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Research Paper

Synthesis and biological evaluation of all A-ring stereoisomers of 5,6-*trans*-2-methyl-1,25-dihydroxyvitamin D_3 and their 20-epimers: possible binding modes of potent A-ring analogues to vitamin D receptor

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Abstract

Background: The secosteroid 1α,25-dihydroxyvitamin D_3 (**1**) has a wide variety of biological activities, which makes it a promising therapeutic agent for the treatment of cancer, psoriasis and osteoporosis. Insight into the structure–activity relationships of the A-ring of **1** is still needed to assist the development of more potent and selective analogues as candidate chemotherapeutic agents, as well as to define the molecular mode of action.

Results: All possible A-ring stereoisomers of 5,6-*trans*-2-methyl-1,25-dihydroxyvitamin D_3 (**6a–h**) and their 20-epimers (**7a–h**) were designed and efficiently synthesized. The dependence of the affinities for vitamin D receptor (VDR) and vitamin D binding protein (DBP), as well as the HL-60 cell differentiation-inducing activity, upon the stereochemistry of the A-ring and at C20 in the side chain was evaluated.

Conclusions: The binding affinities and potency of the 5,6-*trans* and 5,6-*cis* analogues were enhanced by a 2-methyl substituent in a certain orientation. Molecular docking studies based upon the X-ray crystal structure of VDR suggested that the axial 2-methyl group would be accommodated in a pocket surrounded by hydrophobic amino acid residues in the ligand binding domain, resulting in enhanced interaction.

Keywords: Cell differentiation; Chemical synthesis; Molecular modeling; 5,6-*trans*-Vitamin D; Vitamin D receptor

1. Introduction

The hormonally active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (**1**, Fig. 1), dominates the cell cycle in many malignant cells, regulating proliferation, differentiation and apoptosis, in addition to its classical role in calcium and phosphorus homeostasis [1]. Such a wide variety of activities has hampered the clinical application of **1** and has stimulated chemical and biological research to find analogues with selective activity profiles as candidate therapeutic agents, as well as to define its molecular mode of action [2]. Most of the biological activities of **1** are considered to be mediated by a ligand-inducible transcription factor, vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily. The specific interaction of ligands with the ligand binding domain (LBD) of VDR has been the focus of attention in recent years, since it triggers the whole sequence of actions. In order to investigate the structure–activity relationships for this interaction, a number of analogues have been synthesized and biologically evaluated [3]. For the reasons of synthetic convenience and for metabolic studies of the inactivation process, many of the analogues synthesized so far were altered in the side chain, providing useful analogues with high or selective activity. Among them, 20-*epi*-1α,25-dihydroxyvitamin D_3 is noteworthy due to its high activity in

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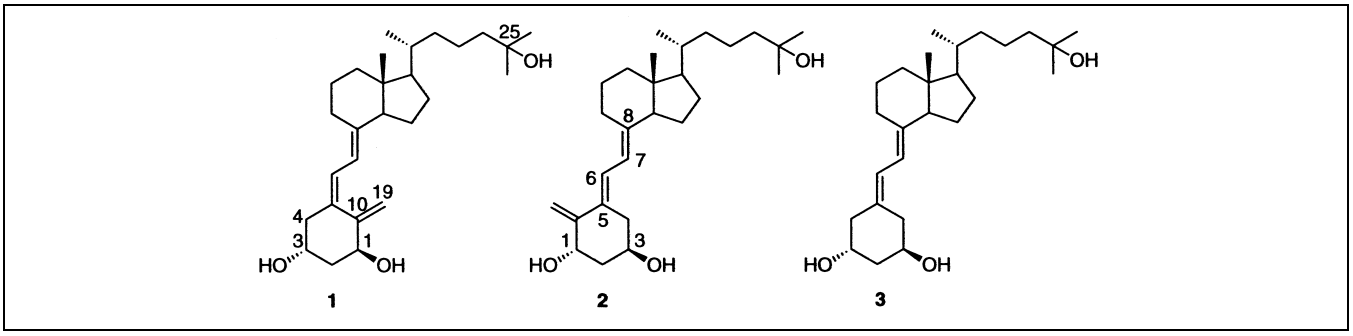


Fig. 1. Structures of 1α,25-dihydroxyvitamin D_3 (**1**), its 5,6-*trans* stereoisomer (**2**) and 19-*nor* analogue (**3**).

cell differentiation with a relatively low calcemic effect, having a clearly distinct biological profile from its parent hormone **1** [4,5]. This 20-epimerization, combined with other modifications in the side chain, led to the highly potent cell differentiation-inducing and immunosuppressive analogue, 20-*epi*-22-oxa-1α,25-dihydroxy-24,26,27-trihomovitamin D_3 (KH-1060) [4].

The A-ring moiety of **1**, which bears two critical hydroxyl groups on the six-membered ring, is of recent interest for metabolic studies as well as for synthesis of analogues [6]. The configurations of the two hydroxyl groups on the A-ring in the natural hormone are 1α and 3β (vitamin D numbering system). Epimerization of either the 1- or 3-hydroxyl group results in a marked reduction of VDR binding activity [7]. Modification in the A-ring provided characteristic analogues, such as 2β-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 (ED-71), which has a strong calcium-regulating activity and is considered a promising candidate for the treatment of osteoporosis [8,42,43]. A known geometric stereoisomer of **1**, 5,6-*trans*-1α,25-dihydroxyvitamin D_3 (**2**, Fig. 1) [9], in which the exocyclic 10(19)-methylene group appears to be transposed to position 4 in a formal sense, has become of interest in connection with the potent A-ring analogue, 1α,25-dihydroxy-19-norvitamin D_3 (**3**, Fig. 1) [10]. In addition, analogues with the 5,6-*trans* motif, in combination with 16-ene modification, were recently reported to be promising antiproliferative agents [11].

Okamura et al. reported in 1974 that the A-ring of **1** is in a rapid equilibrium of two chair conformers, α-form and β-form, in which the C10(19) double bond is orientated above or below the diene plane of the C5–C6–C7–C8 diene, respectively, by definition (Fig. 2) [12,13]. They proposed that the β-form, in which the 1α-hydroxyl group occupies the equatorial position, may be responsible for biological activity, based upon the results obtained with tachysterol and other related analogues [14]. On the other hand, recent studies of 19-*nor* analogues by DeLuca et al. implied that the axial orientation of the 1α-hydroxyl group would be necessary for biological activity of vitamin D compounds [15].

In view of the results of A-ring modification stated above, as well as the conformation–activity relationships in the A-ring, we synthesized all eight possible A-ring diastereomers of 2-methyl-1,25-dihydroxyvitamin D_3 (**4a–h**, Fig. 3), demonstrating that the potency of the analogues varies depending upon the configurations not only of the C1 and C3 hydroxyl groups, but also of the C2 methyl

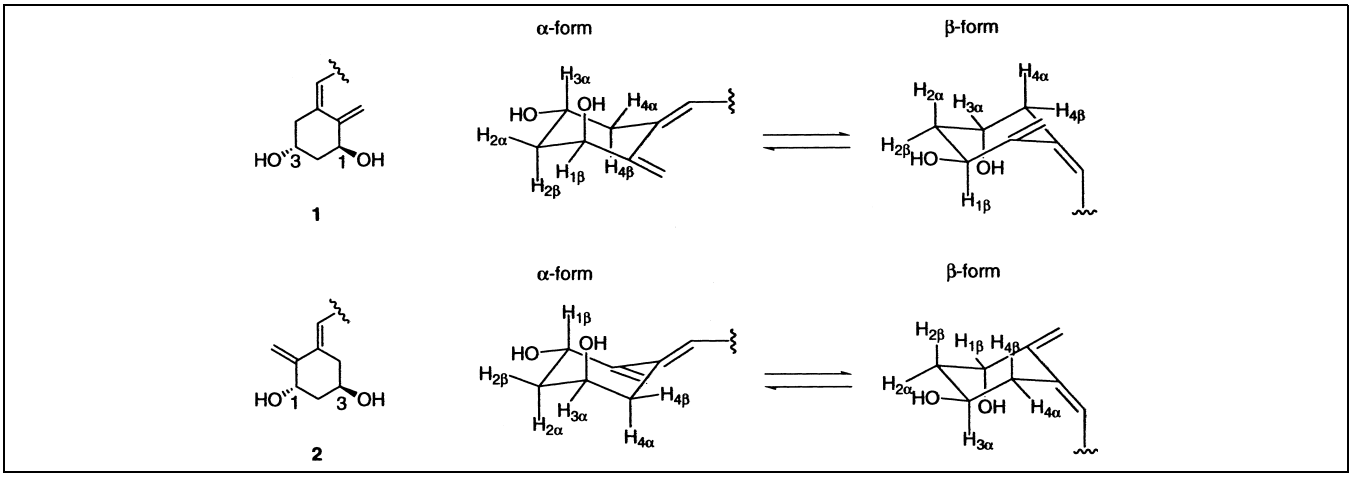


Fig. 2. The A-ring conformational equilibria of **1** and **2**.

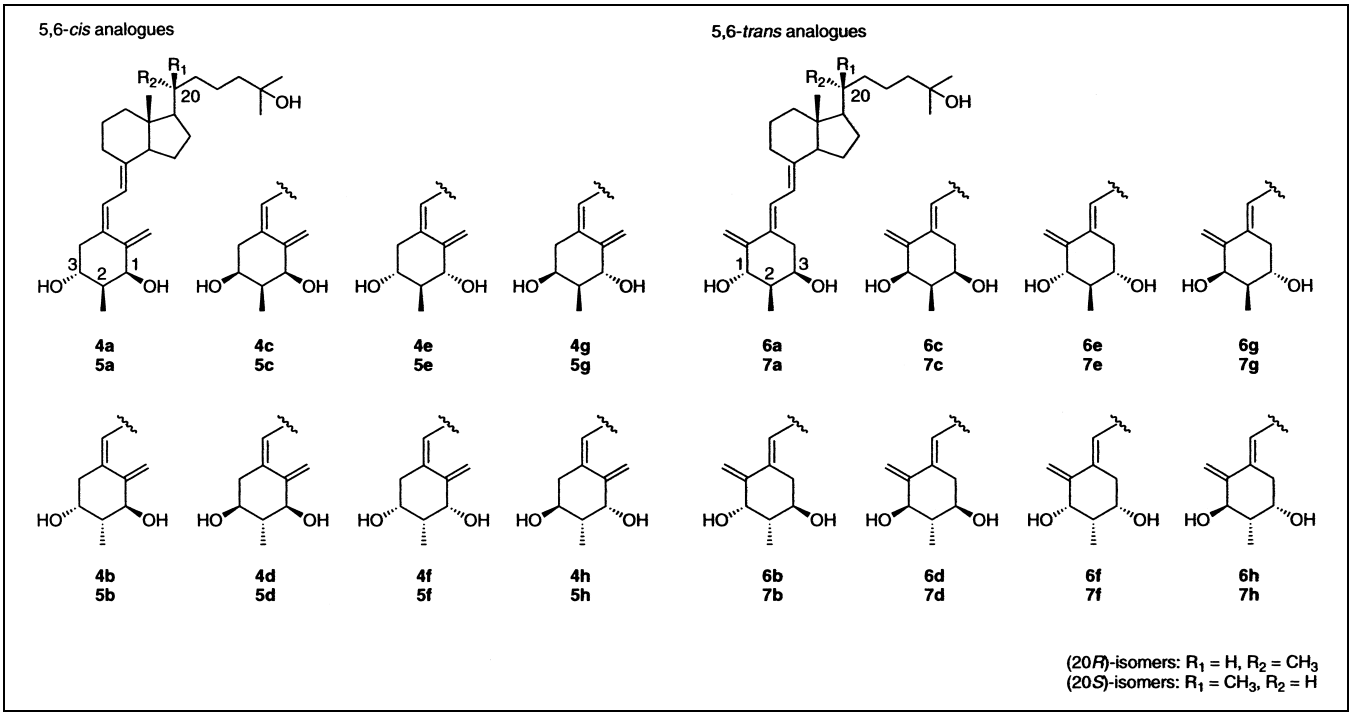
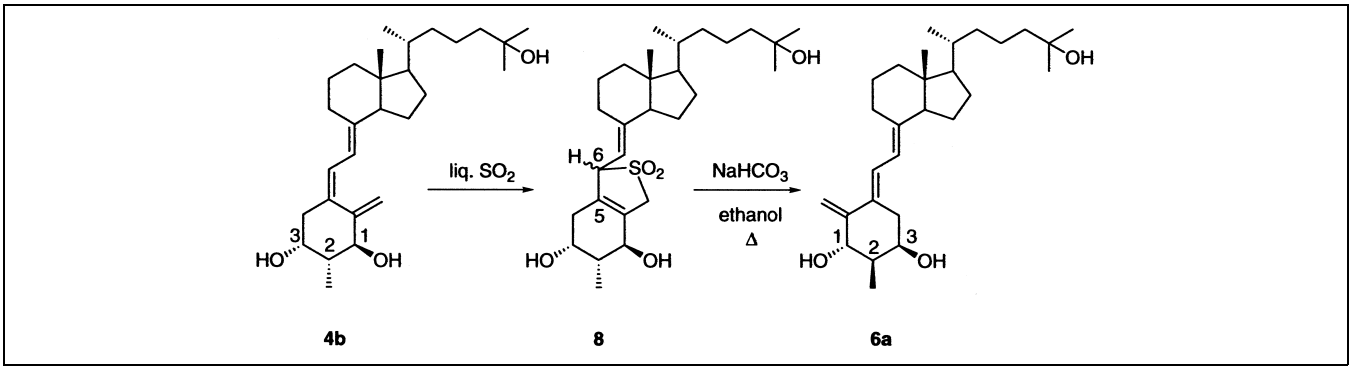


Fig. 3. Structures of 2-methyl-1,25-dihydroxyvitamin D_3 (**4a–h**), 20-*epi*-2-methyl-1,25-dihydroxyvitamin D_3 (**5a–h**), 5,6-*trans*-2-methyl-1,25-dihydroxyvitamin D_3 (**6a–h**) and 5,6-*trans*-20-*epi*-2-methyl-1,25-dihydroxyvitamin D_3 (**7a–h**).

group [16–19]. In particular, 2α-methyl-1α,25-dihydroxyvitamin D_3 (**4a**) exhibited four-fold higher VDR affinity than **1**, while its 2-epimer, 2β-methyl-1α,25-dihydroxyvitamin D_3 (**4b**) showed one-eighth of the affinity of **1**. This simple A-ring modification yielded for the first time an analogue (**4a**), having the natural side chain of **1**, with significantly higher VDR binding activity than the parent hormone. Our research on 2-methyl-20-*epi* analogues (**5a–h**, Fig. 3) also revealed that the combination of 2α-methyl introduction and 20-epimerization resulted in exceptionally high potency: in the case of **5a**, the 20-epimer of **4a**, 12-fold higher in VDR binding, six-fold in in-vivo calcium-regulating activity and 590-fold in HL-60 cell differentiation-inducing activity [17–20]. Introduction of the 2-methyl group was shown to alter the conformational equilibria in the A-ring to a certain extent. However, such high VDR affinity following 2α-methyl substitution could not be explained by those conformational effects alone.

In our present work, we have designed and synthesized all possible A-ring stereoisomers of 5,6-*trans*-2-methyl-1,25-dihydroxyvitamin D_3 (**6a–h**, Fig. 3) and their 20-epimers (**7a–h**, Fig. 3) as novel analogues with the 5,6-*trans* modification to gain further insight into the structure–activity relationships of the A-ring, as well as to investigate how these combinations affect the biological activity profiles. We also conducted computational docking of the 2-methyl analogues to the LBD of VDR, based upon the recently solved X-ray crystal structure of a mutant VDR [21] in order to understand the significance of 2-methyl substitution on the A-ring, and we propose possible binding modes of the highly potent methyl analogues to VDR.



Scheme 1.

2. Results and discussion

2.1. Synthesis

The 5,6-*trans* analogues were synthesized from their 5,6-*cis* counterparts via the SO_2 adducts [22,23]. Scheme 1 exemplifies the synthesis of 5,6-*trans*-2β-methyl-1α,25-dihydroxyvitamin D_3 (**6a**) from the 5,6-*cis* isomer (**4b**). Treatment of **4b** with SO_2 gave a 6-epimeric mixture of the adducts **8**. Subsequent removal of SO_2 in **8** by thermolysis in the presence of $NaHCO_3$ afforded **6a** in 66% yield (two steps). In the case of **6a**, the 3β-hydroxyl, 2β-methyl and 1α-hydroxyl groups may function as *pseudo* 1α-hydroxyl, *pseudo* 2α-methyl and *pseudo* 3β-hydroxyl groups, respectively [14]. The 5,6-*trans* analogues **6b**, **6c**, **6d**, **6e**, **6f**, **6g** and **6h** were synthesized via exactly the same sequence of reactions from their 5,6-*cis* counterparts **4a**, **4f**, **4e**, **4d**, **4c**, **4h** and **4g**, respectively. The other 5,6-*trans*-20-*epi* analogues (**7a–h**) were synthesized from the corresponding 5,6-*cis*-20-*epi* analogues (**5a–h**) as described above for the 20-natural analogues. As a reference compound, 5,6-*trans*-1α,25-dihydroxyvitamin D_3 (**2**) was prepared and its molar absorptivity was determined [9]. The hyperchromic effect and red shift observed in the UV spectrum of **2** compared to **1** can be explained in terms of the change of the triene structure. Our results are consistent with those for 5,6-*trans*-25-hydroxyvitamin D_3 [24].

2.2. Conformational analysis

In order to gain information on A-ring conformation–activity relationships, the solution conformations of the 5,6-*trans* analogues were examined by means of ^1H NMR analyses together with molecular mechanics calculation. Since the roles of the two hydroxyl groups at the C1 and C3 positions appear to be exchanged in the 5,6-*trans* analogues, the α-form and β-form are defined as illustrated in Fig. 2, in which the 3-hydroxyl group is axially or equatorially orientated, respectively, in the case of **2**. Firstly, the conformation of **2** was analyzed by ^1H NMR using $CDCl_3$ as a solvent in comparison with **1**. The conformational equilibrium of **1** was estimated to be a ratio of 45:55 in favor of the β-form from the vicinal coupling constant between H(3α)–H(4β) of 6.7 Hz by the method based upon the data reported for cyclohexanol protons ($J_{ax,ax} = 11.1$ Hz, $J_{eq,eq} = 2.7$ Hz) [25], which is widely accepted for analysis of the A-ring of vitamin D compounds [26,27]. In the case of **2**, steric repulsion between H(7) and H(19E) in the 5,6-*cis* compounds is released and the C10(19) exomethylene and C5–C6–C7–C8 diene can approach planarity, which would result in the hyperchromic effect and red shift observed in the UV spectrum of **2** compared to **1**. However, the three vicinal coupling constants of H(1β)–H(2α), H(2β)–H(3α) and H(3α)–H(4β) in **2**, 5.2 Hz, 8.1 Hz and 8.5 Hz, respectively, indicated that both α- and β-forms were involved in a ratio of 30:70.

On the basis of the results with **2**, the ^1H NMR spectra of all the synthesized analogues (**6a–h**, **7a–h**) were analyzed. The ^1H NMR spectra were taken in $CDCl_3$–D_2O to exclude the coupling of H(1) and H(3) with hydroxyl protons in these cases. Analyses of COSY and decoupling experiments allowed the assignment of all A-ring signals and coupling constants. There was no marked difference in chemical shifts between each pair of the (20*R*)- and (20*S*)-isomers, except for the chemical shifts due to the 20-methyl groups. In every case, the A-ring is in an equilibrium between two chair conformations, the α- and β-forms (Fig. 4), as we previously reported in the case of 5,6-*cis* analogues [19]. In the case of 1,3-*anti* diols (**a**, **b**, **g** and **h**), the chair conformers in which the 2-methyl group occupies the equatorial position are favored. On the other hand, the 1,3-*syn* diols (**c**, **d**, **e** and **f**) mainly exist in the chair forms in which the two hydroxyl groups are axially orientated, because of the intramolecular hydrogen bond. The equilibrium ratios shown in Fig. 4 were deduced from the vicinal coupling constants of H(1)–H(2), H(2)–H(3) and H(3)–H(4). Molecular mechanics calculation using MM2* was carried out using model compounds lacking the side chain. As expected, the two chair conformers involved were most stable in each case and the calculated values of energy difference and equilibrium ratios at 25°C, shown in parentheses in Fig. 4, are in good agreement with the experimental data.

The orientations of the two hydroxyl groups and the 2-methyl group affect the equilibrium ratio of the 5,6-*trans* analogues, and as a result, the equilibrium is biased to various extents in a direction that depends upon the A-ring stereochemistry. In the case of isomer **a**, the 2β-methyl substituent favored the α-form, whereas the 2α-methyl substituent of isomer **b** favored the β-form. DeLuca et al. obtained conformationally locked A-ring analogues by 2-methyl introduction into 19-*nor* analogues [15]. The 19-*nor* modification consists of not only deletion of the C10(19) exomethylene group, but also introduction of two protons into the C10 position. They reported that 2α-methyl-1α,25-dihydroxy-19-norvitamin D_3 was essentially completely locked in the α-form, in which the 1α-hydroxyl group is in the axial position, while 2β-methyl-1α,25-dihydroxy-19-norvitamin D_3 was locked in the β-form. On the other hand, our data on the 5,6-*trans*-2-methyl analogues (**6a–h**, **7a–h**), as well as the 5,6-*cis* counterparts (**4a–h**, **5a–h**), show that 2-methyl introduction yields, instead of conformational lock, a mixture of chair conformers, since the native A-ring with the C10(19) exomethylene groups allow the axial 2-methyl orientation to a certain extent due to the planar sp^2 carbon at C10.

2.3. Biological evaluation

The biological activities of the synthesized analogues

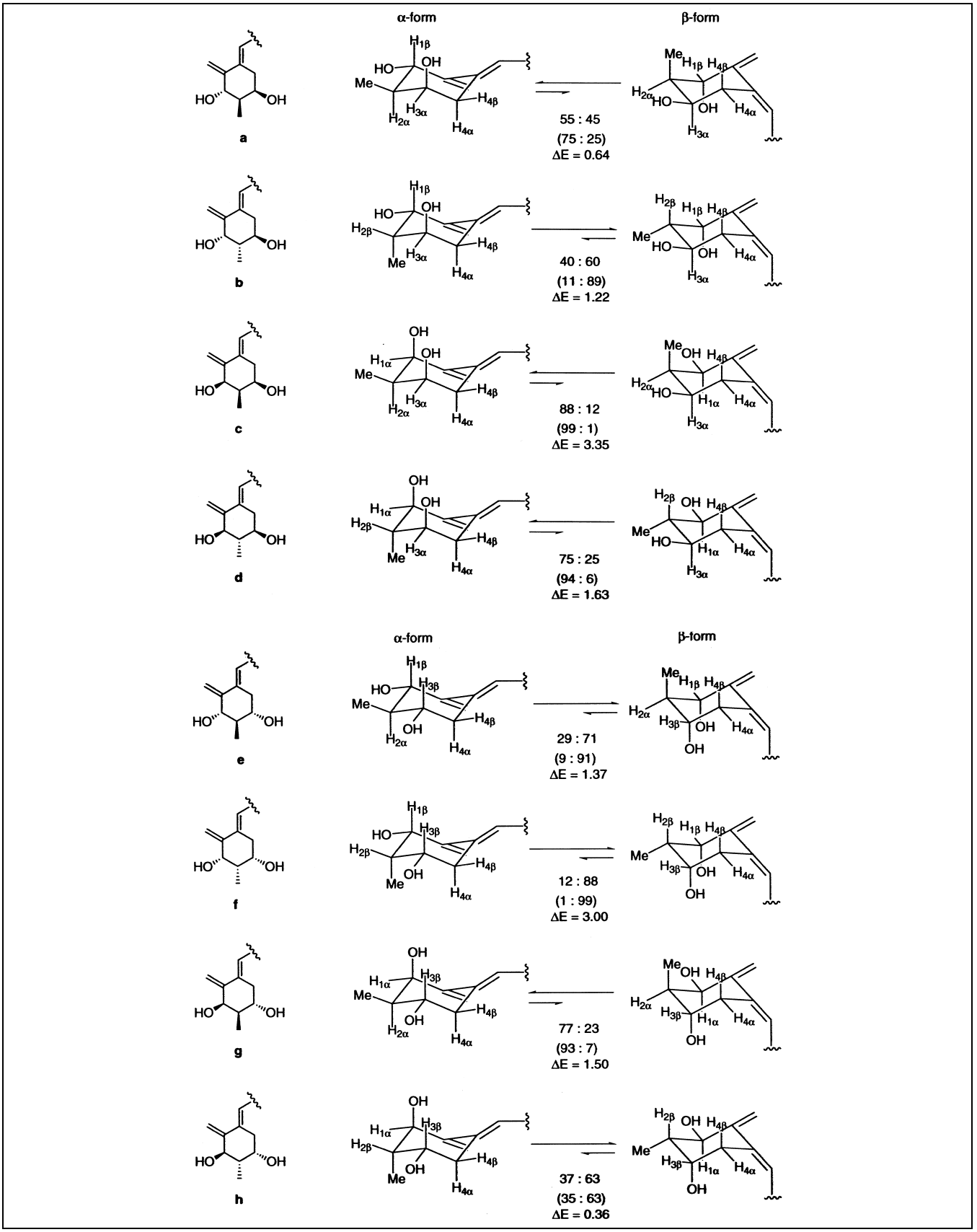


Fig. 4. The A-ring conformational equilibria of the 5,6-*trans*-2-methyl analogues. The numbers show the equilibrium ratio deduced from the ^{1}H NMR analysis. The calculated ratio at 25°C and energy difference values (kcal/mol) are shown in parentheses.

(**6a–h**, **7a–h**) were assessed in comparison with those of the natural hormone **1**, together with its 5,6-*trans* stereoisomer **2**. The results are summarized in Table 1 (the potency of **1** was normalized to 100 in each biological test). The activities were highly dependent upon the stereochemistry not only in the A-ring, but also at C20 in the side chain. On the basis of our previous results with the 5,6-*cis*-2-methyl analogues, the present data indicate that the 5,6-*trans* modification acts as a simple transposition of the C10(19) exocyclic methylene group, and as a result, the roles of the two hydroxyl groups at the C1 and C3 positions appeared to be exchanged in the 5,6-*trans*-2-methyl analogues [16-20], except for the case of vitamin D binding protein (DBP) binding. Thus, these hybrid modifications yielded analogues with unique activity profiles.

In the VDR binding assay using bovine thymus [28], 5,6-*trans*-1α,25-dihydroxyvitamin D_3 (**2**) showed a reduced affinity compared to **1** by approximately 100-fold [9]. In addition, 5,6-*trans* modification of the 2-methyl analogues resulted in a reduction of affinity compared to each structurally-related 5,6-*cis* counterpart; for example, **6a** showed approximately 50-fold lower affinity than **4a** [16–20]. Among the 5,6-*trans*-2-methyl analogues with natural (20*R*)-configuration (**6a–h**), those having the 3β-hydroxyl group (**6a–d**), which would act as a *pseudo* 1α-hydroxyl group, exhibited higher affinity compared to the corresponding 3-epimers (**6e–h**). Introduction of the 2β-methyl group into **2** to produce **6a** elevated the VDR affinity by approximately 10-fold, while introduction of the 2α-methyl group to give **6b** reduced the potency. Thus, as in the case of 2α-methyl introduction into 1α,25-dihydroxyvitamin D_3 (**1**), the *pseudo* 2α-methyl introduction into 5,6-*trans*-1α,25-dihydroxyvitamin D_3 (**2**), such as in **6a**, enhanced the affinity to VDR. These results imply that 2-methyl substitution in 'up' configurations, irrespective of 5,6-*trans* or 5,6-*cis* arrangement, would play an important role in enhancing the VDR binding potency. Transposition of the C10(19) exomethylene group reduced the affinity, but seemed to have little effect on the character of each substituent in the A-ring. Similar trends were found among the 20-*epi* analogues with (20*S*)-configuration. The rank order of the VDR affinity among the 20-*epi* analogues (**7a–h**) was essentially the same as observed among the (20*R*)-counterparts (**6a–h**). The VDR binding potency of 20-*epi*-1α,25-dihydroxyvitamin D_3 using bovine thymus in comparison with **1**, normalized to 100, was estimated to be 400 by us [29] and 500 by others [5]. 20-Epimerization of each 5,6-*trans*-2-methyl isomer elevated the affinity by approximately 3–15-fold. Thus, 2-methyl introduction and 20-epimerization had additive effects on the VDR binding potency.

In the DBP binding assay using rat serum [30], the analogues having the 2α-methyl configuration showed higher affinity than the corresponding 2-epimers, and 20-epimerization of each isomer decreased the affinity. The strongest ligand to DBP among vitamin D metabolites was considered to be 25-hydroxyvitamin D_3, and a 1α-hydroxyl group decreased the affinity to DBP [31]. In the case of 5,6-*trans* analogues, however, isomers having the 3β-hydroxyl group, which would act as a *pseudo* 1α-hydroxyl group, proved to be better binders than those having the 3α-hydroxyl group. The C10(19) exomethylene group of 5,6-*trans* isomers seems to play an important role in the DBP binding and differentiates the roles of the two hydroxyl groups in the A-ring.

Cell differentiation-inducing activity of the analogues towards HL-60 cells at 10^{-8} M was assessed in terms of expression of cell surface CD11b [32]. Fig. 5 indicates that the analogues **6a**, **7a** and **7b** exhibited a potency comparable to or even higher than that of the natural hormone **1**. It is noteworthy that **7e** was significantly active in spite of having the 3α-hydroxyl configuration (i.e., the *pseudo* 1β-hydroxyl configuration), which should impart low or no activity. Recent studies of A-ring analogues, in particular combined with side chain modifications, show that the transcriptional potency is sometimes elevated even when the VDR binding affinity is low [44-46]. In order to examine further the potency as differentiators, the

Table 1
Relative biological activity of the 5,6-*trans*-2-methyl analogues[a]

(20*R*)	VDR[b]	DBP[c]	HL-60[d]	(20*S*)	VDR	DBP	HL-60
1	100	100	100				
2	0.8	14	47				
6a	8.6	7.7	235	**7a**	45	1.0	333
6b	0.4	17	7	**7b**	1	0.7	53
6c	0.1	34	ND[e]	**7c**	0.8	2.1	36
6d	< 0.01	81	ND	**7d**	0.03	1.8	ND
6e	0.04	6.0	ND	**7e**	0.5	< 0.1	38
6f	0.013	11	ND	**7f**	0.2	< 0.1	ND
6g	0.03	8.8	ND	**7g**	0.2	< 0.1	ND
6h	< 0.01	28	ND	**7h**	0.08	0.4	ND

[a]The potencies of 1α,25-dihydroxyvitamin D_3 (**1**) are taken as 100.
[b]Bovine thymus.
[c]Rat serum.
[d]HL-60 cell differentiation was assessed in terms of expression of CD11b.
[e]Not determined.

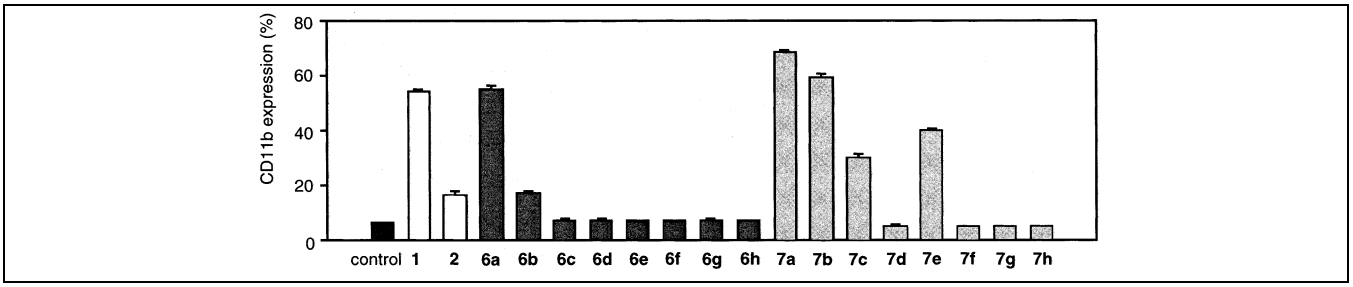


Fig. 5. HL-60 cell differentiation-inducing activity of the 5,6-*trans*-2-methyl analogues. HL-60 cells were treated with 10^{-8} M 1α,25-dihydroxyvitamin D_3 (**1**) or the analogues (**2, 6a–h, 7a–h**) for 72 h. Cell differentiation was assessed in terms of expression of CD11b.

dose dependency of selected analogues, **2**, **6a**, **6b**, **7a**, **7b**, **7c** and **7e**, was investigated (Fig. 6). The data are summarized as relative potencies with respect to **1**, defined as 100, in Table 1. The cell differentiation-inducing potency of 5,6-*trans*-1α,25-dihydroxyvitamin D_3 **2** was approximately half that of the natural hormone **1**. The analogue **6a** showed 2.4-fold higher activity than **1**, whereas its 2-epimer, **6b**, exhibited 7% of the activity of the natural hormone **1**. The potency of the isomers **6a,b** was enhanced by 20-epimerization, but to a lesser extent than in the case of 5,6-*cis*-2-methyl analogues [16–20]. Those results might be explained by a difference in DBP binding, which is believed to correlate with availability of the compounds to target cells [31]. Among the four analogues with (20*S*)-configuration, **7a** exhibited 3.3 times higher potency than the natural hormone **1**. Epimerization of the C1, C2 or C3 substituent in **7a** yields **7c**, **7b** and **7e**, respectively, all of which showed similar cell differentiation-inducing activity. As regards the activity of 20-*epi*-1α,25-dihydroxyvitamin D_3, epimerization of either the C1- or C3-hydroxyl group dramatically decreases cell differentiation-inducing activity by 360 times for C1 and 17 times for C3 [29]. In the case of 5,6-*trans*-2-methyl analogues, however, the beneficial effects of 'up' methyl introduction seemed to be well retained irrespective of C1 or C3 stereochemistry, and as a result, both the 1-epimer (**7c**) and 3-epimer (**7e**) of **7a** showed significant cell differentiation-inducing activity (approximately one-third of the activity of the natural hormone **1**).

2.4. *Docking modes of the A-ring analogues to vitamin D receptor*

Interaction of the side chains of vitamin D analogues with VDR is well documented, and Yamada et al. showed that potential side chain structures, as well as their functions, can be predicted by conformational analysis using molecular mechanics [33]. Molecular modeling studies of the LBD of human VDR have reinforced the above concept [34]. Furthermore, differential interaction of compounds having the natural side chain and their 20-epimers has been demonstrated based upon the X-ray structures of mutant human VDR complexed with 20-*epi* analogues [35]. In contrast, little is known about the structure–activity relationships of the A-ring, irrespective of 5,6-*cis* or 5,6-*trans* structure.

Besides the conformational problems in the A-ring, additional effects of 2-methyl substitution, which gave the analogues characteristic activity profiles, appeared to exist. The present data on the 5,6-*trans*-2-methyl analogues (**6a–h**, **7a–h**) have shed some light on the significance of 2-methyl introduction. Among the epimeric pairs differing only in the configuration at the C2 position, every 2β-methyl (i.e., *pseudo* 2α-methyl) analogue exhibited higher VDR binding potency than the corresponding 2α-methyl (i.e., *pseudo* 2β-methyl) analogue by 10–60-fold. This may mean that an 'upper' methyl group plays an important role in elevation of the VDR binding potency.

The amino acid residues in the LBD of VDR are essentially conserved among mammals, including bovine, porcine and human. The recently solved X-ray crystal structure of a mutant human VDR complexed with **1** demonstrated that the A-ring of **1** was docked to the LBD of VDR in its β-form [21]. The equatorial 1α-hydroxyl group of **1** formed two hydrogen bonds with Ser-237(H3) and Arg-274(H5), while the axial 3β-hydroxyl

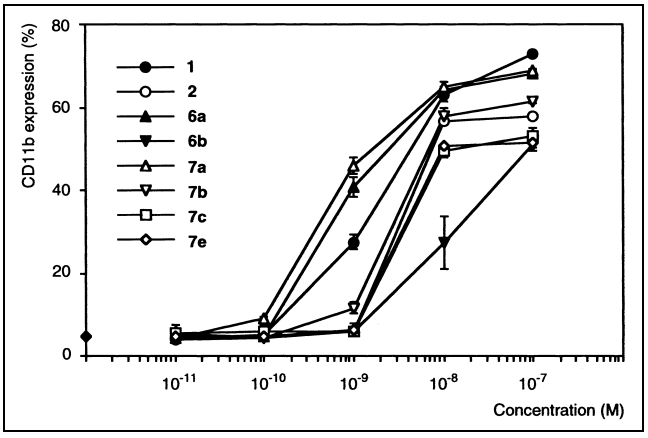


Fig. 6. HL-60 cell differentiation-inducing activity of the 5,6-*trans*-2-methyl analogues. HL-60 cells were treated with between 10^{-11} M and 10^{-7} M 1α,25-dihydroxyvitamin D_3 (**1**) or the analogues (**2, 6a, 6b, 7a, 7b, 7c, 7e**) for 72 h. Cell differentiation was assessed in terms of expression of CD11b. Vehicle average is denoted by a solid diamond on the *y*-axis. The data are summarized as relative potencies with respect to **1**, defined as 100, in Table 1.

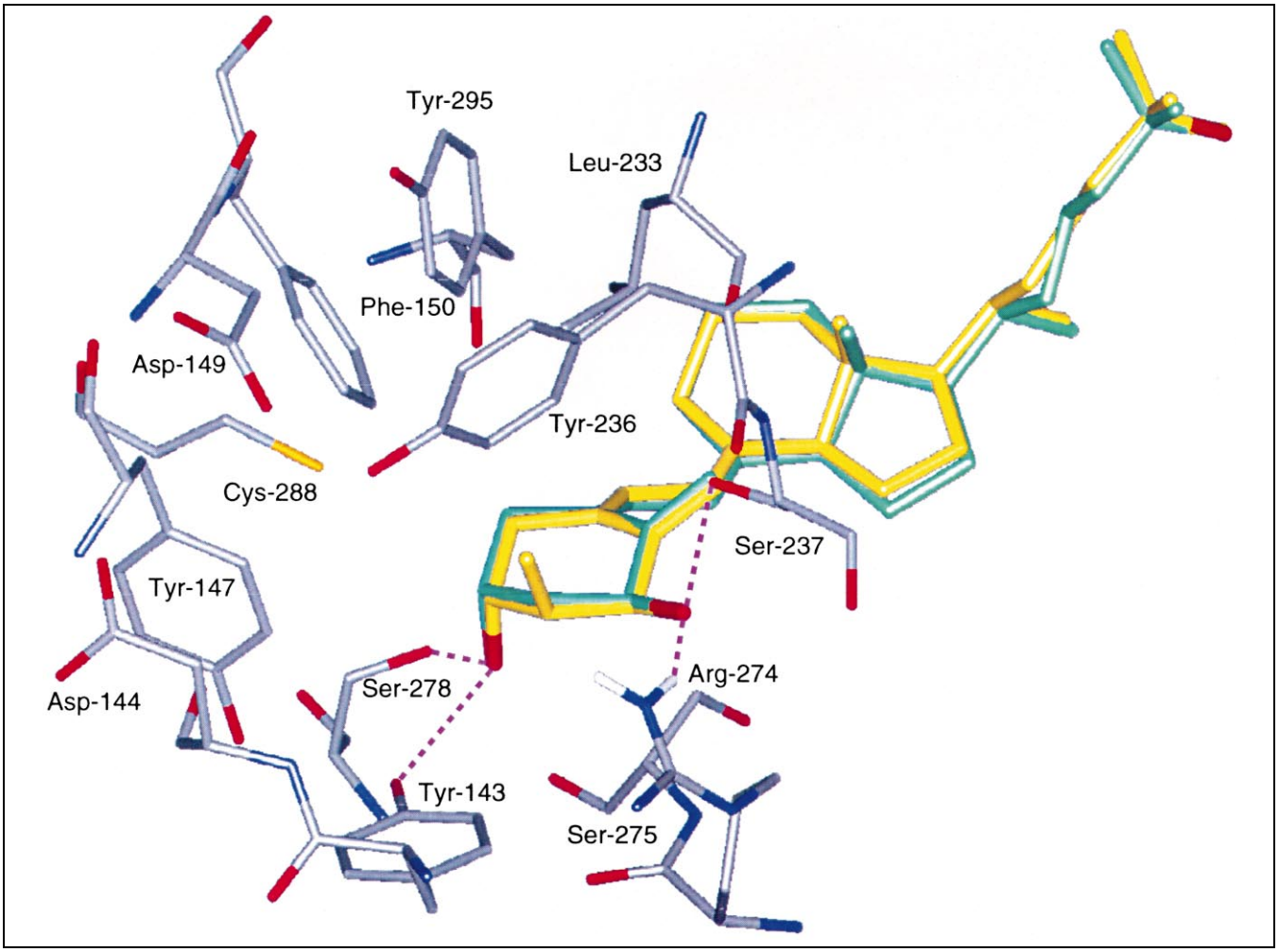


Fig. 7. Overlay of the modeled VDR/2α-methyl-1α,25-dihydroxyvitamin D_3 (**4a**; carbon atoms in yellow) complex with the VDR/1α,25-dihydroxyvitamin D_3 (**1**; carbon atoms in green) structure [21] in the LBD. Hydrogen bonds are drawn as dotted lines. The axial 2-methyl group of **4a** makes a favorable contact with a hydrophobic region surrounded by Leu-233, Tyr-236 and Phe-150. Of the 32 amino acid residues used for the calculation, only those in the close vicinity of the A-ring are included for clarity.

group of **1** formed two hydrogen bonds with Ser-278(H5) and Tyr-143. Interestingly, the experimental data show that there is additional space near the C2 position, which could accommodate a rather bulky substituent. The cavity is long, reaching the surface of the receptor. In the vicinity of the C2 position in the A-ring, the natural hormone **1** is surrounded by hydrophobic amino acid residues, such as Leu-233(H3), Tyr-236(H3) and Phe-150.

Among the 5,6-*cis* analogues, 2β-methyl-1α,25-dihydroxyvitamin D_3 (**4b**), the A-ring of which mainly exists in the β-form, showed a slightly reduced VDR affinity [19]. The VDR binding potencies of 2β-alkyl-substituted analogues were reported by us and by others using bovine thymus VDR. The estimates for 2β-methyl- (**4b**), 2β-ethyl- and 2β-propyl-1α,25-dihydroxyvitamin D_3, relative to **1** normalized to 100, are 13, 10 and 26, respectively [16–19,36]. Since the natural ligand **1** was bound in its β-form with additional space near position 2, these 2β-substituted analogues would interact with the LBD of VDR in their β-forms with the 2β-substituents in the equatorial positions without unfavorable structural dispositions. Indeed, the VDR binding potency was essentially unaltered among those three analogues.

Binding modes of the 2α-substituted 5,6-*cis* analogues, however, could differ from those of 2β-substituted analogues. We have synthesized a series of 2α-substituted analogues having various lengths of 2-alkyl, 2-(ω-hydroxyalkyl) and 2-(ω-hydroxyalkoxy) groups, and found that the 2α-substituted analogues with terminal hydroxyl groups exhibited a marked elevation in VDR binding potency [37,38]. Due to the long 2α-substituent, the A-ring of 2α-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3, which showed 1.8-fold higher VDR affinity than **1**, would adopt the α-form to dock into the LBD of VDR, facilitating an additional hydrogen bond network involving its terminal hydroxyl group with Asp-144 and Tyr-236 [38]. This, combined with two other molecular modeling results on the LBD of VDR [34,39], may mean that vitamin D analogues could be accommodated in the LBD of the receptor in either α- or β-form.

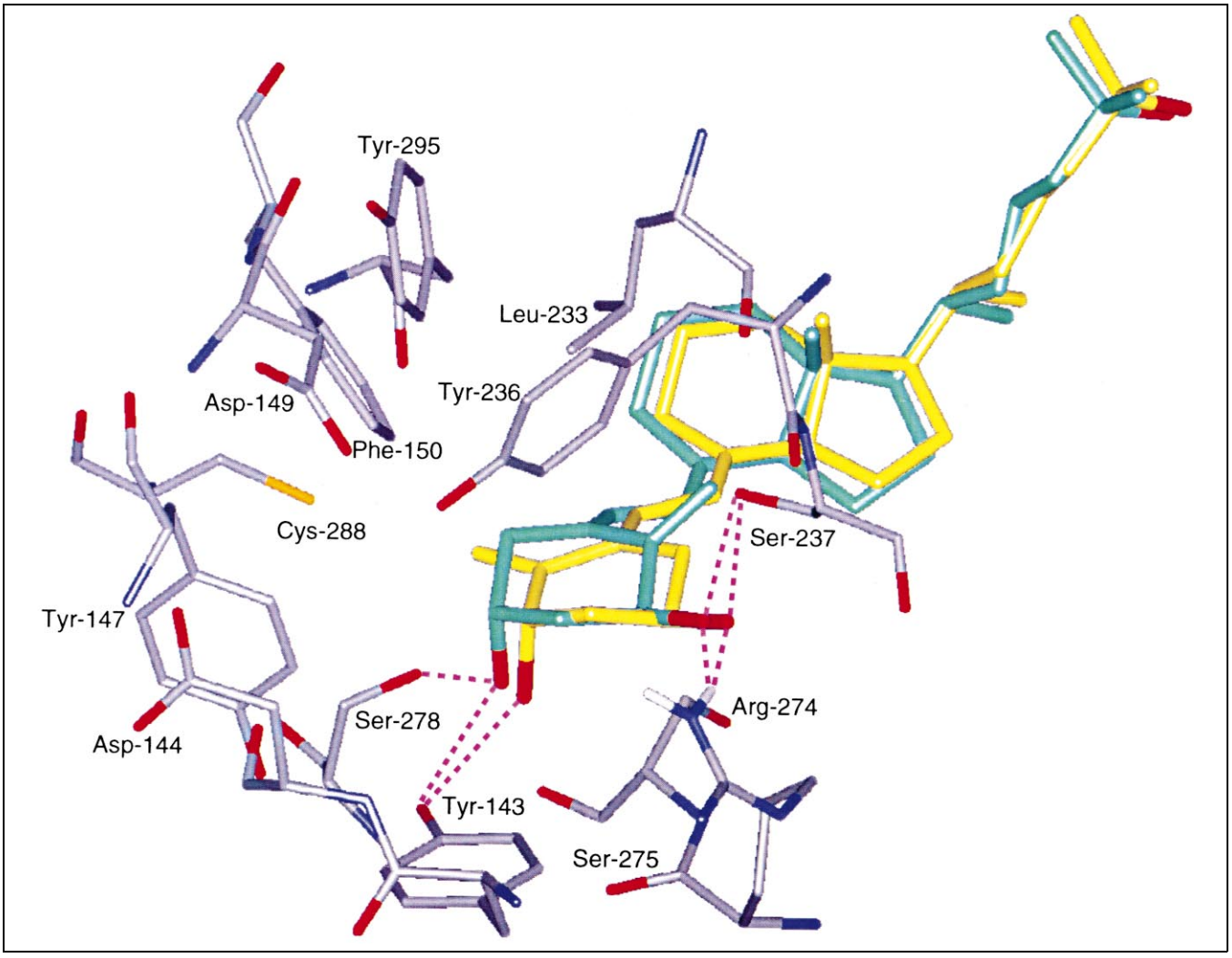


Fig. 8. Overlay of the modeled VDR/5,6-*trans*-methyl-1α,25-dihydroxyvitamin D_3 (**2**; carbon atoms in yellow) complex with the VDR/1α,25-dihydroxyvitamin D_3 (**1**; carbon atoms in green) structure [21] in the LBD. Hydrogen bonds are drawn as dotted lines. Due to the transposed C10(19) exomethylene group, the A-ring of **2** would be forced outward by Cys-288, resulting in a less favorable binding mode in terms of the C1 and C3 hydroxyl groups. Of the 32 amino acid residues used for the calculation, only those in the close vicinity of the A-ring are included for clarity.

The 2α-methyl introduction into the A-ring of **1** (as in **4a**), which favored the α-form, caused a marked elevation in VDR affinity [19]. The VDR binding potencies of 2α-methyl- (**4a**), 2α-ethyl- and 2α-propyl-1α,25-dihydroxyvitamin D_3 relative to **1** (normalized to 100) were estimated to be 400, 40 and 20, respectively, by using bovine thymus VDR [16–19,37]. These 2α-substituted analogues could interact with the LBD of VDR in their α-forms with the 2α substituents in the equatorial positions as stated above in the case of 2α-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3. However, the VDR affinity of 2α-methyl-1α,25-dihydroxyvitamin D_3 (**4a**) was considerably high among the three analogues compared with the corresponding 2β-alkyl-substituted counterparts, each of which showed similar affinity. We speculated that 2α-methyl-1α,25-dihydroxyvitamin D_3 (**4a**) would bind to the LBD in its β-form, essentially retaining the hydrogen bond networks observed in the X-ray-solved structure of **1** in the receptor. In this case, the axial 2α-methyl group would fit into the 'upper' hydrophobic pocket. Molecular mechanics calculations based upon the X-ray crystal structure [21] support the above hypothesis. Fig. 7 depicts a superposition of 2α-methyl-1α,25-dihydroxyvitamin D_3 (**4a**) and the natural hormone (**1**) bound to the LBD of VDR. A noteworthy feature is the lipophilic extension of **4a** provided by the axial 2α-methyl substituent which reaches closer to the 'upper' lipophilic pocket formed by Leu-233(H3), Tyr-236(H3) and Phe-150, leading to enhanced interaction. The axial 2α-methyl group would be in the close vicinity of the aromatic amino acid residues Tyr-236(H3) and Phe-150, which suggests the involvement of CH/π interaction [40], in addition to the steric effects. This binding mode provides a basis for the structure–activity relationships of the 2-methyl-substituted analogues.

The VDR binding potency of 5,6-*trans*-1α,25-dihydroxyvitamin D_3 (**2**) was reduced by approximately 100-fold in

comparison with **1**. This may be explained by unfavorable steric contacts of the transposed exomethylene group with the amino acid residues of the LBD of VDR. Superimposed docking models of **1** and **2** show that the C10(19) exomethylene group of **2** approaches the side chain of Cys-288(β-sheet), which causes a significantly less favorable binding mode (Fig. 8). In the case of 5,6-*trans*-2-methyl analogues, the axial 2-methyl group would contact the 'upper' lipophilic pocket, which indeed resulted in an approximately 10-fold elevation of the VDR affinity as seen in **6a** compared to **2**. In this situation, the A-ring of **6a** would adopt the β-form, retaining the hydrogen bond networks of **2** in terms of the equatorial 3-hydroxyl group and the axial 1-hydroxyl group. Among the 19-*nor* compounds, the VDR binding potency of 2α-methyl-1α,25-dihydroxy-19-norvitamin D_3 was reported to be 22, relative to **1** normalized to 100, and that of its 2-epimer, 2β-methyl-1α,25-dihydroxy-19-norvitamin D_3, to be 2.6 by using porcine intestinal VDR [15]. Similarly, the axial 2-methyl substituent of 2α-methyl-1α,25-dihydroxy-19-norvitamin D_3 could interact with the 'upper' lipophilic pocket. However, the lack of the C10(19) exomethylene group would make the A-ring of the 19-*nor* analogues rather compact and further intrusion into the bottom of the cavity might afford alternative binding modes. Further studies are required to assess the binding modes of the 19-*nor* analogues, taking into account the water molecules involved in this cavity [41].

3. Significance

All possible A-ring stereoisomers of 5,6-*trans*-2-methyl-1,25-dihydroxyvitamin D_3 (**6a–h**) and their 20-epimers (**7a–h**) were designed and efficiently synthesized. Biological evaluation of the 5,6-*trans* analogues revealed that *pseudo* 2α-methyl introduction into the parent 5,6-*trans*-1α,25-dihydroxyvitamin D_3 (**2**) enhanced the potency, as in the case of the 2α-methyl introduction into 1α,25-dihydroxyvitamin D_3 (**1**), which reinforced the idea of a beneficial effect of the 'up' methyl introduction. The combined modification of *pseudo* 2α-methyl introduction and 20-epimerization had additive effects on VDR binding activity. Transposition of the C10(19) exomethylene group, on the other hand, differentially affected DBP binding and HL-60 cell differentiation-inducing activity of the 2-methyl analogues. Molecular docking studies of the analogues in the LBD of VDR based upon the recently solved X-ray crystal structure of mutant VDR suggested that the axial 2-methyl substituent would contact a hydrophobic region, resulting in enhanced interaction. The putative binding modes of the potent 2-methyl analogues well explained the results of biological evaluation of these analogues. These binding models should provide a new design concept for VDR ligands and stimulate structure–function studies focused upon the protein–ligand complex.

4. Materials and methods

4.1. Chemical synthesis

Melting points were determined by using a Yanagimoto hot-stage melting point apparatus and are uncorrected. NMR spectra were recorded on a JEOL GSX-400 spectrometer. Chemical shifts are expressed in ppm relative to tetramethylsilane. Mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded on a JMS-SX 102A. Infrared spectra were recorded on a Jasco FT/IR-8000 spectrometer and are expressed in cm^{-1}. Ultraviolet spectra were recorded with a Shimadzu UV-1600 spectrophotometer. Optical rotations were determined by using a Jasco DIP-370 digital polarimeter. Elemental analyses were carried out in the Microanalytical Laboratory, Faculty of Pharmaceutical Sciences, University of Tokyo, and were within 0.3% of the theoretical values.

4.1.1. (5E,7E)-(1S,3R)-9,10-Seco-5,7,10(19)-cholestatriene-1,3,25-triol (2)

(5*Z*,7*E*)-(1*S*,3*R*)-9,10-Seco-5,7,10(19)-cholestatriene-1,3,25-triol (**1**) (16.5 mg, 0.04 mmol) was dissolved in liquid SO_2 (~10 ml), and the solution was refluxed at the boiling temperature of SO_2 for 30 min. After SO_2 was evaporated, the residue was dissolved in ethanol (2 ml), and $NaHCO_3$ (17 mg, 0.2 mmol) was added. The resulting mixture was heated at 90°C for 4 h. After the solvent was evaporated, brine was added to the mixture and the whole was extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered and concentrated. The crude product was purified by silica gel preparative TLC (ethyl acetate) to give the vitamin (13.7 mg, 83%) as a white solid. Further purification was conducted using reversed-phase recycle HPLC (YMC-Pack ODS column, 20 mm × 150 mm, 9.0 ml/min, acetonitrile:water = 8:2), followed by recrystallization from ethyl acetate to give analytically pure vitamin as colorless fine prisms: mp 175–176°C (recryst. from ethyl acetate); $[\alpha]_D^{27}$ +159 (c = 0.059, $CHCl_3$); UV (EtOH) λ_{max} 274 nm (ε: 23 500), λ_{min} 230 nm (ε: 4900); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.56 (3 H, s), 0.94 (3 H, d, J = 6.1 Hz), 1.22 (6 H, s), 2.28 (1 H, dd, J = 13.4, 8.5 Hz), 2.86 (2 H, m), 4.23 (1 H, dddd, J = 8.4, 8.1, 4.0, 3.7 Hz), 4.49 (1 H, dd, J = 5.2, 4.0 Hz), 4.97 (1 H, s), 5.12 (1 H, s), 5.88 (1 H, d, J = 11.6 Hz), 6.58 (1 H, d, J = 11.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.1 (q), 18.8 (q), 20.8 (t), 22.3 (t), 23.5 (t), 27.6 (t), 29.1 (t), 29.2 (q), 29.4 (q), 33.6 (d), 36.1 (t), 37.8 (t), 40.4 (t), 42.0 (t), 44.4 (t), 46.0 (s), 56.5 (d), 56.6 (d), 65.9 (d), 71.1 (d), 73.4 (s), 109.6 (t), 115.9 (d), 123.4 (d), 132.0 (s), 132.6 (d), 151.7 (s); FTIR (neat) 3422, 2936, 2874, 2361, 2334, 1716, 1651, 1558, 1456, 1375, 1261, 1153, 1051, 908 cm^{-1}; MS 416 $[M]^+$, 398 $[M-H_2O]^+$, 380 $[M-2H_2O]^+$; HRMS calcd. for $[C_{27}H_{44}O_3]$ 416.3263, found 416.3260; anal. calcd. for $C_{27}H_{44}O_3 \cdot 1/4H_2O$: C, 77.00; H, 10.65. Found: C, 76.91; H.10.43.

4.1.2. (5E,7E)-(1S,2R,3R)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (6a)

(5*Z*,7*E*)-(1*S*,2*R*,3*R*)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**4b**) (7.0 mg, 0.016 mmol) was dissolved in liquid SO_2 (~10 ml), and the solution was refluxed at the boiling temperature of SO_2 for 1 h. After SO_2 was evaporated, the residue was dissolved in ethanol (2 ml), and $NaHCO_3$ (6.8 mg, 0.081 mmol) was added. The resulting mixture was heated at 90°C for 1 h. After the solvent was evaporated, brine was added to the

mixture and the whole was extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered and concentrated. The crude product was purified by silica gel preparative TLC (ethyl acetate) to give **6a** (4.6 mg, 66%) as a colorless solid. Further purification for biological evaluation was conducted by using reversed-phase recycle HPLC (YMC-Pack ODS column, 20 mm × 150 mm, 9.0 ml/min, acetonitrile:water = 8:2): UV (EtOH) λ_{max} 272 nm, λ_{min} 230 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.56 (3 H, s), 0.94 (3 H, d, J = 6.4 Hz), 1.08 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.94 (1 H, ddq, J = 7.2, 3.3, 7.0 Hz), 2.56 (1 H, dd, J = 13.7, 3.4 Hz), 2.60 (1 H, dd, J = 14.6, 6.7 Hz), 2.83 (1 H, dd, J = 11.5, 4.4 Hz), 4.13 (1 H, d, J = 7.2 Hz), 4.14 (1 H, dt, J = 6.7, 3.4 Hz), 5.01 (1 H, s), 5.15 (1 H, s), 5.87 (1 H, d, J = 11.6 Hz), 6.61 (1 H, d, J = 11.6 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3446.

*4.1.3. (5E,7E)-(1S,2S,3R)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**6b**)*

This compound was obtained from **4a** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 273 nm, λ_{min} 230 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.57 (3 H, s), 0.95 (3 H, d, J = 6.4 Hz), 1.14 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.83 (1 H, ddq, J = 9.0, 3.2, 7.0 Hz), 2.13 (1 H, dd, J = 13.9, 9.0 Hz), 2.85 (1 H, dd, J = 12.0, 4.2 Hz), 3.02 (1 H, dd, J = 14.0, 4.3 Hz), 3.85 (1 H, dt, J = 4.6, 9.0 Hz), 4.29 (1 H, d, J = 3.1 Hz), 4.93 (1 H, s), 5.12 (1 H, d, J = 1.8 Hz), 5.89 (1 H, d, J = 11.6 Hz), 6.55 (1 H, dd, J = 11.6, 0.9 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3447.

*4.1.4. (5E,7E)-(1R,2R,3R)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**6c**)*

This compound was obtained from **4f** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 274 nm, λ_{min} 231 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.57 (3 H, s), 0.94 (3 H, d, J = 6.4 Hz), 1.22 (6 H, s), 1.24 (3 H, d, J = 7.0 Hz), 1.92 (1 H, ddq, J = 2.4, 2.5, 7.0 Hz), 2.27 (1 H, dd, J = 14.7, 3.1 Hz), 2.88 (1 H,dd, J = 12.8, 3.7 Hz), 3.05 (1 H, dd, J = 14.6, 3.7 Hz), 3.97 (1 H, ddd, J = 2.4, 3.1, 3.7 Hz), 4.21 (1 H, d, J = 2.5 Hz), 4.90 (1 H, d, J = 1.8 Hz), 5.10 (1 H, d, J = 1.8 Hz), 5.91 (1 H, d, J = 11.3 Hz), 6.67 (1 H, d, J = 11.3 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3449.

*4.1.5. (5E,7E)-(1R,2S,3R)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**6d**)*

This compound was obtained from **4e** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 271 nm, λ_{min} 229 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.57 (3 H, s), 0.95 (3 H, d, J = 6.2 Hz), 1.03 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.89 (1 H, ddq, J = 5.5, 4.8, 7.0 Hz), 2.06 (1 H, dd, J = 15.0, 5.8 Hz), 2.65 (1 H, dd, J = 15.0, 4.8 Hz), 2.87 (1 H, dd, J = 12.2, 3.7 Hz), 3.71 (1 H, dt, J = 5.8, 4.8 Hz), 3.98 (1 H, d, J = 5.5 Hz), 4.97 (1 H, s), 5.17 (1 H, s), 5.89 (1 H, d, J = 11.7 Hz), 6.62 (1 H, d, J = 11.7 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3448.

*4.1.6. (5E,7E)-(1S,2R,3S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**6e**)*

This compound was obtained from **4d** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 271 nm, λ_{min} 229 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.57 (3 H, s), 0.95 (3 H, d, J = 6.4 Hz), 1.03 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.91 (1 H, ddq, J = 5.1, 4.8, 7.0 Hz), 2.61 (1 H, dd, J = 15.0, 4.4 Hz), 2.65 (1 H, dd, J = 15.0, 5.1 Hz), 2.86 (1 H, dd, J = 11.9, 3.7 Hz), 3.74 (1 H, dt, J = 4.4, 5.1 Hz), 4.00 (1 H, d, J = 5.1 Hz), 4.97 (1 H, s), 5.18 (1 H, d, J = 1.6 Hz), 5.90 (1 H, d, J = 11.6 Hz), 6.62 (1 H, d, J = 11.6 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3444.

*4.1.7. (5E,7E)-(1S,2S,3S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**6f**)*

This compound was obtained from **4c** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 274 nm, λ_{min} 231 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.57 (3 H, s), 0.95 (3 H, d, J = 6.4 Hz), 1.22 (6 H, s), 1.24 (3 H, d, J = 7.0 Hz), 1.93 (1 H, ddq, J = 2.4, 2.1, 7.0 Hz), 2.28 (1 H, dd, J = 14.6, 2.4 Hz), 2.88 (1 H, dd, J = 12.2, 3.7 Hz), 3.06 (1 H, dd, J = 14.6, 3.7 Hz), 3.98 (1 H, ddd, J = 3.7, 2.4, 2.1 Hz), 4.21 (1 H, d, J = 2.1 Hz), 4.91 (1 H, d, J = 1.8 Hz), 5.12 (1 H, d, J = 1.8 Hz), 5.92 (1 H, d, J = 11.3 Hz), 6.67 (1 H, d, J = 11.3 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3448.

*4.1.8. (5E,7E)-(1R,2R,3S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**6g**)*

This compound was obtained from **4h** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 275 nm, λ_{min} 231 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.57 (3 H, s), 0.95 (3 H, d, J = 6.4 Hz), 1.14 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.83 (1 H, ddq, J = 9.2, 3.1, 7.0 Hz), 2.13 (1 H, dd, J = 14.0, 9.2 Hz), 2.85 (1 H, dd, J = 11.9, 4.0 Hz), 3.01 (1 H, dd, J = 14.0, 4.5 Hz), 3.87 (1 H, dt, J = 4.5, 9.2 Hz), 4.30 (1 H, d, J = 3.1 Hz), 4.93 (1 H, d, J = 1.8 Hz), 5.11 (1 H, d, J = 1.8 Hz), 5.89 (1 H, d, J = 11.6 Hz), 6.55 (1 H, d, J = 11.6 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3442.

*4.1.9. (5E,7E)-(1R,2S,3S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**6h**)*

This compound was obtained from **4g** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 274 nm, λ_{min} 232 nm; ^{1}H NMR (400 MHz, $CDCl_3$–D_2O) δ 0.57 (3 H, s), 0.95 (3 H, d, J = 6.4 Hz), 1.08 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.93 (1 H, ddq, J = 8.0, 3.4, 7.0 Hz), 2.53 (1 H, dd, J = 14.3, 3.4 Hz), 2.61 (1 H, dd, J = 14.3, 5.8 Hz), 2.85 (1 H, dd, J = 12.2, 3.7 Hz), 4.15 (2 H, m), 5.01 (1 H, s), 5.15 (1 H, s), 5.86 (1 H, d, J = 11.3 Hz), 6.60 (1 H, d, J = 11.3 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3443.

*4.1.10. (5E,7E)-(1S,2R,3R,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7a**)*

This compound was obtained from **5d** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 272 nm, λ_{min} 230 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.56 (3 H, s), 0.86 (3 H, d, J = 6.4 Hz), 1.09 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.88 (1 H, m), 1.95 (1 H, ddq, J = 7.6, 3.4, 7.0 Hz), 2.53 (1 H, dd, J = 14.3, 4.0 Hz), 2.60 (1 H, dd, J = 14.6. 7.0 Hz), 4.13 (1 H, d, J = 7.6 Hz), 4.17 (1 H, ddd, J = 7.0, 4.0, 3.4 Hz), 5.01 (1 H, s), 5.16 (1 H, s), 5.87

(1 H, d, J = 11.6 Hz), 6.61 (1 H, d, J = 11.6 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 379 $[M-H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3445.

*4.1.11. (5E,7E)-(1S,2S,3R,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7b**)*

This compound was obtained from **5a** by the same procedure as described for **6a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.56 (3 H, s), 0.86 (3 H, d, J = 6.4 Hz), 1.14 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.83 (1 H, m), 3.01 (1 H, dd, J = 14.3, 5.2 Hz), 3.84 (1 H, m), 4.29 (1 H, m), 4.93 (1 H, d, J = 2.1 Hz), 5.12 (1 H, d, J = 2.1 Hz), 5.89 (1 H, d, J = 11.4 Hz), 6.54 (1 H, d, J = 11.4 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3473.

*4.1.12. (5E,7E)-(1R,2R,3R,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7c**)*

This compound was obtained from **5f** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 274 nm, λ_{min} 231 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.57 (3 H, s), 0.86 (3 H, d, J = 6.4 Hz), 1.21 (6 H, s), 1.24 (3 H, d, J = 7.0 Hz), 2.28 (1 H, br. d, J = 14.6 Hz), 2.88 (1 H, dd, J = 12.4, 3.7 Hz), 3.04 (1 H, dd, J = 15.0, 4.0 Hz), 3.97 (1 H, m), 4.21 (1 H, m), 4.90 (1 H, d, J = 1.5 Hz), 5.10 (1 H, d, J = 1.8 Hz), 5.91 (1 H, d, J = 11.6 Hz), 6.66 (1 H, d, J = 11.6 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3423.

*4.1.13. (5E,7E)-(1R,2S,3R,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7d**)*

This compound was obtained from **5e** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 270 nm, λ_{min} 230 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.57 (3 H, s), 0.86 (3 H, d, J = 6.4 Hz), 1.03 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 1.87 (1 H, m), 2.60 (1 H, dd, J = 15.3, 6.1 Hz), 2.64 (1 H, dd, J = 15.3, 4.6 Hz), 2.87 (1 H, dd, J = 11.9, 4.0 Hz), 3.71 (1 H, m), 3.98 (1 H, m), 4.97 (1 H, s), 5.17 (1 H, d, J = 1.8 Hz), 5.90 (1 H, d, J = 11.6 Hz), 6.61 (1 H, d, J = 11.6 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 397 $[M-H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3465.

*4.1.14. (5E,7E)-(1S,2R,3S,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7e**)*

This compound was obtained from **5d** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 271 nm, λ_{min} 229 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.56 (3 H, s), 0.86 (3 H, d, J = 6.4 Hz), 1.03 (3 H, d, J = 7.4 Hz), 1.21 (6 H, s), 2.62 (2 H, m), 2.86 (1 H, m), 3.75 (1 H, m), 4.00 (1 H, m), 4.97 (1 H, s), 5.18 (1 H, d, J = 1.8 Hz), 5.90 (1 H, d, J = 11.6 Hz), 6.62 (1 H, d, J = 11.9 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 394 $[M-2H_2O]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3442.

*4.1.15. (5E,7E)-(1S,2S,3S,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7f**)*

This compound was obtained from **5c** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 274 nm, λ_{min} 231 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.57 (3 H, s), 0.86 (3 H, d, J = 6.4 Hz), 1.22 (6 H, s), 1.24 (3 H, d, J = 7.3 Hz), 2.27 (1 H, br. d, J = 14.1 Hz), 2.87 (1 H, dd, J = 12.5, 4.0 Hz), 3.06 (1 H, dd, J = 14.0, 3.7 Hz), 3.98 (1 H, m), 4.20 (1 H, m), 4.91 (1 H, d, J = 1.8 Hz), 5.12 (1 H, d, J = 1.8 Hz), 5.92 (1 H, d, J = 11.6 Hz), 6.66 (1 H, d, J = 11.9 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 397 $[M-H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3453.

*4.1.16. (5E,7E)-(1R,2R,3S,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7g**)*

This compound was obtained from **5h** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 275 nm, λ_{min} 231 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.57 (3 H, s), 0.86 (3 H, d, J = 6.7 Hz), 1.14 (3 H, d, J = 7.0 Hz), 1.22 (6 H, s), 2.13 (1 H, dd, J = 13.7, 8.5 Hz), 2.85 (1 H, dd, J = 11.9, 4.0 Hz), 3.01 (1 H, dd, J = 14.0, 4.6 Hz), 3.86 (1 H, dt, J = 4.9, 8.5 Hz). 4.29 (1 H, d, J = 2.7 Hz), 4.92 (1 H, d, J = 1.2 Hz), 5.10 (1 H, d, J = 1.8 Hz), 5.88 (1 H, d, J = 11.6 Hz), 6.55 (1 H, d, J = 11.3 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 397 $[M-H_2O-Me]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447, found 430.3444.

*4.1.17. (5E,7E)-(1R,2S,3S,20S)-2-Methyl-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol (**7h**)*

This compound was obtained from **5g** by the same procedure as described for **6a**: UV (EtOH) λ_{max} 274 nm, λ_{min} 231 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.56 (3 H, s), 0.86 (3 H, d, J = 6.4 Hz), 1.08 (3 H, d, J = 7.0 Hz), 1.21 (6 H, s), 2.53 (1 H, dd, J = 14.3, 3.1 Hz), 2.60 (1 H, dd, J = 14.3, 5.8 Hz), 2.85 (1 H, dd, J = 12.5, 4.3 Hz), 4.16 (2 H, m), 5.01 (1 H, d, J = 1.2 Hz), 5.15 (1 H, d, J = 1.2 Hz), 5.86 (1 H, d, J = 11.6 Hz), 6.60 (1 H, d, J = 11.3 Hz); MS 430 $[M]^+$, 412 $[M-H_2O]^+$, 397 $[M-H_2O-Me]^+$, 379 $[M-2H_2O-Me]^+$; HRMS calcd. for $[C_{28}H_{46}O_3]$ 430.3447. found 430.3446.

4.2. Binding to VDR

Bovine thymus 1α,25-dihydroxyvitamin D_3 receptor was obtained from Yamasa Biochemical (Chiba, Japan) and dissolved in 0.05 M phosphate buffer (pH 7.4) containing 0.3 M KCl and 5 mM dithiothreitol just before use. The receptor solution (500 μl, 0.23 mg protein) was pre-incubated with 50 μl of ethanol solution of 1α,25-dihydroxyvitamin D_3 or an analogue at various concentrations for 60 min at 25°C. Then, the receptor mixture was left to stand overnight with 0.1 nM [^{3}H]-1α,25-dihydroxyvitamin D_3 at 4°C. The bound and free [^{3}H]-1α,25-dihydroxyvitamin D_3 were separated by treatment with dextran-coated charcoal for 30 min at 4°C and centrifuged at 3000 rpm for 10 min. The supernatant (500 μl) was mixed with ACS-II (9.5 ml) (Amersham, UK) and the radioactivity was counted. The relative potency of the analogues was calculated from their concentration needed to displace 50% of [^{3}H]-1α,25-dihydroxyvitamin D_3 from its receptor compared with the activity of 1α,25-dihydroxyvitamin D_3 (assigned a 100% value).

4.3. Binding to DBP

Serum from vitamin D-deficient Wistar male rats was diluted (×70 000) with 3.5 mM barbiturate buffer (pH 8.6) containing 0.13 M NaCl and 0.1% (w/v) bovine serum albumin (BSA) and used as a source of DBP. The diluted serum (1 ml) was incubated with 0.1 nM [^{3}H]-25-hydroxyvitamin D_3 and 100 μl of ethanol solution of 1α,25-dihydroxyvitamin D_3 or an analogue at various concentrations for 60 min at 4°C. Unbound [^{3}H]-25-hydroxyvitamin D_3 was removed by treatment with dextran-coated charcoal for 10 min at 4°C followed by centrifugation at 3000 rpm for

10 min. The radioactivity of the supernatant (1 ml) was measured. The relative potency of the analogues was calculated from their concentration needed to displace 50% of [^{3}H]-25-hydroxyvitamin D_3 from its binding protein compared with the activity of 1α,25-dihydroxyvitamin D_3 (assigned a 100% value).

4.4. Cell surface antigen expression analysis

HL-60 cells were seeded at 10^5 cells/well in 24-well plates, and incubated for 72 h with between 10^{-11} M and 10^{-7} M (or 10^{-8} M) 1α,25-dihydroxyvitamin D_3 or an analogue at 37°C in a humidified atmosphere of 5% carbon dioxide in air. The cells were then washed with PBS and diluted with 50 µl of Diluent solution (PBS (minus Mg^{2+} minus Ca^{2+}) containing 1% BSA and 1% NaN_3). Aliquots of cell suspension (50 µl) were incubated with 5 µl of the human monoclonal fluorescein isothiocyanate-conjugated CD11b antibody for 30 min at room temperature in the dark. The cells were washed once with 1 ml of Diluent solution and then fixed in 300 µl of PBS containing 2% paraformaldehyde. Fluorescence was read on a Becton Dickinson FACSan™ at an excitation wavelength of 490 nm and emission wavelength of 520 nm. Results were recorded as the mean fluorescence index, which is the product of the percent fluorescence and the mean fluorescence intensity, with 10^4 cells being counted per treatment. Results were expressed as percentage activity (at 50% of the dose-response) in comparison with 1α,25-dihydroxyvitamin D_3 (assigned a 100% value).

4.5. Molecular modeling

4.5.1. Conformational analysis

The A-ring conformations of the vitamin D analogues were calculated using model compounds lacking the side chain at C22 by MacroModel™ version 6.5 (Schroedinger) on a Silicon Graphics O2 workstation. Conformational search was performed with the Monte Carlo method of MacroModel™.

4.5.2. Docking studies

The coordinates of mutant human VDR complexed to **1** were taken from the Brookhaven Protein Data Bank (PDB code 1DB1) and hydrogen atoms were added computationally at appropriate positions. The following 32 amino acid residues in the LBD, which are in the vicinity of **1**, were used for the calculation: Tyr-143, Asp-144, Tyr-147, Asp-149, Phe-150, Leu-227, Leu-230, Leu-233, Val-234, Tyr-236, Ser-237, Ile-268, Ile-271, Met-272, Arg-274, Ser-275, Ser-278, Trp-286, Cys-288, Tyr-293, Tyr-295, Val-300, Ala-303, His-305, Leu-309, Leu-313, His-397, Tyr-401, Leu-404, Leu-414, Val-418 and Phe-422. The residues were constrained with a force constant of 100.0 kJ. Structures of vitamin D analogues bound to VDR were constructed by molecular dynamics (MD) simulation and molecular mechanics (MM) energy minimization. The starting position of the ligand was determined manually: the A-ring moiety of the ligand was superimposed onto its crystallographic counterpart of **1** in the LBD. MD simulation was performed during 10 ps at 300 K. A hundred structures sampled from MD simulation were minimized by MM calculation based upon the AMBER* force field. MD simulation performed during 100 ps at 3000 K, with a force constant of 10 000 kJ, gave similar results. All calculations were performed by MacroModel™ version 6.5 (Schroedinger) on a Silicon Graphics O2 workstation.

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