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Structure–activity relationships of 19-norvitamin D analogs having a fluoroethylidene group at the C-2 position

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Abstract—We have synthesized four new geometric isomers of 1α,25-dihydroxy-2-(2′-fluoroethylidene)-19-norvitamin D analogs **1** and **2** having a 20*R*- and 20*S*-configuration, whose structures are correlated with 2MD possessing high potencies in stimulating bone formation in vitro and in vivo. The *E*-isomers of (20*R*)- and (20*S*)-2-fluoroethylidene analogs **1a** and **1b** were comparable with the natural hormone 1α,25-$(OH)_2D_3$ in binding to the vitamin D receptor (VDR), while two *Z*-isomers **2a** and **2b** were about 15–20 times less active than the hormone. In inducing expression of the vitamin D responsive element-based luciferase reporter gene, the *E*-isomers **1a** and **1b** were 1.2- and 8.6-fold more potent than the hormone, respectively, while the *Z*-isomers **2a** and **2b** had 27–55% of the potency. On the basis of the biological activities and a docking simulation based on X-ray crystallographic analysis of the VDR ligand-binding pocket, the structure–activity relationships of the fluorinated 19-norvitamin D analogs are discussed.

1. Introduction

1α,25-Dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], the active metabolite of vitamin D_3, is a major component in the regulation of calcium and phosphorus homeostasis and bone mineralization.[1] In addition, 1α,25-$(OH)_2D_3$ plays a role in controlling differentiation and growth inhibition of a variety of cell types including malignant cells and keratinocytes, and has immunomodulatory activity on B and T cells.[1,2] Most of the biological effects of the natural hormone, 1α,25-$(OH)_2D_3$, are mediated by the vitamin D receptor (VDR), a ligand-activated transcription factor.[3] Binding of the hormone causes a conformational change in the VDR, and the activated VDR forms a complex with the retinoid X receptor (RXR). The VDR/RXR heterodimer binds to the promoter region on the target genes and initiates gene transcription. It is known that the VDR is widely distributed in human tissues except for skeletal muscle, cerebrum, and cerebellum.[4,5]

1α,25-$(OH)_2D_3$ has significant therapeutic potential in the treatment of osteoporosis, rickets, secondary hyperparathyroidism, psoriasis, and renal osteodystrophy.[1] However, use of 1α,25-$(OH)_2D_3$ itself is limited because it induces significant hypercalcemia. A number of 1α,25-$(OH)_2D_3$ analogs have therefore been synthesized, and some of them have been shown to have desirably low calcemic activity (Fig. 1).[6] One of these analogs, 1α,25-dihydroxy-19-norvitamin D_3 (19-ND), lacks the 19-exomethylene group of 1α,25-$(OH)_2D_3$.[7,8] Paricalcitol has been developed and used to treat secondary hyperparathyroidism and psoriasis.[9] Recently, Shevde et al. reported that 2MD with a methylene group at the C-2 position and a 20*S*-configuration stimulated bone formation in vitro and in vivo.[10,11] This was expected to be a promising candidate for the treatment of osteoporosis.

Over the last 5 years, we have been studying the structure–activity relationships (SAR) of 19-norvitamin D

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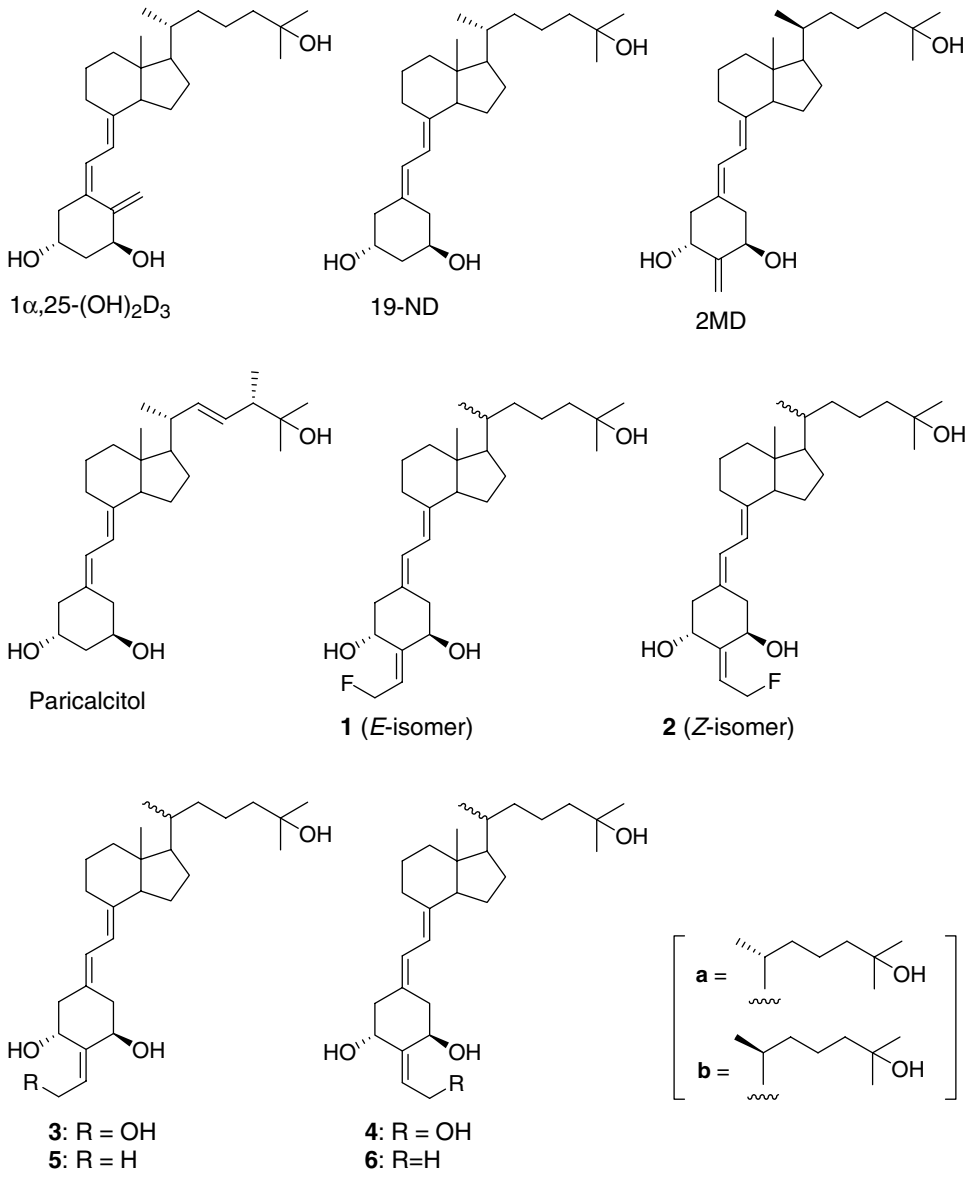

Figure 1. The structures of 1α,25-$(OH)_2D_3$ and its 19-norvitamin D analogs.

analogs and reported that structural modifications at the C-2 position of the A-ring, or on both the A-ring and the side chain, produced more than 100 synthetic analogs, some of which have interesting biological profiles (Fig. 1).[12–16] Among them, we found that 2-hydroxyethylidene analogs **3a** and **3b** with a 2*E*-configuration showed strong binding affinity to the VDR (~2-fold) and ligand-dependent transcriptional activity (2- to 13-fold) compared with the natural ligand 1α,25-$(OH)_2D_3$.[13,16] On the other hand, DeLuca's group has developed 2-ethylidene-19-norvitamin D analogs **5** and **6**, and our 2-hydroxyethylidene analogs **3** and **4** exhibited biological potencies similar to those of the 2-ethylidene analogs.[17]

In view of the results reported for 19-norvitamin D analogs with alkylidene groups attached at the C-2 position, we focused our attention on the synthesis of 2-(2′-fluoroalkylidene)-19-norvitamin D analogs. These analogs containing a fluorine atom are capable of hydrophobic interaction with the amino acid residues in the VDR ligand-binding pocket (LBP), and these lipophilic fluorinated analogs are expected to have different biological activities from the 2-hydroxyethylidene and 2-ethylidene analogs. We report herein the synthesis of 1α,25-dihydroxy-2-(2′-fluoroethylidene)-19-norvitamin D_3 analogs **1** and **2** using a strategy based on a convergent approach using the Wittig-Horner reaction of the A-ring phosphine oxide with the 25-hydroxy-Grundmann's ketones. We also examine the biological activity of the synthetic fluoroethylidene derivatives on the affinity of binding to the VDR and in activating gene transcription. On the basis of the biological activities and docking studies based on the crystal structure of VDR LBP, we discuss the SAR of 2-(2′-alkylidene)-19-norvitamin D analogs.

2. Results and discussion

2.1. Synthesis

Recently, we reported the efficient convergent synthesis of 2-(2′-hydroxyethylidene) analogs **3** and **4**,[13,16] and decided to adopt the same strategy for synthesis of the corresponding 2-(2′-fluoroethylidene) analogs **1** and **2**. Our plan was to synthesize the fluorinated compounds based on the introduction of the fluorine atom to the target position at a late stage of the synthesis, and a scheme of this for **1** and **2** is summarized in Figure 2. The protected 2-(2′-hydroxyethylidene)-19-nor-compounds **11a** and **11b** were prepared by the method described previously.[13,16] Briefly, the protected 25-hydroxy-Grundmann's ketones **7** (steroid numbering) and the known A-ring phosphine oxide **8** were synthesized following the published procedures.[12,18–21] The coupling of **7** with **8** followed by selective deprotection of the TMS-protecting group afforded **9** as an approximately 3:2 diastereomeric mixture. Swern oxidation of **9** gave the 2-keto compounds, which were treated with diethyl(cyanomethyl)phosphonate [$(EtO)_2P(O)CH_2CN$] to yield the 2-cyanomethylene derivatives **10** as an approximately 1:1 mixture of *E*- and *Z*-isomers. The compounds **10** were reduced with diisobutylaluminum hydride (DIBAL-H) followed by sodium borohydride ($NaBH_4$) to give the 2-(2′-hydroxyethylidene) derivatives **11**.

The reaction of the nucleophilic fluorinating reagent, (diethylamino)sulfur trifluoride (DAST), with the alco-

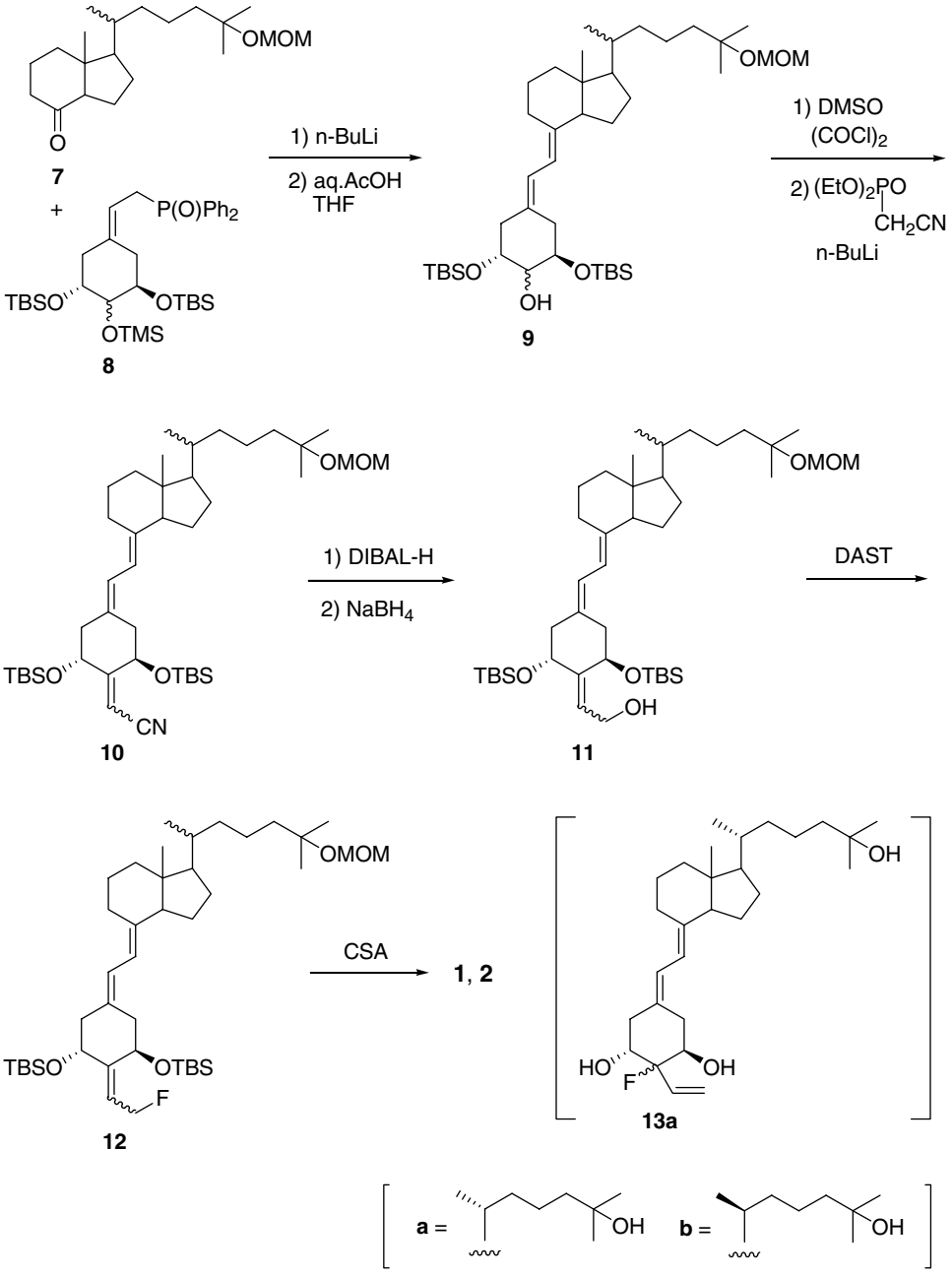

Figure 2. Synthesis of 2-fluoroethylidene-19-norvitamin D analogs (**1** and **2**).

hol to replace the hydroxyl group with the fluorine appears to be a general reaction. The fluorination of **11a** with DAST in CH_2Cl_2 at −78 °C produced a complex reaction mixture, which was treated with camphorsulfonic acid (CSA) to remove the protecting silyl and methoxymethyl groups. Careful column chromatography, then separation by preparative HPLC, gave the desired fluorinated *E*- and *Z*-isomeric products **1a** and **2a** at 22% yields, together with significant amounts of the rearranged product **13a** (18% yield). The desired position of the newly introduced fluorine atom in **1a** and **2a** was deduced from ^1H and ^{19}F NMR spectra: ^1H NMR spectra showed a doublet of multiplets for the methylene protons coupled with the fluorine atom (5.07 and 5.99 ppm for **1a**, 5.10 and 5.12 ppm for **2a**) and a large vicinal coupling constant J_{vic} of ∼47 Hz. The fluorine signals of **1a** and **2a** appeared as −209.1 and −208.7 ppm, respectively, as a triplet of multiplets with a coupling constant of ∼47 Hz. The *Z*-isomer **2a** was shifted slightly downfield compared with its counterpart **1a**. The stereochemistry of the 2-hydroxyethylidene moieties in **3** and **4** has already been established by NOE experiments as reported previously.[13,15,16] The geometry of the 2-fluoroethylidene groups in **1a** and **2a** was determined based on the NMR data for **3** and **4**, and the independent 2D NOESY spectra: a NOE was detected between H-1 and the vinyl proton in **1a**, while a correlation cross peak was observed between H-3 and the vinyl proton in **2a**.

The DAST-induced fluorination reaction of the hydroxyl group occurs rapidly even at low temperature, and often causes double bond rearrangement and dehydration when the allylic alcohol is used as a substrate. It is reported that the fluorination reaction of crotyl alcohol with DAST in a less polar solvent or diglyme gives the desired monofluorides at high yields.[22] Fluorination of the alcohol **11b** was carried out at −78 °C by using DAST in diglyme. A shorter reaction time (∼3 min) and a large excess of the fluorinating reagents afforded considerably better results and high yields (61%; 81% based on the recovered **11b**) of the desired derivatives **12b**, and virtually no rearranged products. Silyl- and methoxymethyl-protecting groups in **12** were deprotected using CSA to give the 2-fluoroethylidene analogs **1** and **2**.

2.2. Biological activity

First, we evaluated the binding affinity of the 2-fluoroethylidene-19-norvitamin D analogs **1** and **2** to the VDR. The affinity for the VDR was determined by a competitive binding assay between radioactive 1α,25-$(OH)_2D_3$ and the analogs using rat recombinant VDR LBD,[23] and the results are summarized in Table 1.

The relative affinity of the *E*-isomers **1a** and **1b** for the VDR was approximately 15 times greater than that of the corresponding *Z*-isomers **2a** and **2b**, and was similar to that of the natural ligand, 1α,25-$(OH)_2D_3$. When compared with the binding affinity of the 2-hydroxyethylidene analogs **3** and **4**,[13,16] the *E*-isomers of the 2-fluoroethylidene analog showed decreased affinities, but the *Z*-isomers showed higher binding potency. The binding affinity of all the 2-fluoroethylidene analogs **1** and **2** (**1a** vs **5a**; **1b** vs **5b**; **2a** vs **6a**, and **2b** vs **6b**) was found to be less active than that of the parent 2-ethylidene analogs **5** and **6**.[10] In the series of 2-hydroxyethylidene and 2-ethylidene derivatives, 20-epimerization decreases the binding potency of the *E*-isomers. In the case of 2-fluoroethylidene analogs, 20*S*-isomers **1b** and **2b** have higher affinity than the corresponding 20*R*-isomers **1a** and **2a**, consistent with the reported findings.[6]

Table 1. Relative VDR affinity and transcriptional activity of 2-(2′-fluoroethylidene)-19-norvitamin D analogs[a]

Compound	VDR affinity	Transcription or cell differentiation
1a	0.77[b]	1.2[d]
1b	1.08[b]	8.6[d]
2a	0.05[b]	0.27[d]
2b	0.07[b]	0.55[d]
3a	2.0[c]	2.0[d]
3b	1.6[c]	12.5[d]
4a	0.007[c]	0.3[d]
4b	0.02[c]	4.2[d]
5a	2.5[e]	1.3[e]
5b	2.0[e]	11.1[e]
6a	0.08[e]	0.6[e]
6b	1.25[e]	16.7[e]

[a] Activities are shown as percentages of that of 1α,25-$(OH)_2D_3$.
[b] Rat VDR LBD.
[c] Thymus full-length VDR.
[d] Activity was assessed in terms of EC_{50}.
[e] Ref. 17.

To investigate the cause of the decreased binding affinity of 2-fluoroethylidene analogs compared with the 2-ethylidene analogs, dockings of both analogs with *E*-geometry to the VDR LBP were studied using the Sybyl molecular modeling software package (Tripos) (Fig. 3). The crystal structures of human or rat VDR LBD complexed with the natural ligand and its analogs (13 compounds) revealed that three hydroxyl groups (1α-OH, 3β-OH, and 25-OH) in these ligands interact with the same amino acid residues in the VDR LBP.[24–29] The X-ray crystallographic data also demonstrated the presence of water molecules near the A-ring of the ligands. In the 1α,25-$(OH)_2D_3$/VDR LBD complex, three water molecules (W1 and W2 near the C-2 position, and an additional W3 interacted with W2) create a water molecule network around the A-ring of VDR ligands and form a water channel. Our docking studies demonstrated a difference in ligand docking between **1a** and **5a** (Fig. 3). In the docking simulation for VDR LBD/**5a** (Fig. 3A), the substituent at the C-2 position was accommodated in the LBP without any disruption of the ordered network formed by the three water molecules. The fluorine atom has a van der Waals radius of 1.47 Å, as compared with 1.20 Å for the hydrogen atom, which indicates that the fluorine causes more steric perturbation than the hydrogen. As shown in Figure 3B, because of the bulkiness of the fluoromethyl group in **1a** relative to the methyl group in **5a**, the water molecule (W2) in the LBP is excluded by the fluoromethyl group. The loss of water in the water channel may explain why

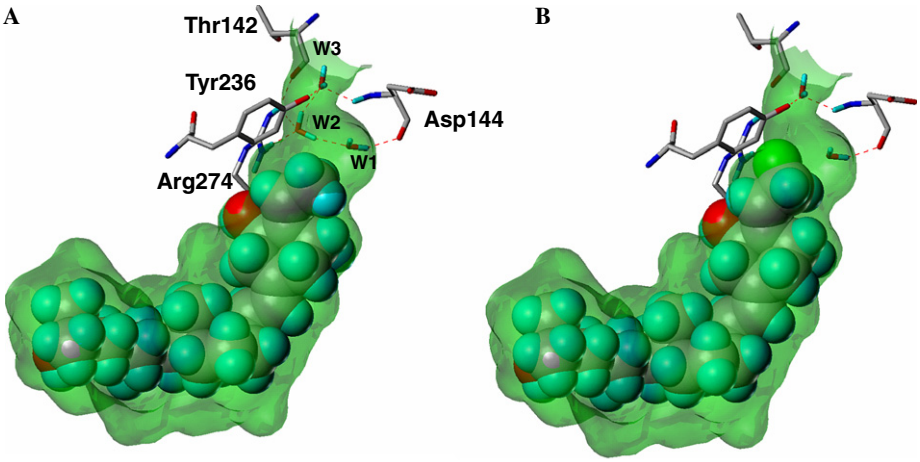

Figure 3. (A) 2-Ethylidene analog (**5a**) and (B) 2-fluoroethylidene analog (**1a**) docked in VDR ligand-binding pocket (LBP), and interacting amino acid residues with water molecules in the water channel. The Connolly channel surface of the VDR LBP is displayed in translucent green.

1a has weaker binding affinity than the parent compound **5a**. Although the water molecule network is partly broken by the bulky 2-fluoroethylidene moiety, **1a** has slightly weaker but still significant VDR binding affinity in comparison with 1α,25-$(OH)_2D_3$ and a higher binding affinity than 19-ND (Fig. 1), which has no substituent at C-2. There are no amino acid residues that can form strong hydrophobic or van der Waals contacts with the 2-methylene group of 2MD.[27] Additional hydrophobic interaction of the terminal fluoromethyl group in **1** with the amino acid residues surrounding the A-ring of the ligand may compensate for the loss of water (W2), which forms the hydrogen bonds with Arg274 and W3. Quite recently, Moras et al. clarified the crystal structure of human VDR LBD in complex with the selected 2α-substituted vitamin D_3 analogs, and pointed out the importance of the number of contacts and stabilized interaction between the substituents at C-2 and the water molecules present in the LBP.[29]

Next, to test whether 2-fluoroethylidene analogs **1** and **2** induce expression of the vitamin D response element (VDRE)-dependent gene, we used COS-7 cells that were transfected with a mouse osteopontin promoter luciferase reporter (mOPN-LUC). The natural ligand 1α,25-$(OH)_2D_3$ induced mOPN-LUC gene expression with an EC_{50} value of 0.6 nM. The transcriptional activity of the *E*-isomers **1a** and **1b** was 1.2 and 8.6 times more potent, respectively, than that of 1α,25-$(OH)_2D_3$, and showed EC_{50} values of 0.5 and 0.07 nM, respectively. In contrast, the *Z*-isomers **2a** and **2b** were less efficacious than the natural ligand, and exhibited EC_{50} values of 2.2 and 1.1 nM, respectively. The activity enhancement by 20-epimerization is consistent with the findings for 2-hydroxyethylidene or 2-ethylidene analogs. The hydrophobic interaction of ligands with the LBP-lining amino acid residues is one of the important factors for enhancing the transcriptional activity. The synthesized fluorinated analogs **1** and **2** were expected to lead analogs having increased lipophilicity and transcriptional potency than the parent 2-ethylidene analogs **5** and **6**. In the transcriptional assay, we found that four fluorinated analogs **1** and **2** showed equivalent or reduced activities with respect to the corresponding 2-ethylidene analogs. Despite the low binding affinity of the *Z*-isomers **2a** and **2b** to the VDR, the difference in transcriptional activity between them is small, and similar activity profiles were observed for 2-hydroxypropylidene analogs reported recently.[30] 20-Epimerization does not markedly affect the transcriptional activity of *Z*-isomers **2a** and **2b**.

3. Conclusion

We have synthesized new 2-(2′-fluoroethylidene)-19-nor-vitamin D analogs and described their biological evaluation. The isomeric pairs, *E*- and *Z*-isomers **1** and **2**, exhibited distinct biological profiles, and the *E*-isomers were more potent than the *Z*-isomers. The *E*-isomers **1a** and **1b** had similar effects on VDR binding, and had enhanced gene-activating activities in comparison with 1α,25-$(OH)_2D_3$. Contrary to expectation, introduction of a fluorine atom at the terminal position of the 2-ethylidene group did not have strong effects on the VDR binding and transcriptional activity. The weaker biological activities of the 2-fluoroethylidene analogs as compared with the parent 2-ethylidene analogs may be explained by imbalance of the water molecule network in the water channel. The present results suggest that evaluation of the much broader spectrum of activities of the 2-fluoroethylidene analogs **1** and **2** would be warranted, and such studies are now underway in our laboratory.

4. Experimental

^1H NMR spectra were obtained on a Bruker ARX-400 spectrometer, operating at 400 MHz for ^1H and 376 MHz for ^{19}F. Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane as an internal standard (δ 0 ppm) for ^1H NMR and trifluorotoluene as an internal standard (δ 0 ppm) for ^{19}F NMR. Abbreviations used are: singlet (s), doublet (d),

triplet (t), multiplet (m), aromatic (arom), broad signal (br). Low- and high-resolution mass spectra (LR-MS and HR-MS) were obtained with electronic ionization (EI) on a JEOL JMS-AX505HA spectrometer run at 70 eV for EI; *m*/*z* values are given with relative intensities in parentheses. UV spectra were obtained on a Beckmann DU-7500 spectrophotometer. A mixture of diastereomers was separated by HPLC equipped with a Model PU-980 pump, a Rheodyne Model 7125 injector, and a Model MD-910 multiwavelength UV detector from JASCO. Column chromatography was carried out on silica gel (Wako Pure Chem. Ind. Ltd, Wakogel C-200). All reactions were conducted under an atmosphere of argon gas. Methylene chloride (CH_2Cl_2) was distilled from calcium hydride. Digyme was distilled from calcium hydride under reduced pressure. Yields were not optimized.

4.1. (2*E*)- and (2*Z*)-(20*R*)-1α-[(*tert*-Butyldimethylsilyl)-oxy]-2-(2′-fluoroethylidene)-25-hydroxy- 19-norvitamin D_3 *tert*-butyldimethylsilyl ether (12a), (2*E*)-(20*R*)-1α,25-dihydroxy-2-[(2′-hydroxyethylidene)]-19- norvitamin D_3 (1a), (2*Z*)-(20*R*)-1α,25-dihydroxy-2-[(2′-hydroxyethylidene)]-19-norvitamin D_3 (2a), and compounds (13a)

(*E*)- and (*Z*)-(20*R*)-1α-[(*tert*-Butyldimethylsilyl)oxy]-2-[(2′-hydroxyethylidene)]-25-hydroxy-19-norvitamin D_3 *tert*-butyldimethylsilyl ether (**11a**) was prepared by the method reported previously.[13,16]

To a stirred cold (−78 °C) solution of an alcohol **11a** (53 mg, 0.074 mmol, approximately 1:1 isomeric mixture) in dry CH_2Cl_2 (1 ml) was added (diethylamino)sulfur trifluoride (DAST, 24 mg, 0.15 mmol), and the mixture was stirred for 5 min, quenched with saturated sodium bicarbonate ($NaHCO_3$), and extracted with CH_2Cl_2. The organic phase was washed with brine, dried over $MgSO_4$, and evaporated to dryness. The residue was passed through silica gel column (5 g), eluting with 5% ethyl acetate (AcOEt) in hexane. The eluate was evaporated off to give a mixture of desired compounds **12a**, the starting materials, and the rearranged isomeric 2-fluoroethylidene derivatives (46 mg).

The above mixture (46 mg) was dissolved in methanol (MeOH, 1 ml) and 10-camphorsulfonic acid (CSA, 102 mg, 0.44 mmol, based on **11a**) was added. The whole mixture was stirred at ambient temperature for 3 h, poured into cold 5% $NaHCO_3$ solution, and extracted with AcOEt. The AcOEt layer was washed with brine, dried over $MgSO_4$, and evaporated in vacuo. The residue was chromatographed by silica gel (5 g) with 50% AcOEt in hexane to give an isomeric mixture of **1a** and **2a** (7.1 mg, 22%) and **13a** (6.0 mg, 18.2%). The mixture of **1a** and **2a** was further purified by HPLC (YMC Pack ODS-AM SH-342-5, S-5 μm, 150 mm × 20 mm I.D., 20% H_2O in MeOH, 8 ml/min, ambient temperature, 220–340 nm) to afford the *E*-isomer **1a** (1.88 mg) and the *Z*-isomer **2a** (1.98 mg).

4.1.1. *E*-Isomer 1a. ^{1}H NMR ($CDCl_3$) δ: 0.55 (3H, s, H-18), 0.94 (3H, d, *J* = 6.3 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.47 (2H, m, H-4), 2.81 (1H, m, H-9), 3.21 (1H, dd, *J* = 12.7, 5.2 Hz, H-10), 4.47 (1H, m, H-1), 4.87 (1H, m, H-3), 5.07, 5.09 (each 1H, dm, *J* = 47 Hz, CH_2F), 5.90 (1H, d, *J* = 11.2 Hz, H-7), 5.91 (1H, m, CH=C), 6.32 (1H, d, *J* = 11.2 Hz, H-6). ^{19}F NMR ($CDCl_3$) δ: −209.1 (tm, *J* = 47 Hz). LR-MS *m*/*z* (%): 448 (M^+, 11), 430 (11), 428 (15), 410 (60), 392 (100), 374 (36), 313 (20), 299 (15), 281 (29), 263 (27), 245 (29). HR-MS *m*/*z*: 448.3377 (Calcd for $C_{28}H_{45}FO_3$: 448.3353). UV λ_{max} (EtOH): 246, 254, 263 nm.

4.1.2. *Z*-Isomer 2a. ^{1}H NMR ($CDCl_3$) δ: 0.55 (3H, s, H-18), 0.94 (3H, d, *J* = 6.4 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.21 (1H, m, H-4), 2.26 (1 H, br d, *J* = 14.2 Hz, H-10), 2.73 (1H, dd, *J* = 12.6, 5.0 Hz, H-4), 2.81 (1H, m, H-9), 2.90 (1H, dd, *J* = 14.2, 4.6 Hz, H-10), 4.51 (1H, m, H-3), 4.88 (1H, m, H-1), 5.10, 5.12 (each 1H, dm, *J* = 47 Hz, CH_2F), 5.83 (1H, d, *J* = 11.2 Hz, H-7), 5.89 (1H, m, CH=C), 6.43 (1H, d, *J* = 11.2 Hz, H-6). ^{19}F NMR ($CDCl_3$) δ: −208.7 (tm, *J* = 47 Hz). LR-MS *m*/*z* (%): 448 (M^+, 18), 428 (54), 410 (100), 392 (72), 313 (84), 299 (31), 281 (35), 263 (16), 245 (50). HR-MS *m*/*z*: 448.3363 (Calcd for $C_{28}H_{45}FO_3$: 448.3353). UV λ_{max} (EtOH): 243, 252, 261 nm.

4.1.3. Compound 13a. LR-MS *m*/*z* (%): 448 (M^+, 90), 430 (100), 410 (28), 374 (23), 319 (64), 245 (29). HR-MS *m*/*z*: 448.3362 (Calcd for $C_{28}H_{45}FO_3$: 448.3353). UV λ_{max} (EtOH): 243, 251, 261 nm.

4.1.4. Major isomer. ^{1}H NMR ($CDCl_3$) δ: 0.56 (3H, s, H-18), 0.94 (3H, d, *J* = 6.4 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.24 (1H, dd, *J* = 14.2, 4.5 Hz), 2.34 (1H, m), 2.80 (1H, m, H-9), 2.90 (1H, dd, *J* = 13.3, 4.6 Hz), 3.85 (1H, m), 4.00 (1H, m), 5.45 (1H, dt, *J* = 11.2, 1.2 Hz), 5.57 (1H, dt, *J* = 17.5, 1.2 Hz), 5.87 (1H, d, *J* = 11.2 Hz, H-7), 6.14 (1H, m), 6.32 (1H, d, *J* = 11.2 Hz, H-6). ^{19}F NMR ($CDCl_3$) δ: −181.3 (br).

4.1.5. Minor isomer. ^{1}H NMR ($CDCl_3$) δ: 0.54 (3H, s, H-18), 0.94 (3H, d, *J* = 6.4 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.40–2.55 (2H, m), 2.34 (1H, m), 2.80 (2H, m), 3.93 (1H, m), 4.06 (1H, m), 5.48 (1H, dt, *J* = 9.0, 1.2 Hz), 5.60 (1H, dt, *J* = 17.2, 1.2 Hz), 5.83 (1H, d, *J* = 11.0 Hz, H-7), 6.14 (1H, m), 6.40 (1H, d, *J* = 11.2 Hz, H-6). ^{19}F NMR ($CDCl_3$) δ: −176.8 (br).

4.2. (2*E*)-and (2*Z*)-(20*S*)-1α-[(*tert*-Butyldimethylsilyl)-oxy]-2-[(2′-fluoroethylidene)]-25-hydroxy-19-norvitamin D_3 *tert*-butyldimethylsilyl ether (12b)

(*E*)- and (*Z*)-(20*S*)-1α-[*tert*-Butyldimethylsilyl]oxy]-2-[(2′-hydroxyethylidene)]-25-hydroxy-19-norvitamin D_3 *tert*-butyldimethylsilyl ether (**11b**) was prepared by the method reported previously.[13,16]

To a stirred cold (−78 °C) solution of **11b** (17.5 mg, 0.024 mmol, approximately 1:1 mixture) in dry diethylene glycol dimethyl ether (diglyme, 300 μl) was added a solution of DAST (40.0 mg, 0.248 mmol) in dry diglyme (50 μl). After 3-min stirring, the mixture was quenched with saturated $NaHCO_3$ and extracted with AcOEt. The organic layer was washed with brine, dried

with $MgSO_4$, and evaporated to dryness. The residue was purified by column chromatography on silica gel (5 g) with 3% AcOEt in hexane to afford an isomeric mixture of **12b** (10.7 mg, 61%) and an unreacted starting material **11b** (4.2 mg, 24%).

4.2.1. Compound 12b. ^{1}H NMR ($CDCl_3$) δ: 0.07–0.10 (12H, 4× Me–Si), 0.54, 0.55 (ca. 1:1) (3H, s, H-18), 0.827, 0.834 (ca. 1:1) (9H, s, *t*-Bu–Si), 0.85 (3H, d, J = 6.5 Hz, H-21), 0.92, 0.93 (ca. 1:1) (9H, s, *t*-Bu–Si), 1.21 (6H, s, H-26, 27), 3.37 (3H, s, OCH_3), 4.40, 4.51 (ca. 1:1) (1H, m), 4.707, 4.709 (ca. 1:1) (2H, s, OCH_2O), 4.80, 4.85 (ca. 1:1) (1H, m, H-1), 4.97, 5.09 (each 1H, m, CH_2F), 5.78 (1H, m, CH=C), 5.80, 5.86 (ca. 1:1) (1H, d, J = 11.2 Hz, H-7), 6.14, 6.27 (ca. 1:1) (1H, d, J = 11.2 Hz, H-6). ^{19}F NMR ($CDCl_3$) δ: −208.4, −208.2 (ca. 1:1) (tm).

4.3. (2*E*)-(20*S*)-1α,25-Dihydroxy-2-[2′-(fluoroethylidene)]-19- norvitamin D_3 (1b) and (2*Z*)-(20*S*)-1α,25-dihydroxy-2-[2′-(fluoroethylidene)]-19- norvitamin D_3 (2b)

To a stirred solution of **12b** (15.9 mg, 0.022 mmol) in dry MeOH (0.75 ml) was added CSA (30.7 mg, 0.132 mmol). The mixture was stirred at ambient temperature for 10 h, poured into cold saturated $NaHCO_3$ solution, and extracted with AcOEt. The AcOEt extracts were washed with brine, dried over $MgSO_4$, and evaporated in vacuo. The residue was chromatographed by silica gel (5 g) with 50% AcOEt in hexane to give an isomeric mixture of **1b** and **2b** (9.2 mg, 93%). The mixture of **1b** and **2b** was further purified by HPLC (YMC Pack ODS-AM SH-342-5, S-5 μm, 150 mm × 20 mm I.D., 15% H_2O in MeOH, 8 ml/min, ambient temperature, 220–340 nm) to afford the *E*-isomer **1b** (3.62 mg) and the *Z*-isomer **2b** (3.24 mg).

4.3.1. *E*-Isomer 1b. ^{1}H NMR ($CDCl_3$) δ: 0.55 (3H, s, H-18), 0.89 (3H, d, J = 6.5 Hz, H-21), 1.22 (6 H, s, H-26, 27), 2.46 (2H, m, H-4), 2.81 (1H, m, H-9), 3.21 (1H, dd, J = 12.8, 5.1 Hz, H-10), 4.47 (1H, m, H-1), 4.86 (1H, m, H-3), 5.07, 5.09 (each 1H, dm, J = 47 Hz, CH_2F), 5.89 (1H, d, J = 11.2 Hz, H-7), 5.93 (1H, m, CH=C), 6.32 (1H, d, J = 11.2 Hz, H-6). ^{19}F NMR ($CDCl_3$) δ: −209.0 (tm, J = 47 Hz). LR-MS *m*/*z* (%): 448 (M^+, 100), 430 (37), 428 (62), 410 (63), 392 (27), 313 (87), 299 (36), 281 (23), 245 (62). HR-MS *m*/*z*: 448.3337 (Calcd for $C_{28}H_{45}FO_3$: 448.3353). UV λ_{max} (EtOH): 246, 254, 263 nm.

4.3.2. *Z*-Isomer 2b. ^{1}H NMR ($CDCl_3$) δ: 0.55 (3H, s, H-18), 0.86 (3H, d, J = 6.4 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.21 (1H, m, H-4), 2.26 (1H, br d, J = 14.0 Hz, H-10), 2.73 (1H, dd, J = 12.6, 4.8 Hz, H-4), 2.81 (1H, m, H-9), 2.91 (1H, dd, J = 14.0, 4.5 Hz, H-10), 4.51 (1H, m, H-3), 4.88 (1H, m, H-1), 5.11, 5.13 (each 1H, dm, J = 47 Hz, CH_2F), 5.83 (1H, d, J = 11.2 Hz, H-7), 5.88 (1H, m, CH=C), 6.43 (1H, d, J = 11.2 Hz, H-6). ^{19}F NMR ($CDCl_3$) δ: −208.7 (tm, J = 47 Hz). LR-MS *m*/*z* (%): 448 (M^+, 8), 428 (16), 410 (39), 392 (100), 374 (18), 313 (34), 299 (13), 281 (37), 263 (18), 245 (29). HR-MS *m*/*z*: 448.3351 (Calcd for $C_{28}H_{45}FO_3$: 448.3353). UV λ_{max} (EtOH): 246, 254, 264 nm.

4.4. Vitamin D receptor-binding assay

The rat recombinant VDR ligand-binding domain (LBD) (amino acids 115–423) was expressed as an amino-terminal His-tagged protein in *E. coli* BL21 (DE3) pLys S (Novagen).[23] The cells were lysed by sonication. The supernatants were diluted approximately 1000 times in 50 mM Tris buffer (100 mM KCl, 5 mM DTT, and 0.5% CHAPS, pH 7.5) containing bovine serum albumin (100 μg/ml) and were pipetted into glass culture tubes. A solution containing an increasing amount of 1α,25-$(OH)_2D_3$ or the synthetic analogs in 15 μl EtOH was added to the receptor solution in each tube and the mixture was vortexed 2–3 times. The mixture was incubated for 1 h at room temperature. [^{3}H]-1α,25-$(OH)_2D_3$ (specific activity, 6.62 TBq/mmol, ca. 5000 dpm) in 15 μl EtOH was added, vortexed 2–3 times, and the whole mixture was then allowed to stand at 4 °C for 18 h. At the end of the second incubation, 200 μl of dextran-coated charcoal suspension (purchased from Yamasa Shoyu) was added to bind any free ligands (or to remove free ligands) and the sample was vortexed. After 30 min at 4 °C, bound and free [^{3}H]-1α,25-$(OH)_2D_3$ were separated by centrifugation at 3000 rpm for 15 min at 4 °C. Aliquots (500 μl) of the supernatant were mixed with 9.5 ml of ACS-II scintillation fluid (Amersham, Buckinghamshire, UK) and submitted for radioactivity counting. Each assay was performed at least twice in duplicate.

4.5. Transactivation assay

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). Cells were seeded on 24-well plates at a density of $\sim 2 \times 10^4$ cells per well. After 24 h, cells were transfected with a reporter plasmid containing three copies of the mouse osteopontin VDRE (5′-GGTTCAc gaGGTTCA, SPPx3-TK-LUC), a wild-type or mutant hVDR expression plasmid [pCMX-hVDR or pSG5-hVDR (Δ165–215)], and the internal control plasmid containing sea pansy luciferase expression constructs (pRL-CMV) by the lipofection method as described previously.[31] After 4-h incubation, the medium was replaced with fresh DMEM containing 1% FCS (HyClone, UT). The next day, the cells were treated with either indicated concentration of 1α,25-$(OH)_2D_3$, 19-norvitamin D analogs, or ethanol vehicle and cultured for 24 h. Cells in each well were harvested with a cell lysis buffer, and the luciferase activity was measured with a luciferase assay kit (Tokyo Ink, Inc., Japan) according to the manufacturer's instructions. Transactivation measured by the luciferase activity was normalized with the internal control. All experiments were done in triplicate.

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