17\beta$ -dihydroequilin (7-dehydroestriol) and the 8-dehydro isomer have been isolated from the urine of a woman carrying a SLOS fetus, further supporting that human CYP19A1(aromatase) can convert  $\Delta 7$ -androgens to  $\Delta 7$ -estrogens.<sup>48,49,51</sup>

In summary, studies with purified enzymes, organelles and tissues, together with studies on equilin synthesis by pregnant mares and on  $\Delta 7$  steroids in SLOS patients, clearly demonstrate that 7DHC can be converted to 7DHP, 17-hydroxy-7-DHP, 7-dehydroprogesterone, 7-dehydro-DHEA and the estrogens equilin and 17-dihydroequilin, by the same steroidogenic enzymes involved in the classical steroidogenic pathways. No  $\Delta 7$  corticosteroids have been detected suggesting that C21 steroids with a  $\Delta 7$  double bond cannot serve as substrates for CYP21A2. Studies with pregnant mares and SLOS patients suggest that the availability of  $\Delta 7$  -substrates, such as 7DHC and 7-dehydro-DHEA determines the flux through  $\Delta 7$  pathways. An overview of  $\Delta 7$  steroidogenic pathways with the enzymes involved and major product is shown in Figure 1.

**Local  $\Delta 7$  steroidogenesis in the skin.** Skin is the largest organ with powerful metabolic and neuroendocrine activities,<sup>2,52,53</sup> and has a relatively high content of 7DHC which serves as the precursor for vitamin D<sub>3</sub> formation.<sup>40,41</sup> Although skin expresses CYP11A1 at relatively low levels, it has been predicted to be a

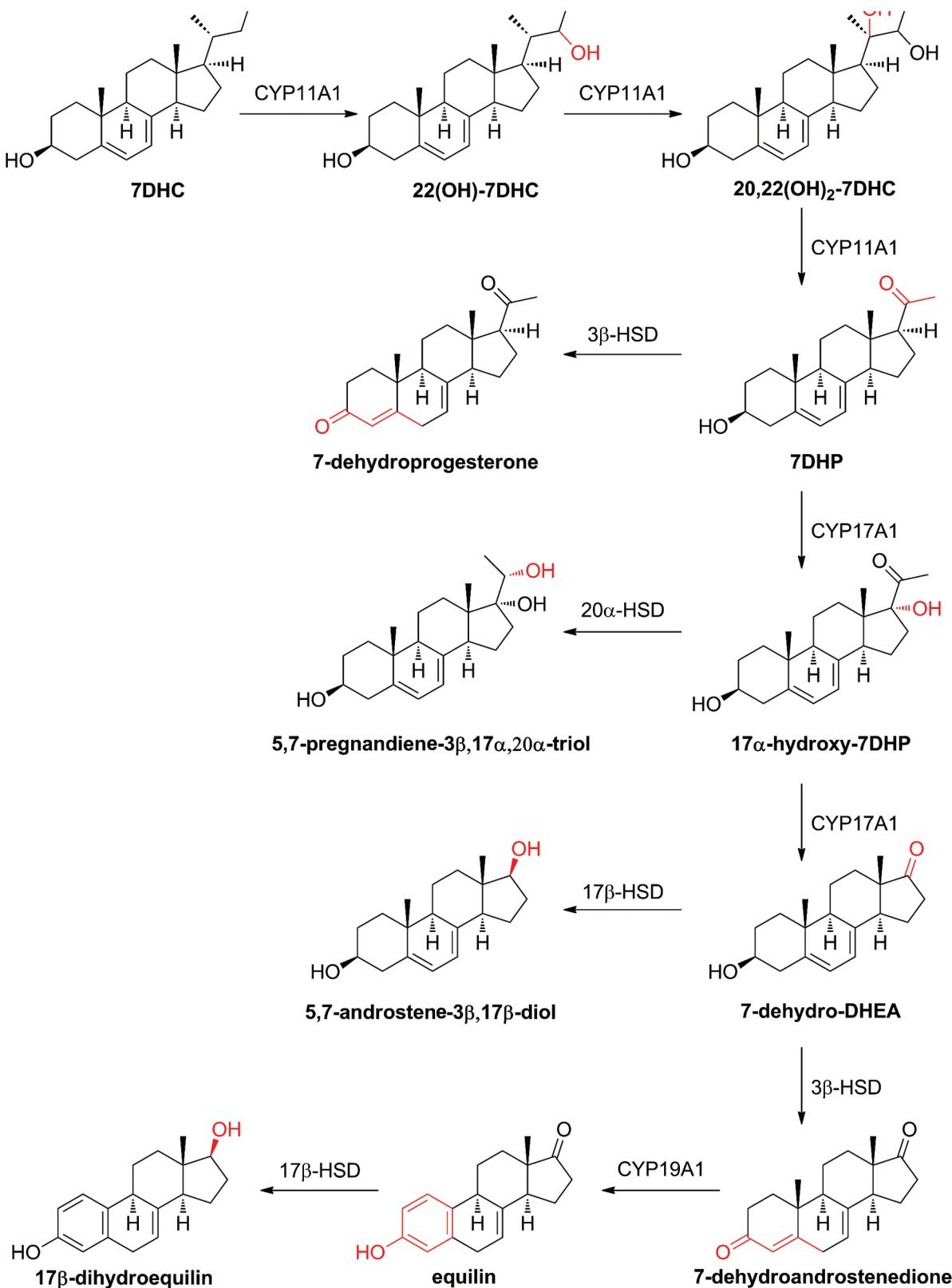
site of  $\Delta 7$  steroid synthesis under non-pathological conditions because of the availability of 7DHC to this enzyme.<sup>4</sup> The first support for this hypothesis came from the incubation of mitochondria isolated from rat skin with 7DHC, which showed that 7DHC is metabolized to 7DHP.<sup>5</sup> Interestingly, rat skin extracts metabolized 7DHP to a more polar  $5,7$ -diene (most likely a hydroxyderivative), while the skin microsomal fraction transformed 7DHP to two products with modified A and B rings, presumably one of them representing a 4,6-diene.<sup>5</sup>

Recently we provided evidence for this novel cutaneous pathway using cultured epidermal keratinocytes and LC/MS techniques.<sup>6</sup> Specifically, immortalized human HaCaT keratinocytes transformed exogenously added 7DHC to  $22(\text{OH})7\text{DHC}$ ,  $20,22(\text{OH})_27\text{DHC}$  and 7DHP.<sup>6</sup> In addition, epidermal keratinocytes from pig skin which contain high concentration of endogenous 7DHC, were able to produce  $22(\text{OH})7\text{DHC}$ ,  $20,22(\text{OH})_27\text{DHC}$  and 7DHP without the addition of exogenous substrate.<sup>6</sup> These studies suggest that skin can metabolize 7DHC, although at a low level, with the following sequence 7DHC  $\rightarrow$   $22(\text{OH})7\text{DHC} \rightarrow 20,22(\text{OH})_27\text{DHC} \rightarrow 7\text{DHP} \rightarrow$  7-dehydroprogesterone and hydroxy 7DHP. Possible production of 7-dehydroprogesterone and hydroxy 7DHP is indicated by the expression of  $3\beta\text{HSD}$  and other steroidogenic enzymes in the skin,<sup>54-56</sup> as well as the detection of 7DHP metabolism by rat skin extracts or microsomes.<sup>5</sup> Such cutaneous  $5,7$ -dienes can be photolytically transformed to the corresponding secosteroids. However, isomerisation to a 4,7-diene as in 7-dehydroprogesterone or to a 4,6-diene, which could potentially occur in the skin, would prevent such phototransformation.

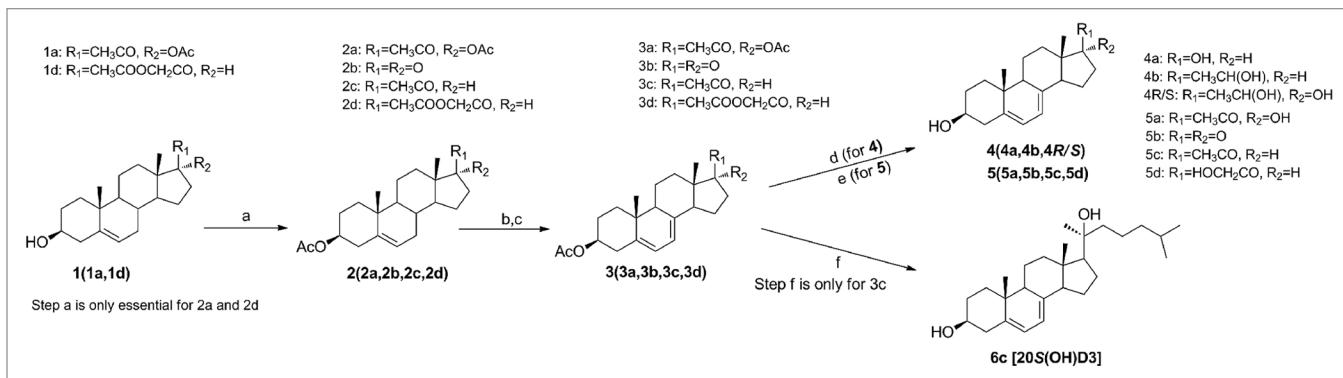
**Chemical synthesis of steroidal 5,7-dienes.** In order to confirm the chemical nature of the isolated metabolites from tissues as well as to test their biological activity several  $5,7$ -diene precursors of secosteroid analogs were synthesized as outlined in Figure 2 and reported previously.<sup>27,34-36,38</sup> Generally, compounds **1(a, d)** were protected by acetylation at C-3 to give compounds **2(a-d)** to be later brominated at the C-7 position followed by dehydrobromination to afford compounds **3(a-d)** with an additional double bond at C7-C8. Compounds **3(a-d)** were used for the generation of the subsequent products **4(a, b, R/S), 5(a-d)** and **6c** under various reaction conditions.

The synthesis of  $20R(\text{OH})7\text{DHC}$  is shown in Figure 3. As described in our previous publication<sup>57</sup> pregnenolone acetate was oxidized to etienic acid **8** by freshly prepared NaOBr from bromine and sodium hydroxide solution. Compound **8** was converted to the Weinreb amide **9** in the presence of HBTU/DIPEA, and HNMeOMe•HCl salt. The  $3\beta$ -hydroxyl group of compound **9** was protected by tERT-butylidiphenylsilylation with TBDPSCl to yield compound **10**. The Weinreb amide moiety of compound **10** was then reacted with (4-methylpentyl)magnesium bromide to produce compound **11**. Removal of the TBDPS group from compound **11** followed by acetylation generated compound **12**. Bromination-dehydrobromination of compound **12** provided the  $5,7$ -diene **13**. Finally, compound **13** was reacted with methylmagnesium bromide to achieve  $20R(\text{OH})7\text{DHC}$  (**14**).

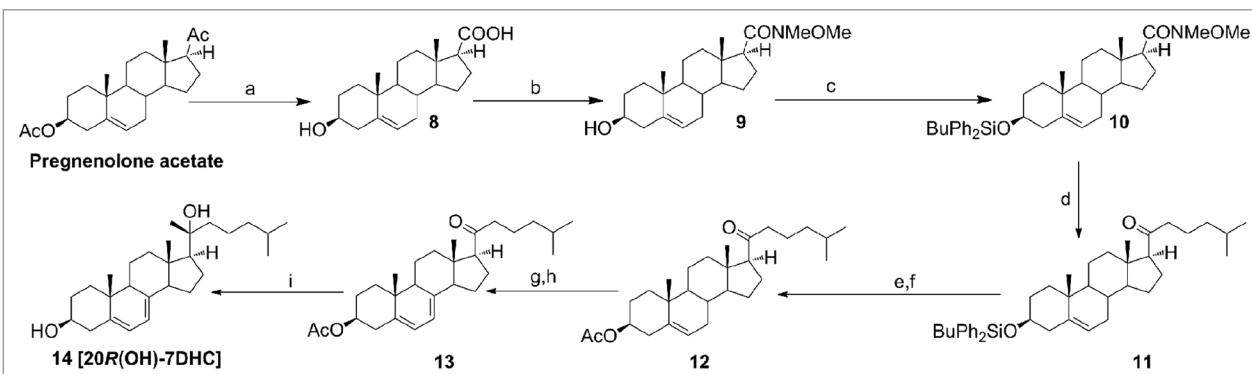
The plausible synthetic route of  $20(\text{OH})\text{ergosterol}$  is shown in Figure 4.<sup>22</sup> The 2,3-dimethylbutyraldehyde **16** will be converted



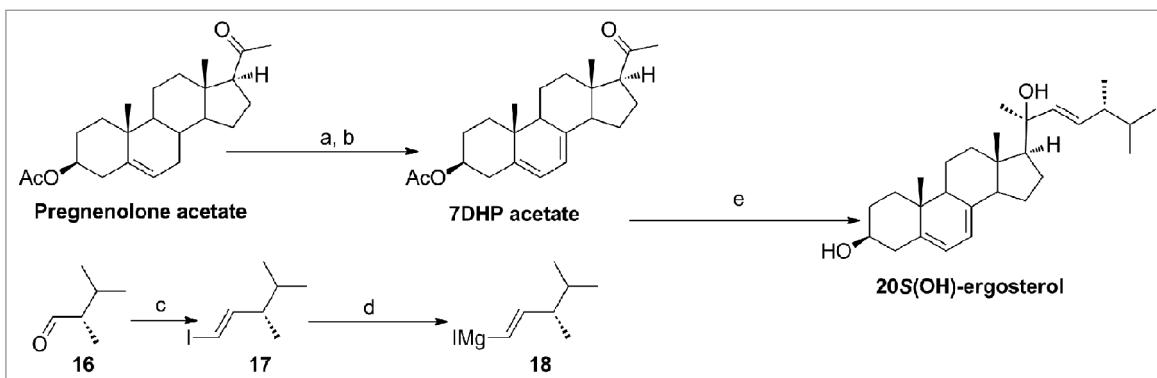
**Figure 1.**  $\Delta 7$  Steroidogenic pathways with the enzymes involved and major products.



**Figure 3. The synthesis of 20R(OH)-7DHC.**



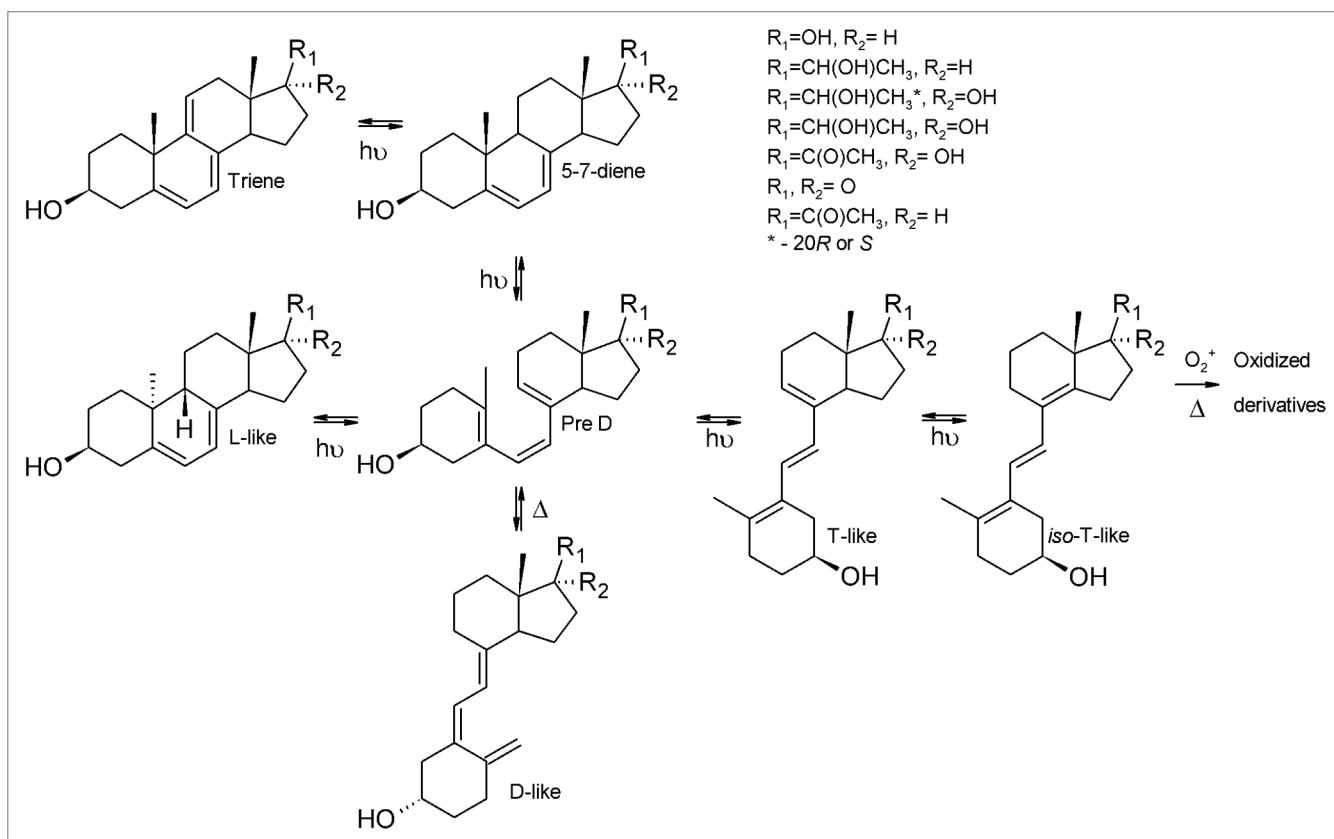
**Figure 4. The plausible synthetic route of 20(OH)ergosterol.**



to the iodo compound **17** followed by reacting with magnesium to produce the Grignard reagent **18**.<sup>58</sup> The coupling of 7DHP acetate with the Grignard reagent **18** will provide 20(OH) ergosterol.

**UVB-induced transformation of steroid 5,7-dienes to secosteroids (Fig. 5).** The UVB-induced photolysis of the B-ring of 7DHC is one of the most fundamental reactions in photobiology.<sup>40,59,60</sup> It was also shown that other 5,7-dien-3 $\beta$ -ols can also

be transformed to the corresponding secosteroids, generating a new family of secosteroids (Fig. 5, pro-D)<sup>36,38,61,62</sup> (Table 1). The initial fast UVB-dependent reaction to break the B ring in 7DHC is followed by a relatively slow isomerization of pre-D<sub>3</sub> to D<sub>3</sub>-like, tachysterol-like and lumisterol-like analogs,<sup>59</sup> with similar reactions being involved in the generation of novel secosteroids from their intact B-ring precursors<sup>36,38,61,62</sup> (Fig. 5). Prolonged UVB-exposure of 7DHC may lead to further transformation



**Figure 5.** UVB-induced transformation of steroidal 5,7-dienes to secosteroids.

into 5,6-transvitamin D<sub>3</sub>, suprasterol I and suprasterol II.<sup>63</sup> On the other hand, isomerization of tachysterol-like compounds may lead to isotachysterol-like compounds and their peroxide or hydroperoxide derivatives.<sup>38</sup> Furthermore, UV irradiation is sufficient for the auto-catalytic conversion of 5,7-dien-3 $\beta$ -ols with or without a short side chain into the corresponding 5,7,9(11)-trien-3 $\beta$ -ols.<sup>61</sup>

#### Biological Activity of $\Delta$ 7 Secosteroids with a Short Side Chain

**Cells of epidermal origin.** *Normal keratinocytes and melanocytes.* Having established chemical/photochemical routes of synthesis for steroids/secosteroids with a short (2C) side chain or no side chain at all at C17, we tested their biological activity in normal skin cells. Specifically, 7DHP and pregnenolone inhibited the proliferation of epidermal HaCaT keratinocytes in a dose-dependent fashion with similar potency, while 20-oxopregnacalciferol (pD) was less potent.<sup>5,62</sup> Similar effects were observed for cultures of immortalized normal epidermal melanocytes (PIG1)<sup>6,64</sup> and normal primary melanocytes (see below).

We also tested the effect of 7DHP, pregnenolone and pD in comparison to 1,25(OH)<sub>2</sub>D<sub>3</sub>, on NF $\kappa$ B activity in HaCaT keratinocytes. All of these compounds inhibited NF $\kappa$ B activity with 7DHP being more efficient than pD, however, pD had similar potency to pregnenolone and 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>5</sup> This

observation indicates the potential of these compounds to exhibit anti-inflammatory properties similar to 1,25(OH)<sub>2</sub>D<sub>3</sub>.

We also tested in detail another compound that was unexpectedly discovered during synthesis of 21(OH)7DHP, namely (5Z,7E)-3 $\beta$ -hydroxyandrosta-5,7-diene-17 $\beta$ -carboxylic acid (17-COOH-7DA). It resulted from the oxidative cleavage of the side chain in a reaction dependent on the presence of oxygen.<sup>34</sup> 17-COOH-7DA showed high antiproliferative potency, without toxicity.<sup>34</sup> For example it inhibited keratinocytes proliferation at doses as low as 10<sup>-11</sup> M. Also, it inhibited proliferation of normal epidermal melanocytes and melanoma cells, and induced leukemia differentiation, however with lower potency than in keratinocytes. The corresponding secosteroidal derivatives remain to be tested.

**Melanomas.** Vitamin D analogs with a short (2C) side chain or the side chain absent possess proven or predicted low hypercalcemic activity.<sup>65-67</sup> Therefore, secosteroids with a shortened and modified side chain were generated (Table 1) and their biological activity tested using a variety of human, mouse and hamster melanoma lines (for recent review see ref. 68). For instance, pD inhibited growth of human SKMEL-188 and hamster AbC1 cell lines in soft agar.<sup>5,62</sup> In addition, vitamin D-like and lumisterol-like derivatives hydroxylated at C-21: (5Z,7E)-3 $\beta$ ,21-dihydroxy-9,10-secopregna-5,7,10(19)-trien-20-one [21(OH)pD] and 3 $\beta$ ,21-dihydroxy-9 $\beta$ ,10 $\alpha$ -pregna-5,7-dien-20-one [21(OH)pL] inhibited growth of human

**Table 1.** List of new secosteroids with shortened side chain and their 5,7-diene precursors

Short name	Parental 5–7 diene	Photoderivative
7DHP	3 $\beta$ -hydroxypregna-5,7-dien-20-one	
pD		(5Z,7E)-3 $\beta$ -hydroxy-9,10-secopregna-5,7,10(19)-trien-20-one
pT		(6E)-3 $\beta$ -hydroxy-9,10-secopregna-5(10),6,8-trien-20-one
pL		3 $\beta$ -hydroxy-9 $\beta$ ,10 $\alpha$ -pregna-5,7-dien-20-one
17 $\alpha$ (OH)7DHP	3 $\beta$ ,17 $\alpha$ -dihydroxypregna-5,7-dien-20-one	
17 $\alpha$ (OH)pD		(5Z,7E)-3 $\beta$ ,17 $\alpha$ -dihydroxy-9,10-secopregna-5,7,10(19)-trien-20-one
17 $\alpha$ (OH)pT		(6E)-3 $\beta$ ,17 $\alpha$ -dihydroxy-9,10-secopregna-5(10),6,8-trien-20-one
17 $\alpha$ (OH)pL		3 $\beta$ ,17 $\alpha$ -dihydroxy-9 $\beta$ ,10 $\alpha$ -pregna-5,7-dien-20-one
20(OH)7DHP	pregna-5,7-diene-3 $\beta$ ,20-diol	
20(OH)pD		(5Z,7E)-9,10-secopregna-5,7,10(19)-triene-3 $\beta$ ,20-diol
20(OH)pL		9 $\beta$ ,10 $\alpha$ -pregna-5,7-diene-3 $\beta$ ,20-diol
20(OH)pT		(6E)-9,10-secopregna-5(10),6,8-triene-3 $\beta$ ,20-diol
7DHEA	androsta-5,7-dien-3 $\beta$ -ol	
aD		(5Z,7E)-9,10-secoandrosta-5,7,10(19)-trien-3 $\beta$ -ol
aL		9 $\beta$ ,10 $\alpha$ -androsta-5,7-dien-3 $\beta$ -ol
aT		(6E)-9,10-secoandrosta-5(10),6,8-trien-3 $\beta$ -ol
17 $\alpha$ (OH)7DHEA	androsta-5,7-diene-3 $\beta$ ,17 $\alpha$ -diol	
17 $\alpha$ (OH)aD		(5Z,7E)-9,10-secoandrosta-5,7,10(19)-triene-3 $\beta$ ,17 $\alpha$ -diol
17 $\alpha$ (OH)aL		9 $\beta$ ,10 $\alpha$ -androsta-5,7-diene-3 $\beta$ ,17 $\alpha$ -diol
17 $\alpha$ (OH)aT		(6E)-9,10-secoandrosta-5(10),6,8-triene-3 $\beta$ ,17 $\alpha$ -diol
17-COOH	(5Z,7E)-3 $\beta$ -hydroxy-androsta-5,7-diene-17 $\beta$ -carboxylic acid	
17-COOH aD3		(5Z,7E)-3 $\beta$ -hydroxy-9,10-secoandrosta-5,7,9(10)-triene-17 $\beta$ -carboxylic acid
17-COOH aT3		(6E)-3 $\beta$ -hydroxy-9,10-secoandrosta-5(10),6,8-triene-17 $\beta$ -carboxylic acid
17-COOH aL3		(5Z,7E)-3 $\beta$ -hydroxy-9 $\beta$ ,10 $\alpha$ -androsta-5,7-diene-17 $\beta$ -carboxylic acid

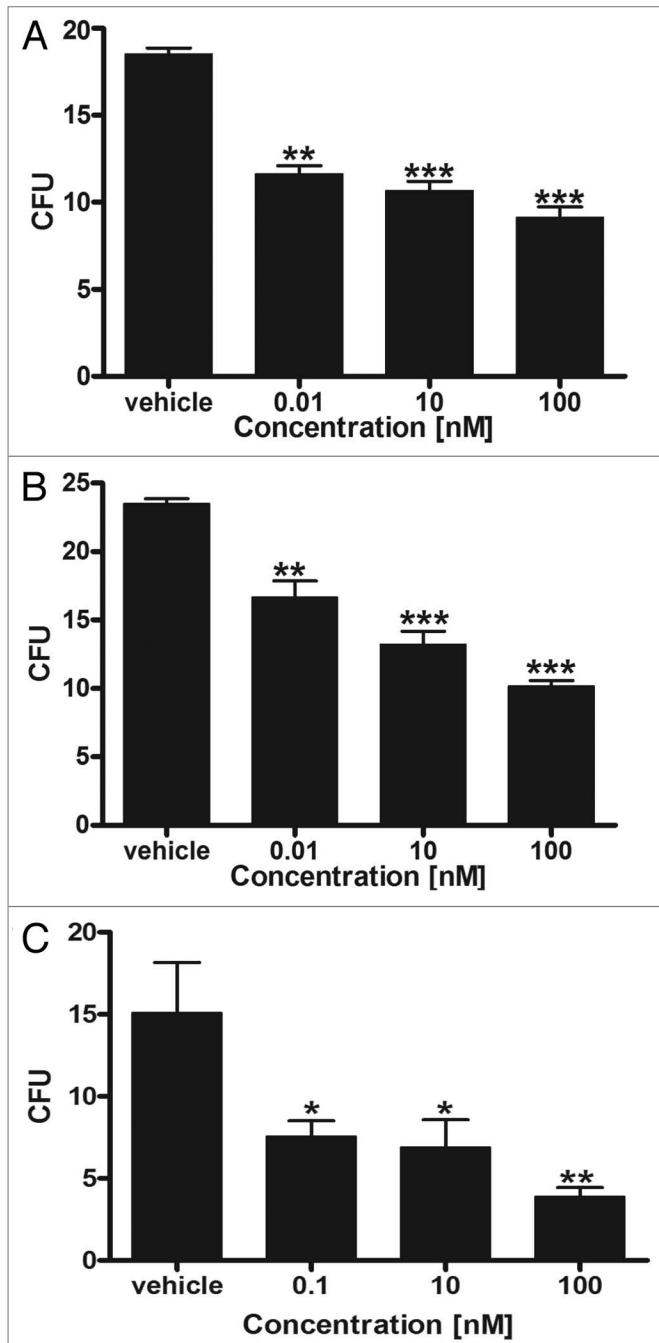
SKMEL-188 melanoma cells in a dose dependent manner, with a potency similar to or even higher than that of 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>38</sup> Moreover, 3 $\beta$ ,21-dihydroxy-9 $\beta$ ,10 $\alpha$ -pregna-5,7-dien-20-one [21(OH)7DHP] precursor and its oxidized isotachysterol-like derivative [21(OH)oxy-piT] showed stronger antiproliferative activity against pigmented SKMEL-188 melanoma cells, while treatment with the parental compound, 21(OH)7DHP, and its UV-photoproduct 21(OH)pD, had similar impact on pigmented and nonpigmented cells.<sup>38</sup> Finally, 21(OH)pD was shown to inhibit colony formation of SKMEL-188 melanoma cells.

Similar effects on colony formation by SKMEL-188 human melanoma cells were also observed for other vitamin D-like [17,20(OH)<sub>2</sub>pD] and lumisterol-like [17,20(OH)<sub>2</sub>pL] compounds derived from pregn-a-5,7-diene-3 $\beta$ ,17 $\alpha$ ,20R-triol and pregn-a-5,7-diene-3 $\beta$ ,17 $\alpha$ ,20S-triol, respectively, which were equally potent to or even more potent than 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>35</sup> We have also shown that 20(OH)pD<sub>3</sub> and 20(OH)pL inhibit proliferation of SKMEL-188 human melanoma cells, and colony formation of AbC1 melanoma cells on soft agar, while (5Z,7E)-9,10-secoandrosta-5,7,10(19)-trien-3 $\beta$ -ol (aD) had a similar inhibitory effect on anchorage independent growth of SKMEL-188 melanoma cells.<sup>62</sup> In addition, lumisterol derivatives including pL and 20(OH)pL inhibited growth of human melanoma

in soft agar (Fig. 6). Interestingly, pD inhibited colony formation on soft agar by PC3 human prostate cancer cells.<sup>62</sup>

Since vitamin D analogs with a short side chain were less efficient in inhibiting the proliferation of normal immortalized epidermal keratinocytes and melanocytes in comparison to melanoma cells,<sup>5,69</sup> we further tested them on normal primary epidermal melanocytes and found that while 1,25(OH)<sub>2</sub>D<sub>3</sub> and 20(OH)D<sub>3</sub> inhibited cell proliferation at 10<sup>-7</sup> M, the effect of 20(OH)pD or pD was minimal or absent (Fig. S1). Also, these compounds had no effect on melanin pigmentation and cell shape. However, when we used a continuous line of transformed melanocytes (PIG1) we observed inhibition of proliferation by 7DHP and pD, however, with lower efficiency in comparison to 25(OH)D<sub>3</sub>, 20(OH)D<sub>3</sub>, 20,23(OH)<sub>2</sub>D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>, indicating some sensitivity of immortalized melanocytes to pD (Fig. 7). Furthermore, 20(OH)pD inhibited proliferation of human melanoma cells without noticeable toxic effect (Fig. S2).

**Dermal fibroblasts.** In contrast to normal epidermal cells, secosteroidal products with a short side chain significantly inhibited total collagen and hyaluronan synthesis in dermal fibroblasts induced by TGF- $\beta$ .<sup>25</sup> Interestingly, the S isomer of 17,20(OH)<sub>2</sub>pD was more potent than the R form or the corresponding 17,20-dihydroxypregnalumisterols [17,20(OH)<sub>2</sub>pL] or even 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>25</sup> We also tested the ability of 7DHP,



**Figure 6.** pL (3 $\beta$ -hydroxy-9 $\beta$ ,10 $\alpha$ -pregna-5,7-dien-20-one) and 20(OH)pL (9 $\beta$ ,10 $\alpha$ -pregna-5,7-diene-3 $\beta$ ,20-diol) inhibit the anchorage independent growth (ability to form colonies in soft agar) of human melanoma cells in a dose-dependent manner. SKMel-188 human melanoma cells were grown in soft agar in the presence of graded concentrations of 20(OH)pL (**A and B**) or pL (**C**) as described previously.<sup>24</sup> After three weeks colonies with a diameter larger than 0.2 mm (**A**) or 0.5 mm (**B and C**) were counted. Data are shown as mean  $\pm$  SD ( $n = 4$ ); statistical significance was estimated using the t-test and is presented as \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

pD and 17,20R(OH)<sub>2</sub>DHP and 17,20S(OH)<sub>2</sub>DHP [precursors to corresponding 17,20(OH)<sub>2</sub>pD and 17,20(OH)<sub>2</sub>pL] to inhibit collagen synthesis (Fig. 8). All of these compounds

inhibited TGF- $\beta$ -induced collagen synthesis, with pD being more potent than 7DHP. Thus, 7DHP and 17,20(OH)<sub>2</sub>DHP and their corresponding photoderivatives pD, 17,20(OH)<sub>2</sub>pD and 17,20(OH)<sub>2</sub>pL are identified as excellent inhibitors of collagen synthesis (Fig. 8).<sup>25</sup>

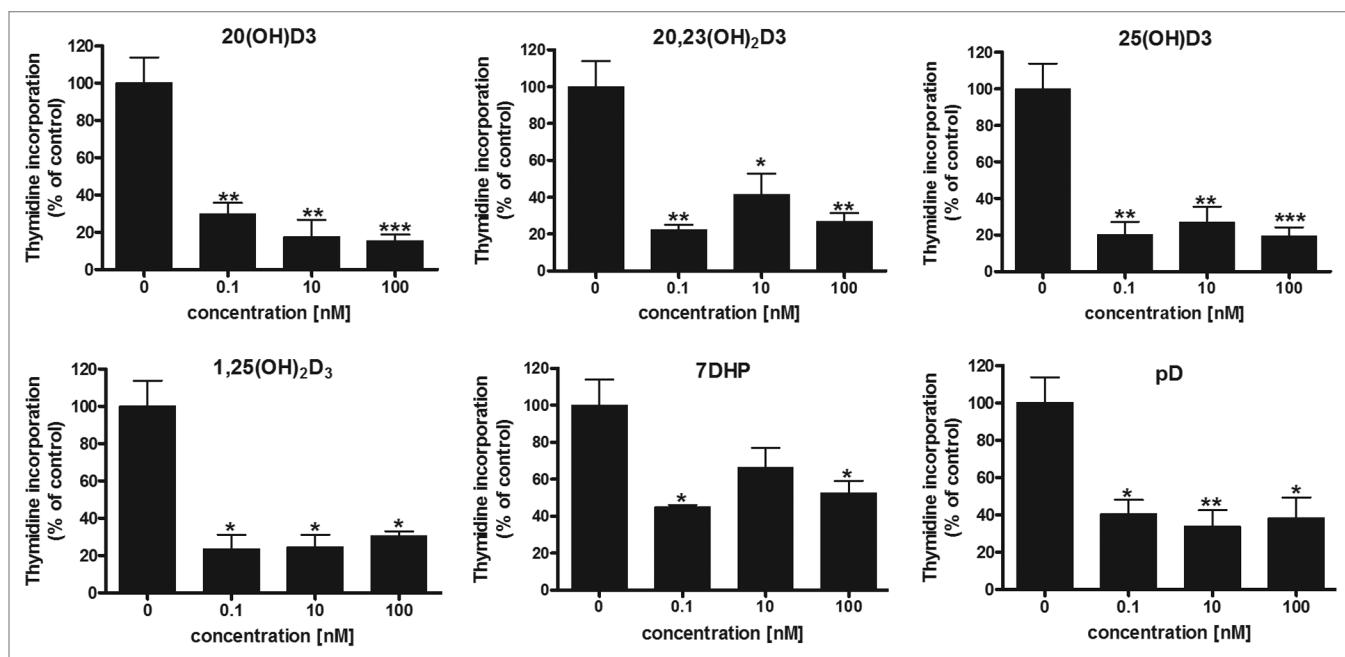
**Leukemias.** Selected compounds with a short side chain [7DHP, pD, pL, 20(OH)7DHP, 20(OH)pD] were also tested against leukemia cells in comparison to vitamin D<sub>3</sub> hydroxy-derivatives with a full 8 carbon side-chain.<sup>26</sup> In general, they inhibited proliferation and induced erythroid differentiation of K562 human chronic myeloid and MEL mouse leukemia cells, being only slightly or moderately less potent in comparison to novel vitamin D<sub>3</sub> hydroxy-derivatives with a full side-chain or to 1,25(OH)<sub>2</sub>D<sub>3</sub>. With HL-60 promyelocytic and U937 pro-monocytic human leukemia cells, pD and pL compounds were significantly less potent at inhibiting proliferation and stimulating monocytic differentiation in comparison to 20(OH)D<sub>3</sub>, 20,23(OH)<sub>2</sub>D<sub>3</sub>, 1,20(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>26</sup>

**Overview of biological activity of secosteroids with a full-length side chain.** The biological activity of vitamin D hydroxy-derivatives with a full-length, 8 carbon side chain has been extensively studied in normal epidermal keratinocytes, melanocytes, fibroblasts and melanoma cells, as well as other malignant tumors.<sup>6,8,12,17,22,23,26,28-31,38,57,61,62,70,71</sup> Below is short overview.

In keratinocytes, 20(OH)D<sub>3</sub>, 20,23(OH)<sub>2</sub>D<sub>3</sub> and 17,20,23(OH)<sub>3</sub>D<sub>3</sub> inhibited DNA synthesis and colony formation, caused cell cycle arrest, and stimulated the differentiation program with potencies comparable to that of 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>29,31,62,70</sup> 20(OH)D<sub>3</sub> and 20,23(OH)<sub>2</sub>D<sub>3</sub> inhibited NF $\kappa$ B activity in normal and immortalized keratinocytes, and in melanoma,<sup>28-30</sup> and had both anti-inflammatory and anti-fibrinogenic properties.<sup>30,62,71</sup> In additional studies using normal human peripheral blood mononuclear cell (PBMC) cultures, we found that 20(OH)D<sub>3</sub> markedly reduced TNF $\alpha$  production induced by LPS (10 pg/ml) [vehicle = 6002  $\pm$  1479 pg/ml; 20(OH)D<sub>3</sub> 10<sup>-8</sup> M = 2609  $\pm$  1961 pg/ml  $p < 0.01$ ] (description of methodology is in Supplemental file).

20(OH)D<sub>3</sub> and 20,23(OH)<sub>2</sub>D<sub>3</sub> have potent anti-melanoma and anti-cancer activities.<sup>23,24,26,28,62</sup> They act as partial agonists of the VDR, as demonstrated by gene silencing experiments.<sup>29,31</sup> 1 $\alpha$ -Hydroxy-derivatives of 20(OH)D<sub>3</sub> and 20,23(OH)<sub>2</sub>D<sub>3</sub> show similar inhibition of keratinocyte proliferation, and stimulation of differentiation and VDR expression to that of their precursors. The related CYP11A1-derived 22(OH)<sub>2</sub>D<sub>3</sub> and 20,22(OH)<sub>2</sub>D<sub>3</sub> show antiproliferative and prodifferentiation effects, being less potent than 20(OH)D<sub>3</sub> and 20,23(OH)<sub>2</sub>D<sub>3</sub>.<sup>12</sup> Chemically synthesized 20S(OH)D<sub>3</sub> has the same properties as that generated enzymatically<sup>27</sup> but a different effect is exerted by 20R(OH)D<sub>3</sub> at low concentrations.<sup>57</sup> Interestingly, 20S(OH)7DHC (a precursor to 20S(OH)D<sub>3</sub>) also inhibited cell proliferation (Fig. 9).

Studies on the structurally related secosteroid, 20(OH)D<sub>2</sub>, have also demonstrated its ability to induce the cell differentiation program mediated, at least in part, through activation of the VDR. This is illustrated by the attenuation of cell proliferation after silencing of the VDR, by enhancement of the inhibitory effect through stable overexpression of the VDR and by



**Figure 7.** Comparison of the effects of vitamin D derivatives having a full-length site chain to those of 7DHP and pD, on the proliferation of the immortalized line, PIG1, of human melanocytes. The immortalized melanocytes were cultured as described previously.<sup>82</sup> To study DNA synthesis, immortalized melanocytes were plated in 96-well plates at 10,000 cells/well. After overnight incubation, 20(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, 20,23(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, pD, 7DHP or ethanol as a control were added to the cells which were processed as described previously.<sup>22</sup> Briefly, after 12 h of incubation [<sup>3</sup>H]-thymidine was added to a final concentration of 1  $\mu$ Ci/mL medium. After an additional 12 h, media were discarded, cells washed with cold PBS, lysed and processed for final measurement of the incorporated <sup>3</sup>H-radioactivity into DNA with a  $\beta$  counter.<sup>22</sup> Significant differences between treated and non-treated (ethanol control) cells were measured using the t-test (\*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001). Data are expressed as % of control.

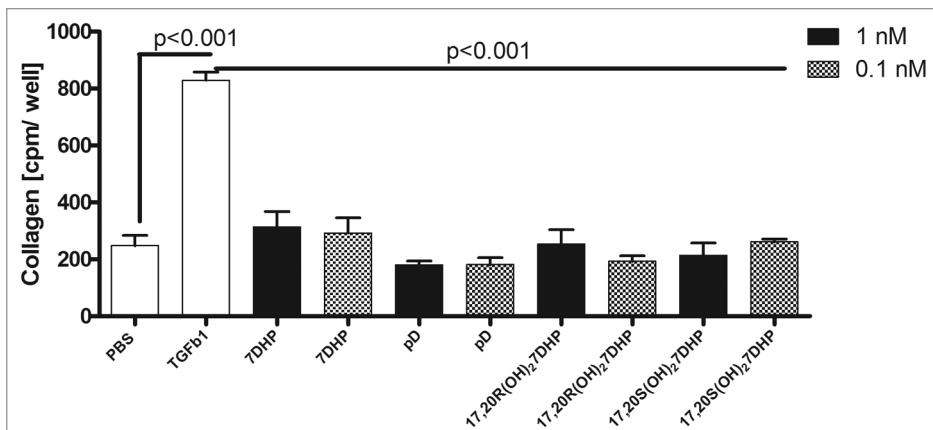
the demonstration that 20(OH)D<sub>3</sub> induces time-dependent translocation of VDR from the cytoplasm to the nucleus at a comparable rate to 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>22</sup> 20(OH)D<sub>3</sub> did not require 1 $\alpha$ -hydroxylation for biological activity. In addition, we have demonstrated that ergosterol hydroxy-derivatives have the potential to inhibit proliferation and induce differentiation of keratinocytes.<sup>8</sup>

Importantly, 20(OH)D<sub>3</sub> at a dose as high as 3.0  $\mu$ g/kg had no calcemic activity in rats whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> at lower doses raised calcium to 16.0  $\pm$  1.2 mg/dL.<sup>26</sup> The addition of a 1 $\alpha$ -hydroxyl group to 20(OH)D<sub>3</sub> conferred some calcemic activity to the derivative.<sup>26</sup> We repeated this testing and administered 20(OH)D<sub>3</sub> doses as high as 30  $\mu$ g/kg to C57BL/6 mice daily for 14 d and found no significant differences in sera Ca<sup>2+</sup> levels compared with control mice and no toxicity as determined by serum chemistry and histological analyses of heart, liver, spleen and kidney.<sup>23</sup> 20(OH)D<sub>2</sub> is also non-calcemic in rats at doses at least up to 4  $\mu$ g/kg,<sup>22</sup> and 20,23(OH)<sub>2</sub>D<sub>3</sub> is non-calcemic in mice at 3  $\mu$ g/kg.<sup>71</sup>

**Secosteroids with long side chain are better ligands for the vitamin D receptor than secosteroids with short side chain.** Using pLenti-CMV-VDR-EGFP-pgk-puro constructs,<sup>22,70</sup> we tested the ligand-induced translocation of VDR from the cytoplasm to the nucleus.<sup>22</sup> Vitamin D<sub>3</sub> hydroxy-derivatives with a full-length (8C) side chain and hydroxy-seco-steroids with a shortened side (2C) chain (pD) stimulated VDR translocation and inhibited proliferation, however, the former were

more potent than pDs. Molecular modeling of the binding of secosteroids to the VDR genomic binding pocket (G-pocket) correlated well with the experimental data for VDR translocation. In contrast, docking scores for the non-genomic binding site [A-pocket] of the VDR were poor, suggesting that they do not act on the A-pocket.<sup>70</sup> For example, 20(OH)D<sub>3</sub> bound to the G-pocket in a manner that overlapped well with the native ligand 1,25(OH)<sub>2</sub>D<sub>3</sub>. Both of these secosteroids posed the side chains toward the surface of VDR and buried the secocholesta head deeply inside the pocket bottom. The glide score of 20(OH)D<sub>3</sub> was -11.746 compared with that of -12.321 for 1,25(OH)<sub>2</sub>D<sub>3</sub>.

**Anti-oxidative effects of 5,7-dienes.** Since it has been proposed that cholesterol and vitamin D act as membrane anti-oxidants,<sup>72,73</sup> we compared anti-oxidative properties of 7DHC, 7DHP, vitamin D<sub>3</sub>, vitamin D<sub>2</sub> and cholesterol by measuring 2,4-dinitrophenylhydrazine (DNPH) reactive protein-bound carbonyls in isolated rat liver mitochondria treated with iron/ascorbate (for methodology see Supplemental file). As shown in Figure 10A, 7DHC and 7DHP significantly attenuated oxidative protein modification being more effective than cholesterol, vitamin D<sub>3</sub> and vitamin D<sub>2</sub>. 7DHP at concentrations of 1  $\mu$ M and 10  $\mu$ M decreased the protein carbonyl content by 26.7% (\*\*p < 0.01) and 26.3% (\*\*p < 0.01), respectively, compared with mitochondria treated only with iron/ascorbate. 7DHC at the same concentrations was more potent than 7DHP and attenuated oxidative protein modification by 37.8% (\*\*p < 0.01) and 34.3% (\*\*p < 0.01), respectively.

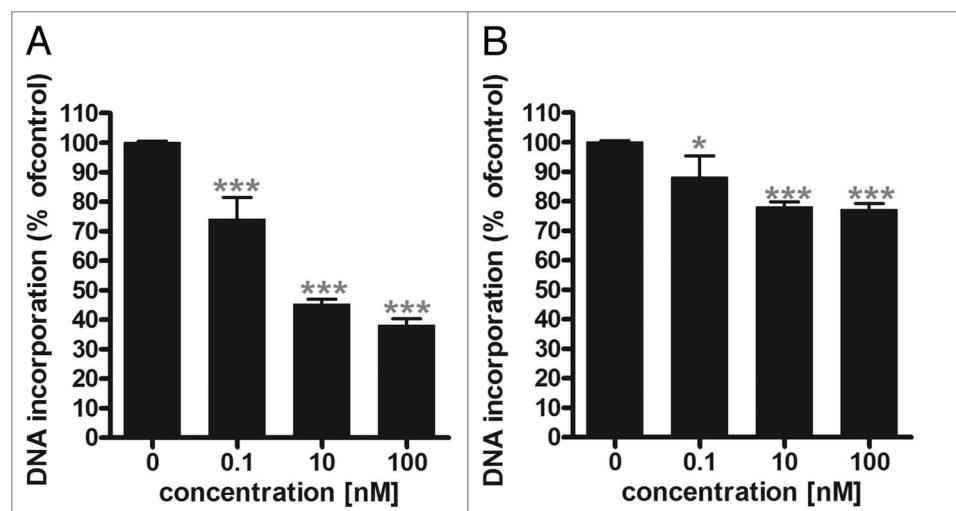


**Figure 8.** 7DHP, pD, 17,20R(OH) $_2$ 7DHP and 17,20S(OH) $_2$ 7DHP inhibit TGF- $\beta$ -induced collagen synthesis. Human dermal fibroblasts grown from explant skin cultures at less than 10 subpassages were used as described previously.<sup>25</sup> After a 2 h preincubation with the compounds being tested at concentrations of 10 $^{-9}$  or 10 $^{-10}$  M, or the vehicle control (ethanol), TGF- $\beta$ 1 (R and D systems) was added to each well (except control) at a final concentration of 5 ng/ml. After 48 h of culture, plate wells were pulsed with 1  $\mu$ Ci  $^3$ H-proline. After 24 h, culture supernatants were harvested and collagenase sensitive protein was determined.<sup>25,71</sup> Data are shown as mean  $\pm$  SD ( $n = 4$ ); statistical significance was estimated using the t-test and showed that TGF- $\beta$ 1 stimulated collagen synthesis, while the 5,7-dienes and pD inhibited it ( $p < 0.001$ ). pD was more potent than its precursor, 7DHP, at both concentrations: 1 nM ( $p < 0.01$ ) and 0.1 nM ( $p < 0.05$ ).

We also compared the antioxidant ability of 7DHC and 7DHP to that of the powerful mitochondrial antioxidants such as GSH, as well other endogenous anti-oxidants such as melatonin and its metabolites [6-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK)]<sup>74-76</sup> and its precursor N-acetylserotonin (NAS). While 6-hydroxymelatonin was the most effective in preventing oxidative damage to the proteins, 7DHC and 7DHP also showed prominent antioxidant properties, similar to that of AFMK and NAS (Fig. 10B). Furthermore,

which are based on the catalytic activity of CYP11A1 on the side chain of 7DHC, ergosterol and vitamin D. Although production of 5,7-diene steroids was anticipated because of SLOS, our<sup>4</sup> and Guryev and coworker's<sup>39</sup> studies were the first to document a crucial role of CYP11A1 in the generation of  $\Delta$ 7-steroids. Importantly, our laboratories were the first to indicate UVB induced transformation of such  $\Delta$ 7-steroids in the skin to androsta- and pregra-calciferol, lumisterol or tachysterol-like compounds that are biological active. Of equal significance is the P450scc-mediated generation of novel biologically active hydroxy-derivatives of vitamins D $_3$  and D $_2$ . Recently, evidence was provided for their production in vivo under physiological conditions.<sup>6,13</sup> We have also established biochemical and chemical routes of synthesis of these secosteroids and defined their structures.

Importantly, 20(OH)D $_3$ , 20,23(OH) $_2$ D $_3$  and 20(OH)D $_2$ , as well as 17,20(OH) $_2$ pD, are non-toxic and non-calcemic in rodents. These and related novel secosteroids show anti-proliferative, pro-differentiation, anti-cancer, anti-fibrosing and anti-inflammatory properties that are determined by their chemical structure and the lineage of the target cells. Therefore, they should serve as excellent therapeutic agents for hyperproliferative, fibrosing or



**Figure 9.** 20S(OH)7DHC (precursor to 20S(OH)D $_3$ ) inhibits proliferation of neonatal human epidermal keratinocytes (HEKn). HEKn in their third passage were treated with graded concentrations of 20S(OH)7DHC for 24 h (A) or 48 h (B), as described previously.<sup>57</sup> Incorporation of radioactive thymidine ( $^3$ H) into DNA was determined 4 h after incubation. Data are presented as mean  $\pm$  SD ( $n = 4$ ). DNA incorporation was calculated as a percentage of the control (ethanol treated cells). Statistical significance was measured using one-way ANOVA presented as \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

**Figure 10.** 7DHC and 7DHP attenuate oxidative protein damage in isolated rat liver mitochondria treated with iron/ascorbate. (A) 7DHC and 7DHP are more effective than cholesterol, vitamin D<sub>3</sub> and vitamin D<sub>2</sub> in attenuating oxidative protein modification. (B) Relative antioxidant abilities of 7-DHC and 7-DHP in comparison to GSH, melatonin and its metabolites (6-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine(AFMK) and its precursor, N-acetylserotonin (NAS). The carbonyl content of mitochondrial proteins following treatment with iron/ascorbate was measured as described in the Supplemental file. Data are presented as means  $\pm$  SEM ( $n = 3$ ), statistical significance was estimated using the t-test. The basal level of protein-bound carbonyls in intact mitochondria was  $2.20 \pm 0.13$  nmol/mg protein. The content of protein carbonyls increased 3.6 times over the basal level after incubation of mitochondria in the iron/ascorbate system for 45 min.

inflammatory disorders, or as major drugs or adjuvants in cancer therapy.

Although we have already provided evidence that these new secosteroids can act as partial agonists on the vitamin D receptor, the future challenge is to identify alternative nuclear receptors that are activated by these compounds. Furthermore, mechanism(s) of action of steroid 5,7-dienes remain(s) to be clarified, since they also demonstrate antiproliferative and antifibrotic activities. Lastly, measurement of the relative concentrations of the new vitamin D analogs in body fluids as well as the CYP11A1 dependent rates of production of  $\Delta 7$ -steroids in the skin *in vivo* await further experimentation.

#### Disclosure of Potential Conflicts of Interest

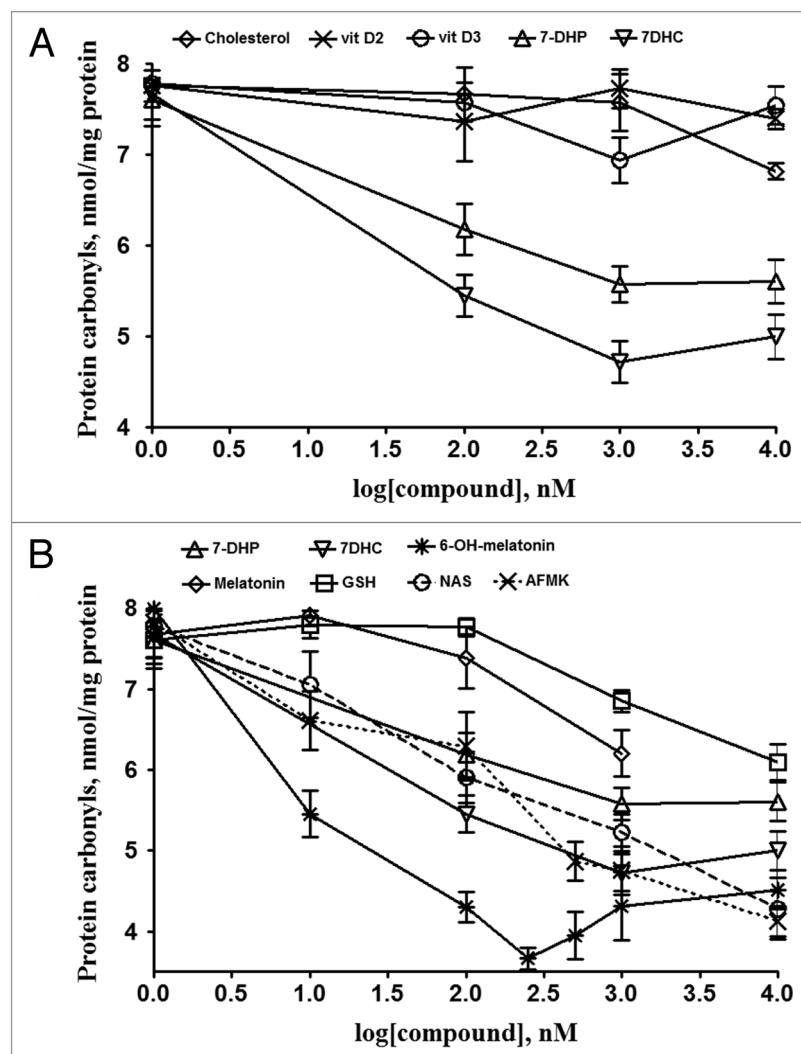
No conflicts of interest were disclosed.

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#### Supplemental Material

Supplemental material may be downloaded here:  
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