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Two-dimensional alanine scanning mutational analysis of the interaction between the vitamin D receptor and its ligands: studies of A-ring modified 19-norvitamin D analogs☆,☆☆

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Abstract

To clarify the structure–function relationship (SFR) of vitamin D analogs in terms of their interaction with the vitamin D receptor (VDR), we have proposed a new approach, two-dimensional alanine scanning mutational analysis (2D-ASMA). In this paper, attention was focused on the interactions around the A-ring of vitamin D. For this purpose, we synthesized four new 2-substituted 19-norvitamin D derivatives (**3**–**6**). The VDR affinity (**3**–**6**: 1, 5, 2 and 1/140, respectively) and transcriptional activity (**3**–**6**: 10, 30, 2 and 0.3, respectively) of the four compounds were evaluated relative to 1,25-$(OH)_2D_3$ (**5**) (normalized to 1). Then, the transcriptional activities of wild-type and 18 mutant VDRs induced by the four compounds (**3**–**6**) were investigated. The results of this 18 × 4 2D-ASMA were presented as a patch table, and the effects of the mutations were analyzed in comparison with the natural hormone (**1**) and 2-methylene-19-nor-20-epi-1,25-$(OH)_2D_3$ (2MD, **2**). Of the four A-ring analogs, the 2α-hydroxyethoxy derivative (**3**) showed striking differences in the pattern on the patch table. From the results, we suggest a docking mode of this compound (**3**) in which the A-ring adopts the α conformation.

Keywords: Alanine scanning mutation; Vitamin D receptor; 19-Norvitamin D analogs; Structure–function relationship; Chemical synthesis

1. Introduction

The biological actions of 1,25-$(OH)_2D_3$ (**1**) are mostly mediated through the vitamin D receptor (VDR, NR1I1) [1,2], a member of the nuclear receptor (NR) superfamily [3,4]. Since 1995 a number of three-dimensional structures of the ligand binding domains (LBD) of NRs have been determined by X-ray crystallographic analysis, and this has greatly improved our understanding of the structural bases of the functional mechanism of NRs [5]. The three-dimensional (3D) structure of hVDR-LBD has also been determined by use of a genetically engineered deletion mutant, hVDR LBD (Δ165–215) [6,7]. Although some ambiguities still remain because of the deletion of 51 residues from the loop between helices 1 and 3 [8], this achievement has afforded a great deal of information on the structure–function relationship (SFR) of vitamin D analogs [9,10]. 3D structures of proteins, however, afford only positional information, rather than functional information for each amino acid residue. For example, we readily determine from the 3D structure of the VDR the residues that interact with the ligand 1,25-$(OH)_2D_3$, but we cannot determined the function of each of these residues.

We have been studying the SFR of vitamin D ligands and have proposed a simple SFR theory for vitamin D side chain derivatives [11–18]. In this approach, the SFR of vitamin D side chain analogs can be presented visually based on the active space group concept derived from conformational analysis of the vitamin D side chain. Our SFR study of vitamin D is now focused on the interaction between the VDR and its ligands [9,10]. For this purpose, we need to know the role of each amino acid residue interacting with the ligand inside the ligand binding pocket (LBP). The VDR LBP is lined with about 30 amino acid residues. To investigate the functional interaction of the VDR with its ligands, we have performed complete alanine scanning mutational studies of the LBP residues. In addition, we have extended this alanine scanning mutational analysis (ASMA) by concomitantly varying

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the ligand structure; we term this approach two-dimensional (2D) ASMA. In 2D-ASMA, the transactivation potencies of the VDR and its mutants induced by various vitamin D ligands were studied in series, and the 2D activity data were analyzed by presentation as a patch table. This profiling of the 2D-ASMA results provides us with precise information on the interaction of the VDR with its ligands. In a previous paper, we reported 2D-ASMA (18 × 12) of 18 mutants and 12 ligands including the natural hormone [9].

In this paper, we focused on VDR-ligand interactions around the A-ring. For this purpose, we synthesized four new 2-substituted 19-nor-1,25-$(OH)_2D_3$ derivatives: 2α- and 2β-hydroxyethoxy-19-nor-20-epi-1,25-dihydroxyvitamin D_3 (2αHEOED **3** and 2βHEOED **4**) and (2*E*)- and (2*Z*)-2-hydroxyethylidene-19-nor-1,25-dihydroxyvitamin D_3 (2*E*HED **5** and 2*Z*HED **6**) (Fig. 1A). 2D-ASMA of these compounds was studied in comparison with the natural hormone (**1**) and the closely related 19-norvitamin D analog, 2MD (**2**) [19]. 19-Norvitamin D derivatives [19–22], which were first developed by DeLuca's group, are versatile analogs of vitamin D that are chemically stable and also easy to synthesized.

2. Materials and methods

2.1. Vitamin D compounds

2MD was kindly donated by Dr. DeLuca. Four new 19-norvitamin D derivatives (**3**–**6**) were synthesized in our laboratory following the scheme shown in Fig. 1B. Spectral properties of the four compounds (**3**–**6**) are as follows. Experimental data for the synthesis of the four compounds (**3**–**6**) is available as supplementary material.

2.1.1. (20S)-1α,25-Dihydroxy-2α-(2-hydroxyethoxy)-19-norvitamin D_3 (3)

^{1}H NMR ($CDCl_3$) δ: 0.55 (3H, s, H-18), 0.85 (3H, d, J = 6.5 Hz, H-21), 1.21 (6H, s, H-26, 27), 2.18 (1H, dd, J = 13.2, 9.8 Hz, H-4), 2.20 (1H, m, H-10), 2.33, 2.41, 2.56 (each 1H, br. s, 3 × OH), 3.72–3.83 (4H, m, OCH_2CH_2O), 3.96 (1H, m, H-3), 4.14 (1H, m, H-1), 5.83 (1H, d, J = 11.2 Hz, H-7), 6.34 (1H, d, J = 11.2 Hz, H-6).

LR-MS *m*/*z*: 464 (M^+, 46), 446 (88), 428 (100), 410 (28), 366 (35), 348 (70). HR-MS *m*/*z*: 464.3485 (Calcd for $C_{28}H_{486}O_5$: 464.3502). UV λ_{max} (EtOH): 243, 252, 261 nm.

2.1.2. (20S)-1α,25-Dihydroxy-2β-(2-hydroxyethoxy)-19-norvitamin D_3 (4)

^{1}H NMR ($CDCl_3$) δ: 0.54 (3H, s, H-18), 0.85 (3H, d, J = 6.5 Hz, H-21), 1.21 (6H, s, H-26, 27), 2.35 (1H, br. d, J = 14.2 Hz, H-4), 2.48 (1H, dm, J = 14.2 Hz, H-4), 3.29 (1H, dd, J = 8.7, 2.7 Hz, H-2), 3.67–3.89 (5H, m, H-1 and OCH_2CH_2O), 4.17 (1H, m, H-3), 5.84 (1H, d, J = 11.2 Hz, H-7), 6.28 (1H, d, J = 11.2 Hz, H-6).

LR-MS *m*/*z*: 464 (M^+, 35), 446 (96), 428 (100), 410 (22), 366 (30), 348 (55). HR-MS *m*/*z*: 464.3497 (Calcd for $C_{28}H_{486}O_5$: 464.3502). UV λ_{max} (EtOH): 243, 252, 261 nm.

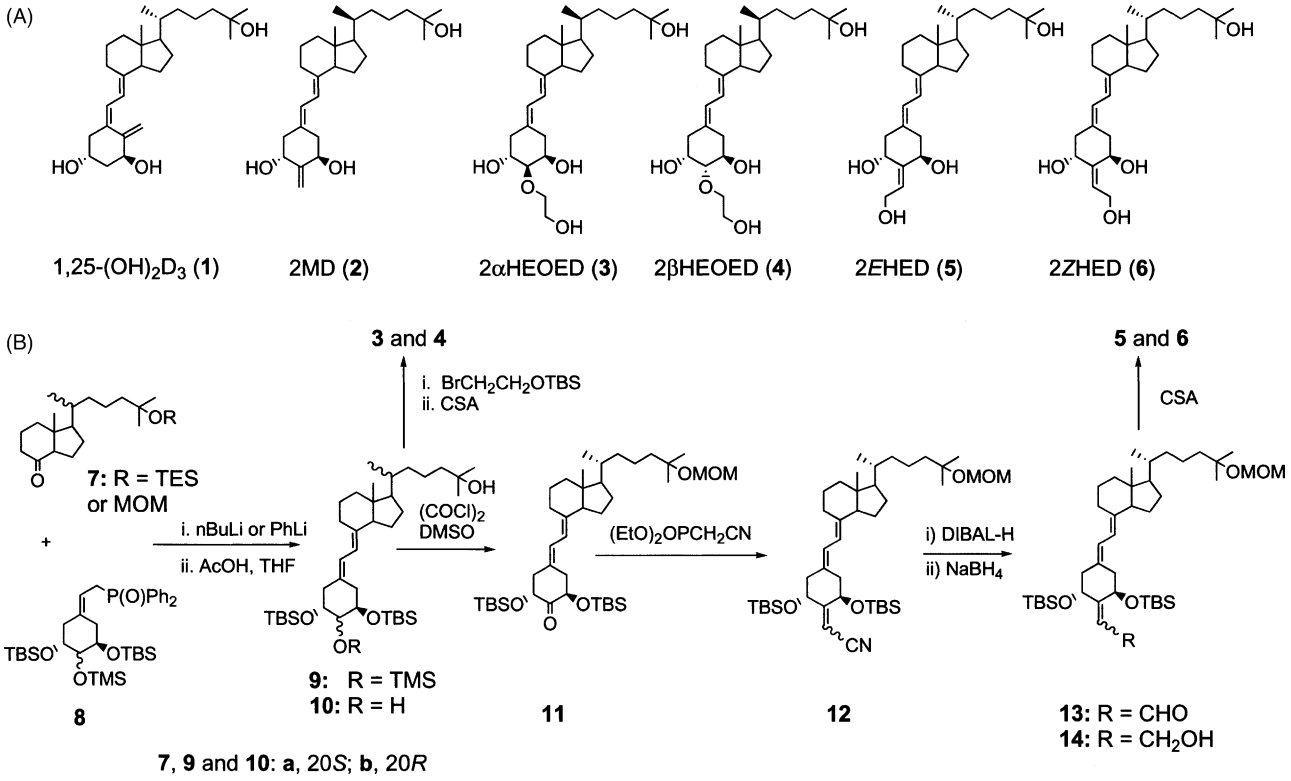

Fig. 1. Chemical structures of vitamin D compounds (A) and synthetic scheme of four 2-substituted 19-norvitamin D derivatives (**3**–**6**) (B).

*2.1.3. (E)-1α,25-Dihydroxy-2-(2-hydroxyethylidene)-19-norvitamin D_3 (**5**)*

^{1}H NMR (CD_3OD) δ: 0.58 (3H, s, H-18), 0.97 (3H, d, J = 6.3 Hz, H-21), 1.17 (6H, s, H-26, 27), 1.86 (1H, t, J =∼ 12 Hz, H-10), 2.35, 2.42 (each 1H, br. d, J = 13.7 Hz, H-4), 4.24 (2H, m, CH_2OH), 4.33 (1H, m, H-1), 4.83 (1H, m, H-3), 5.79 (1H, m, CH=C), 5.91 (1H, d, J = 11.1 Hz, H-7), 6.22 (1H, d, J = 11.1 Hz, H-6).

LR-MS *m/z* (%): 446 (M^+, 14), 428 (17), 410 (42), 392 (100), 374 (70), 281 (48), 263 (85). HR-MS *m/z*: 446.3395 (Calcd for $C_{28}H_{46}O_4$: 446.3396). UV λ_{max} (EtOH): 246 (ε 30,700), 254 (ε 34,800), 263 (ε 23,000) nm.

*2.1.4. (Z)-1α,25-Dihydroxy-2-(2-hydroxyethylidene)-19-norvitamin D_3 (**6**)*

^{1}H NMR ($CDCl_3$) δ: 0.56 (3H, s, H-18), 0.94 (3H, d, J = 6.2 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.19 (1H, $J3\alpha$, 4β =∼ 10.9 Hz, H-4), 4.21 and 4.36 (each 1H, m, CH_2OH), 4.45 (1H, m, $w/2$ =∼ 20 Hz, H-3), 4.86 (1H, br. s, $w/2$ =∼ 10 Hz, H-1), 5.81 (1H, m, CH=C), 5.84 (1H, d, J = 11.1 Hz, H-7), 6.40 (1H, d, J = 11.1 Hz, H-6).

LR-MS *m/z* (%): 446 (M^+, 66), 428 (51), 410 (85), 392 (100), 374 (30), 299 (21), 281 (63), 263 (47), 245 (41). HR-MS *m/z*: 446.3390 (Calcd for $C_{28}H_{46}O_4$: 446.3396). UV λ_{max} (EtOH): 246, 254, 263 nm.

2.2. Binding assay for VDR

We evaluated the affinity of the newly synthesized compounds (**3**–**6**) for the VDR using the receptor from bovine thymus (Yamasa, Chiba, Japan) as described previously [13]. Each assay was performed at least twice in duplicate.

2.3. Transfection and transactivation assay

Transient transcription assays were conducted in COS-7 cells by using a reporter plasmid containing three copies of the mouse osteopontin VDRE (5′-GGTTCAcgaGGTTCA, SPPx3-TK-Luc), a wild-type or mutant hVDR expression plasmid (pCMX-hVDR), and the internal control plasmid containing sea pansy luciferase expression constructs (pRL-CMV) as described previously [9,10]. Transactivation measured by the luciferase activity was normalized with the internal control. All experiments were done in triplicate.

3. Results and discussion

3.1. Synthesis

*3.1.1. (20S)-1α,25-Dihydroxy-2α-(2-hydroxyethoxy)-19-nor-20-epivitamin D_3 (**3**) and (20S)-1α,25-dihydroxy-2β-(2-hydroxyethoxy)-19-nor-20-epivitamin D_3 (**4**)*

Synthesis was started with (3*R*, 5*R*)-3,4,5-trihydroxy A-ring phosphine oxide derivative (**8**, a 1:1 isomeric mixture at C(2)), which was synthesized enantioselectively from D-glucose as reported [23]. 25-Hydroxy-20-epi-Grundmann's ketone (**7a**) was treated with **8** in the presence of *n*-BuLi (−78 °C) to yield **9a** (66%) which was deprotected and separated to two C(2) epimers (**10a** and **10′a**) by silica gel column chromatography. The isomers **10a** and **10′a** were allowed to react with 2-bromoethanol TBS ether in the presence of NaH (73%) and then all protecting groups were removed by treatment with camphor sulfonic acid (CSA) to yield **3** and **4**.

*3.1.2. (E)- and (Z)-1α,25-dihydroxy-2-(2-hydroxyethylidene)-19-norvitamin D_3 (**5** and **6**)*

2-Hydroxy-19-norvitamin D derivative **10b**, which was obtained similarly by the reaction of **7b** with **8**, was oxidized to 2-ketone **11** (99%) under Swern's conditions. The ketone **11** was treated with diethyl (cyanomethyl)phosphonate (*n*-BuLi, THF, −40 °C) to yield **12** (91%) which was reduced with DIBAL-H followed by $NaBH_4$ to give 2-hydroxyethylidene derivative **14** (60%) as a 1:1 mixture of *E* and *Z* isomers at C(2). Deprotection (CSA, MeOH) of **14** afforded (*E*)- and (*Z*)-hydroxyethylidene 19-norvitamin D derivatives (**5** and **6**) as a 1:1 mixture which was separated by HPLC. The structures, including the stereochemistry, of all compounds described were confirmed by their spectral data (see supplementary material).

3.2. VDR affinity and transcriptional activity of four A-ring analogs (**3**–**6**)

We evaluated the VDR affinity of the four new vitamin D derivatives (**3**–**6**) for the bovine thymus receptor. The relative affinities of **3**–**6** for the VDR compared with 1α,25-$(OH)_2D_3$ (**1**) were 1-, 5-, 2- and 1/140-fold, respectively, of that of **1**. It is noteworthy that 2β-hydroxyethoxy derivative **4** was about five-fold as active as the 2α-isomer **3** [23]. This result is in contrast with the finding for 2-alkyl- and 2-hydroxyalkyl-1,25-$(OH)_2D_3$ derivatives [18] for which the 2α-isomers are more potent than the corresponding 2β-isomers. When comparing the environments above and below the A-ring, that above the A-ring is hydrophobic, surrounded by L233, F150 and the phenyl ring of Y236, whereas that below the A-ring is rather hydrophilic, surrounded by R274, S275 and Y143. Therefore, the oxygen substituted at the 2-position would be more stabilized in the β-configuration than in the α-configuration. The volume of the cavities above and below the A-ring may also be related to this activity difference: the cavity below the A-ring is smaller than that above the A-ring, so the less bulky oxygen would be easier to accommodate in the smaller space below the A-ring than the more bulky methylene. As described below for the 2D-ASMA study, 2α-hydroxyethoxy compound (**3**) docks in the LBP with a different conformation from that of the β-isomer and the natural hormone.

Of the two geometrical isomers **5** and **6**, the *E*-isomer **5** is much more active than the *Z*-isomer **6**. The reason may be that, in the free state, the *E*-isomer **5** adopts exclusively the A-ring β-conformation whereas the *Z*-isomer **6** takes predominantly (98%) the α-conformation, as their NMR spectra show: The coupling constant (12 Hz) between H-1β and H-10α of **5** indicates that the 1α-hydroxyl group is exclusively in an equatorial conformation and hence the A-ring of **5** adopts the β-conformation. Similarly, the coupling constant of H-4β and H-3α of **6** (10.9 Hz) indicates that the 3β-hydroxyl group takes mostly (98%) the equatorial conformation and the A-ring adopts the α-conformation. Sicinski et al. reported similar conformational population for two geometrical isomers of 2-ethylidene-19-nor-1,25-$(OH)_2D_3$ [20]. It has been established by X-ray crystallographic analysis of three VDR–vitamin D complexes [6,7] that vitamin

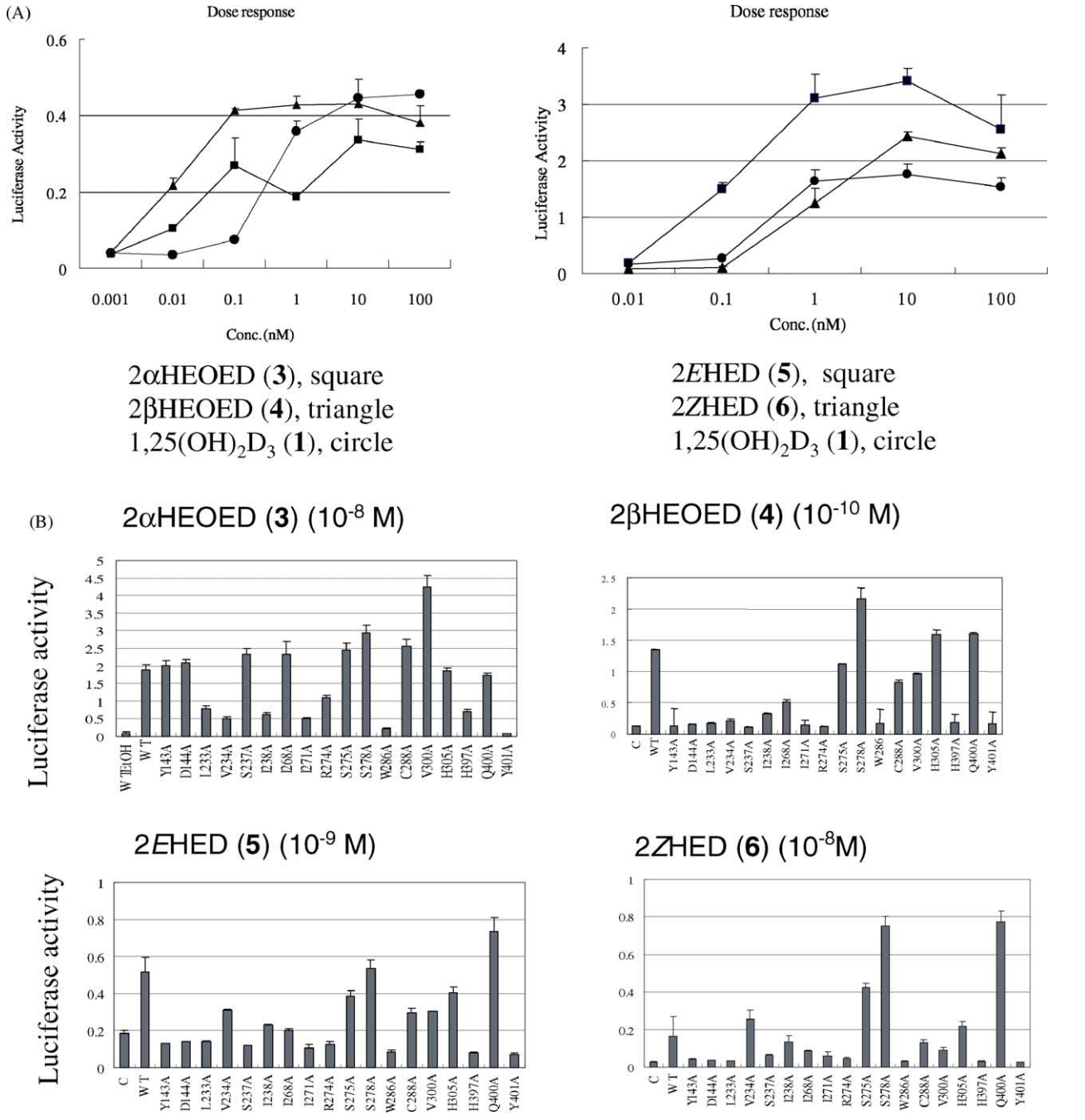

Fig. 2. Biological activity of 2-substituted 19-norvitamin D derivatives (**3**–**6**). Dose response of four vitamin D analogs (**3**–**6**) in transactivation activity. The activities were evaluated by dual luciferase assay using a full-length hVDR expression plasmid (pCMX-hVDR) and a luciferase reporter gene with a mouse osteopontin VDRE at the promoter (SPPx3-TK-Luc) in COS7 cells. (B) Transcriptional activities of wtVDR and 18 one-point mutants induced by four 19-norvitamin D compounds (**3**–**6**). The assays were conducted as described above in (A).

Patch table of 2D alanine scanning mutational analysis (2D-ASMA)

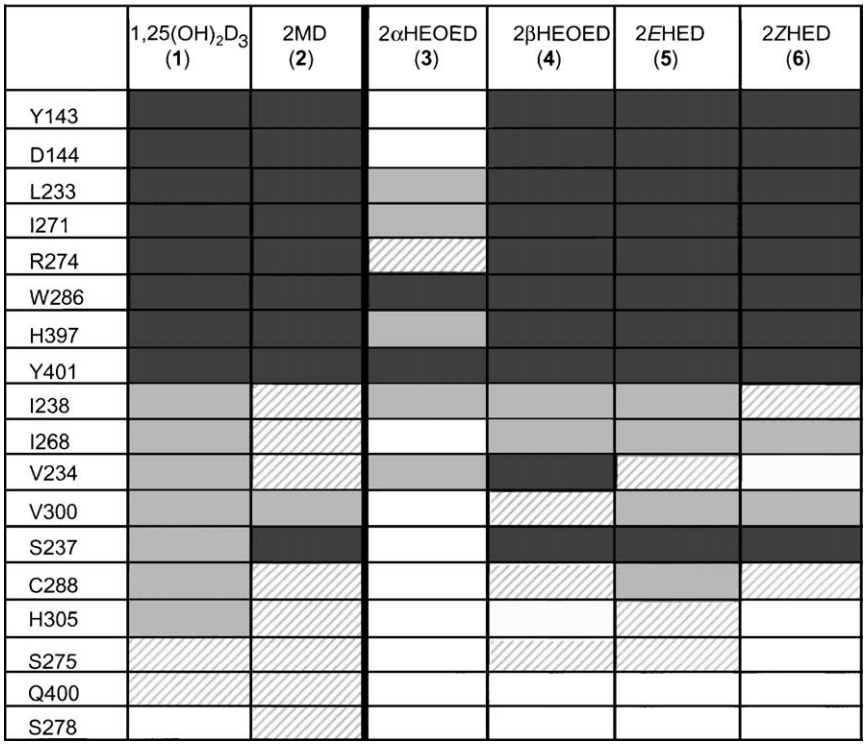

Fig. 3. Patch table presentation of 2D-ASMA. Whole sets of transcriptional assay results shown in Fig. 2B are presented in a patch table, where the effects of mutations are categorized into four groups and displayed by four kinds of patches: <25% of the original activity of wtVDR, dark gray; moderately reduced (25–60%), light gray; slightly affected (61–90%), hatched; and similar or elevated (>90%), unshaded. The columns and rows show mutants and ligands, respectively.

D compounds adopt the β-conformation in the LBP. Thus, the *E*-isomer (**5**) can be accommodated in the LBP with the most stable conformation. From the 2D-ASMA study, the two isomers (**5** and **6)** are considered to adopt the same A-ring β-conformation in the LBP. If this is true, the *Z*-isomer (**6**) has to adopt the less stable β-conformation in the LBP.

Transcriptional activities of the four compounds were determined by luciferase assay with mouse osteopontin VDRE (Fig. 2A). The relative activities of **3**–**6**, as determined by their ED50 concentrations, were 10-, 30-, 2- and 0.3-fold of that of **1**.

3.3. 2D-Alanine scanning mutational analysis (2D-ASMA)

We used the same 18 alanine mutants of amino acid residues lining the VDR LBD as in our previous paper [9,10]. These were: three pairs of residues forming hydrogen bonds with the three hydroxyl groups of vitamin D, namely the 1α-hydroxyl group (R274A and S237A), 3β-hydroxyl group (Y143A and S278A) and 25-hydroxyl group (H305A and H397A); residues having hydrophobic interactions with ligands (L233A, W286A, V234A, I268A, I271A, C288A and V300A); and residues having a role in the folding (D144A, I238A, Q400A and Y401A). We performed 2D-ASMA with the two isomeric pairs (**3**/**4** and **5**/**6**). The 2D assay results are shown in Fig. 2B. The results are also presented as a patch table (Fig. 3) in comparison with those of natural hormone (**1**) and 2MD (**2**). In this way the effects of mutations can be appreciated easily. The 2D-ASMA patterns of the 2-substituted 19-norcompounds **4**–**6** are similar to 2MD (**2**), in contrast to that of 2αHEOED (**3**). The upper eight residues, Y143, D144, L233, I271, R274, W286, H397 and Y401, are essential as their Ala mutations significantly reduce (<25%) the activity. In addition compounds **4**–**6** require S237 as does 2MD (**2**). As reported, S237 is essential for 19-norvitamin D derivatives, probably because the hydrogen bond between S237 and the 1α-hydroxyl group becomes more important to compensate for the loss of the van der Waals contact at $C(19)H_2$ with L233. 2αHEOED (**3**) shows striking differences in the 2D-ASMA table. Of the eight residues important for the activity of the natural hormone (**1**), only W286 and Y401 remain essential for 2αHEOED (**3**): the residues, which are essential to anchor the 1α-, 3β- and 25-hydroxyl groups of the natural hormone (**1**) with hydrogen bonds, R274, Y143 and H397, are no longer important with **3**. These results indicate that the manner of hydrogen-bonding of compound **3** with LBP residues is totally different from that of the natural hormone and 2MD (**2**). It is possible that 2αHEOED (**3**) adopts the A-ring α-form, in which the 1α-hydroxyl group and 2α-substituent take axial and

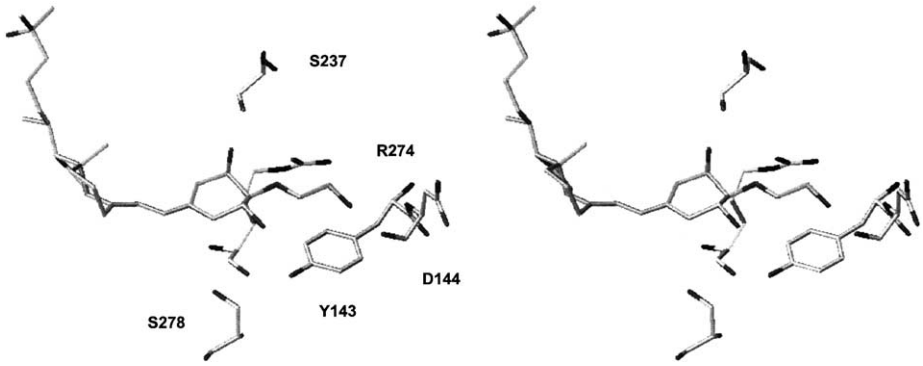

Fig. 4. Docking model of 2α-hydroxyethoxy-19-nor-20-epi-1,25-$(OH)_2D_3$ (2αHEOED, **3**) in the VDR LBD (stereo view).

equatorial conformations, respectively. The ^{1}H NMR of **3** indicates that the A-ring of 2αHEOED (**3**) adopts predominantly (85%) the α-conformation ($J_{3\alpha,4\beta} = 9.8$ Hz) in the free solution state. Significantly, however, regardless of this difference in the binding mode, this compound has the same VDR affinity as **1** and 10 times higher transactivation potency than **1**. We examined docking of 2αHEOED (**3**) in the α-form in the VDR LBD (1DB1) as shown in Fig. 4. It is clear that the 1α-hydroxyl group in the axial conformation cannot form a hydrogen bond with R274. The terminal hydroxyl group of the substituent at C(2) can form hydrogen bond with the backbone carbonyl group of D144. This hydrogen bond may be the key interaction to anchor the ligand.

In conclusion, in a 2D-ASMA study of A-ring modified 19-norvitamin D analogs (**3**–**6**), we found a compound (**3**) that is accommodated in the LBP in a distinct manner from that of the natural hormone. We suggest that this compound (**3**) adopts an A-ring α-conformation in the VDR LBP. A more extensive 2D-ASMA analysis is in progressing in our laboratory, using mutants of all LBP residues, and is likely to provide us with intriguing results.

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