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Novel nonsecosteroidal vitamin D_3 carboxylic acid analogs for osteoporosis, and SAR analysis

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ABSTRACT

Novel vitamin D_3 analogs with carboxylic acid were explored, focusing on a nonsecosteroidal analog, LG190178, with a bisphenyl skeleton. From X-ray analysis of these analogs with vitamin D receptor (VDR), the carboxyl groups had very unique hydrogen bonding interactions in VDR and mimicked 1α-hydroxy group and/or 3β-hydroxy group of 1α,25-dihydroxyvitamin D_3. A highly potent analog, **6a**, with good in vitro activity and pharmacokinetic profiles was identified from an SAR study. Compound **6a** showed significant prevention of bone loss in a rat osteoporosis model by oral administration.

1. Introduction

1α,25-Dihydroxyvitamin D_3, [1,25$(OH)_2D_3$], **1**, is a known classic ligand of vitamin D receptor (VDR) which is synthesized in skin, liver, and kidney and plays an essential role in calcium homeostasis.[1–3] It stimulates calcium absorption in intestine, regulates bone formation and resorption, enhances calcium reabsorption in kidney, and inhibits the synthesis and secretion of parathyroid hormone (PTH) in the parathyroid gland.[4] Physiological actions of **1** are exhibited by binding to VDR and the consequent formation of a complex with several co-factors which binds to vitamin D responsive element (VDRE) in the promoter moiety of target genes controlling the transcription.[5–9]

Over three decades, derivatizations of vitamin D_3 have focused on the secosteroidal skeleton. Chemical modifications have led to a wide range of derivatives, especially around the side chain moiety and the A ring moiety of secosteroidal vitamin D_3 structure. Accordingly, more potent VDR agonists than 1,25$(OH)_2D_3$ (**1**) have been reported.[10]

Meanwhile, there has been a growing interest in nonsecosteroidal VDR agonists, since a bisphenyl compound, LG190178 (**2**), showed characteristics of a vitamin D_3 analog in vitro.[11–16]

Recently the crystal structure of YR301 (**3**), which is a stereoisomer of **2**, with VDR was reported and it has revealed that the secondary hydroxyl group in the A-part and the secondary hydroxyl group in the side chain of **3** worked like the 1-OH and 25-OH groups of **1**, respectively.[17–19]

Hydrogen bonding of the 2-OH group in the A-part of **3** with Ser237 and Arg274 would be of especial interest, because Arg274 in VDR is an essential amino acid which binds the 1-OH group of **1**, and indeed the lack of this interaction, such as in 25-OH vitamin D_3, decreased the binding affinity to VDR. However, the structure–activity relationships (SAR) of the A-part of **2** is still unclear.

Here we describe the SAR of nonsecosteroidal carboxylic acid derivatives focusing on an interaction of the A-part of the ligand with Arg274. We attempted to introduce a carboxyl group with methylene linker into the A-part of the bisphenyl skeleton (Fig. 1). The SAR study revealed the carboxyl group and methylene linker length significantly influence the VDR agonistic activity. As a result, we successfully confirmed that our carboxyl group had a salt bridge interaction to Arg274 in VDR from X-ray analysis of these derivatives.

We evaluated these analogs in the reporter gene activity of VDRE and osteocalcin production activity as a VDR agonist in vitro. Also, in vivo effects on bone and serum calcium levels were evaluated using a rat osteoporosis model, which is commonly used in the evaluation of the activity of vitamin D_3 analogs.[20,21]

2. Chemistry

The synthesis of compounds **4a–d** and **6a** is shown in Scheme 1. Compound **7** was prepared from bisphenol by the method reported

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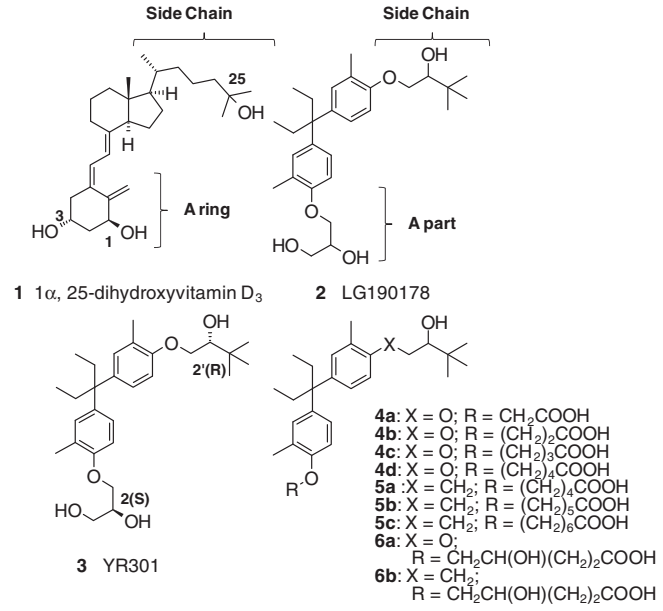


Figure 1. Structures of secosteroids $1,25(OH)_2D_3$ (**1**), nonsecosteroidal analog LG190178 (**2**) and YR301 (**3**), and novel nonsecosteroidal carboxylic acid derivatives (**4a–d**, **5a–c**, **6a**, and **6b**). Part labeled A of **2** (upper right) shows the structural correspondence to the A ring of secosteroids (upper left).

by Boehm.[11] Alkylation of **7** with K_2CO_3 and various alkylbromides gave corresponding ester (**8a**, **8c**, and **8d**) in moderate yields. Hydrolysis of ester and the following reduction of ketone using $NaBH_4$ gave carboxylic acid analogs in good yields (**4a**, **4c**, and **4d**).

Compound **4b** was prepared from alcohol **9**, which was obtained from the alkylation of phenol **7** using 3-bromo-1-propanol. In the case of direct alkylation of phenol **7** using 3-bromopropionoic acid ester, no product was obtained because of dominant β-elimination. Pyridinium dichromate (PDC) oxidation of alcohol **9** afforded carboxylic acid **10**. Reduction of ketone group of **10** using $NaBH_4$ gave carboxylic acid **4b**. Alkylation of phenol **7** with tosylate **11** afforded lactone **12** and was then treated with $NaBH_4$ to give alcohol **13**. Compound **13** was hydrolyzed with 1 N NaOH to afford gamma hydroxycarboxylic acid **6a** in very good yield.

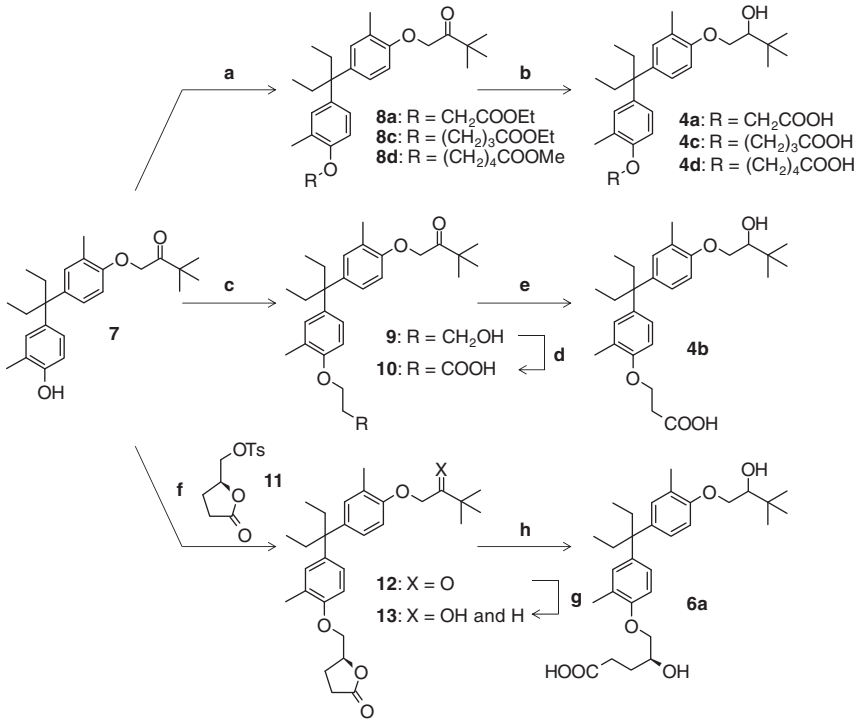
Carbon link side chain analogs were prepared from bisphenol **14**, which was also reported by Boehm[11] (Scheme 2). Treatment of **14** with triflic anhydride and pyridine afforded mono triflate **15**. Palladium-catalyzed Sonogashira coupling of triflate **15** with **16** gave acetylene **17** and the following hydrogenation gave phenol **18** in moderate yield. Alkylation of phenol group using K_2CO_3 and alkyl halides and tosylate **11** afforded corresponding ester **19a–c** and lactone **20** in very good yield. Every ester or lactone was hydrolyzed in the usual manner and carboxylic acid derivatives **5a–c** and gamma hydroxycarboxylic acid **6b** were obtained, respectively.

3. Results and discussion

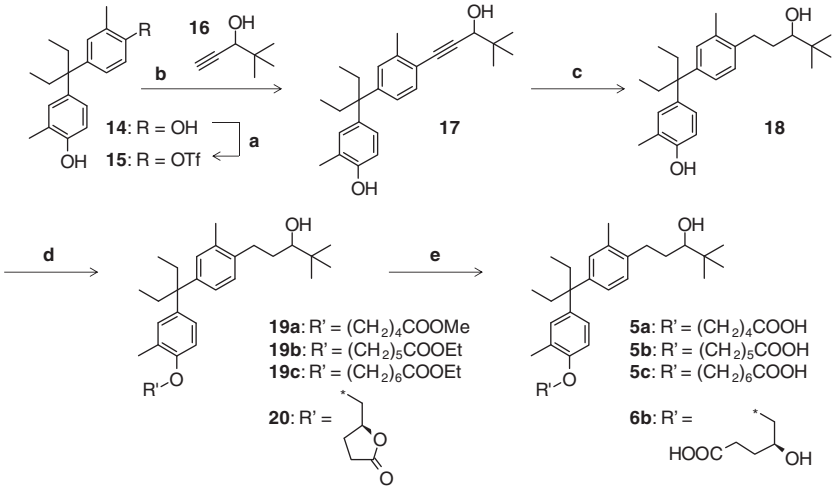
All synthesized compounds were evaluated by reporter gene assay in MG-63 cells, which contained VDRE sequence derived from mouse osteopontin promoter for vitamin D agonistic activity. The osteocalcin production activity in MG-63 cells was evaluated to measure bone formation activity of functional vitamin D agonistic effect.[22–24] VDRE reporter gene activity and osteocalcin activity are represented by a relative EC_{50} value which is the EC_{50} value of $1,25(OH)_2D_3$ assigned as 100% (a larger figure means stronger activity).

At first, we examined the biological activity to see the impact of linker length between carboxyl group and bisphenyl ring on VDR agonistic activity (**4a–d**, Table 1).

As expected, the carboxylic acid derivatives exhibited VDR agonistic activity. In addition, we found that there is an optimal linker length (**4d** and **5a**), which showed more potent activity than

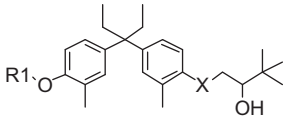


Scheme 1. Reagents and conditions: (a) K_2CO_3, $Br(CH_2)_nCO_2R$, 70 °C, DMF, 31–52%; (b) (1) 1 N NaOH, 60 °C, MeOH; (2) $NaBH_4$, room temperature, MeOH–THF, 74–88% (for two steps); (c) K_2CO_3, 3-bromo-1-propanol, 50 °C DMF, 50%; (d) PDC, room temperature, DMF, 9.5%; (e) $NaBH_4$, room temperature, MeOH, 67%; (f) K_2CO_3, **11**, 70 °C, DMF, 49%; (g) $NaBH_4$, room temperature, MeOH, 41%; (h) 1 N NaOH, room temperature, MeOH–THF, 74%.



Scheme 2. Reagents and conditions: (a) Tf_2O, pyridine, 0 °C to room temperature, CH_2Cl_2, 37%; (b) **16**, CuI, $Pd(PPh_3)_4$, Et_3N, 80 °C, CH_3CN, 34%; (c) 10% Pd/C, H_2, room temperature, ethyl acetate, 98%; (d) K_2CO_3, $Br(CH_2)_nCO_2R$ or **11**, 70 °C, DMF, 78–88%; (e) 1 N NaOH, 60 °C, MeOH, 70–84%.

Table 1
VDRE reporter gene activity and osteocalcin induction activity of carboxylic acid analogs



Compound	R_1	X	Ratio of VDRE reporter activity[#] (%)	Ratio of osteocalcin activity[#] (%)
4a	HOOC–CH_2–	O	1.4	<13
4b	HOOC–$(CH_2)_2$–	O	6.2	49
4c	HOOC–$(CH_2)_3$–	O	2.6	<13
4d	HOOC–$(CH_2)_4$–	O	19	205
5a	HOOC–$(CH_2)_4$–	CH_2	31	368
5b	HOOC–$(CH_2)_5$–	CH_2	16	168
5c	HOOC–$(CH_2)_6$–	CH_2	2.2	9.3
6a	(*S*)–HOOC–$(CH_2)_2$CH(OH)CH_2–	O	20	484
6b	(*S*)–HOOC–$(CH_2)_2$CH(OH)CH_2–	CH_2	52	68
LG190178	HO–CH_2CH(OH)CH_2–	O	6.7	185
$1,25(OH)_2D_3$			100	100

[#] $1,25(OH)_2D_3$ ratio of EC_{50}. $1,25(OH)_2D_3$ was assigned to 100% (a larger figure means stronger activity).

LG190178 in the VDRE reporter gene and osteocalcin assays. The shorter methylene analogs (**4a**–**c**) exhibited weaker activities than **4d**, but the activities were not proportional to the linker length in either VDRE reporter gene activity or osteocalcin activity. The longer five-methylene linker analog (**5b**) showed moderate activities; however, a six-methylene linker analog (**5c**) dramatically reduced both activities. Introduction of a hydroxyl group into the gamma position of the carboxyl group in **4d** and **5a** maintained efficacy in VDRE reporter gene activity and osteocalcin activity (**6a** and **6b**).

To confirm the detailed interaction of a carboxyl group with VDR, we cocrystallized the ligand-binding domain for human VDR with **4a**, **4d** and **6b** in accordance with a method previously published by Moras et al.[25,26] (Figs. 2–4). The X-ray structure, determined to a resolution of 2.0 Å, shows the overall structure of **4d** was consistent with that of YR301 (Fig. 2). The hydroxyl group in the side chain moiety interacted with His305 and His397 and these interactions were seen in the crystal structures of **4a** and **6b** with VDR in the same manner. The diethyl moiety between bisphenyl rings made a good CH–π interaction with Trp278. The diethyl moieties were buried in the hydrophobic pocket formed by Leu227, Val234, Leu404 and Val418. It should be noted that the carboxyl group of **4d** forms a salt bridge with Arg274 and also has a hydrogen bonding interaction with Ser237. However **4d** has no hydrogen bonding interaction with Ser278 and Tyr134 in the way that the 3-OH group of $1,25(OH)_2D_3$ interacts with them. On the other hand, shorter methylene linker analog **4a** had another pattern of interaction (Fig. 3). The carboxyl group of **4a** has hydrogen bonding interactions with Ser278 and Tyr134; however, with Ser237 and Arg274, **4a** shows an indirect hydrogen bonding interaction via crystal water. In the case of **6b**, the carboxyl group has the same hydrogen bonding interactions with Ser237 and Arg274 of VDR as **4d** and the additional gamma hydroxyl group makes indirect hydrogen bonding interactions with Ser278, Tyr134 and Arg274 via crystal water (Fig. 4).

From these results, the interactions of a ligand with Ser237 and Arg274 might contribute to enhanced VDR agonistic activity. In VDRE reporter gene activity, **4d** and **6b**, which had tight binding to Ser 237 and Arg274, indicated stronger activity than **4a** which had no direct binding to Ser237 and Arg274 and showed weak activity. On the other hand, **6a** and **6b**, which could have interactions with Ser278 and Tyr134 via crystal water, showed similar VDRE reporter gene activity to corresponding analogs (**4d** and **5a**). It also suggested that the indirect hydrogen bonding interactions with Ser278 and Tyr134 could not be associated with VDR agonistic activity.

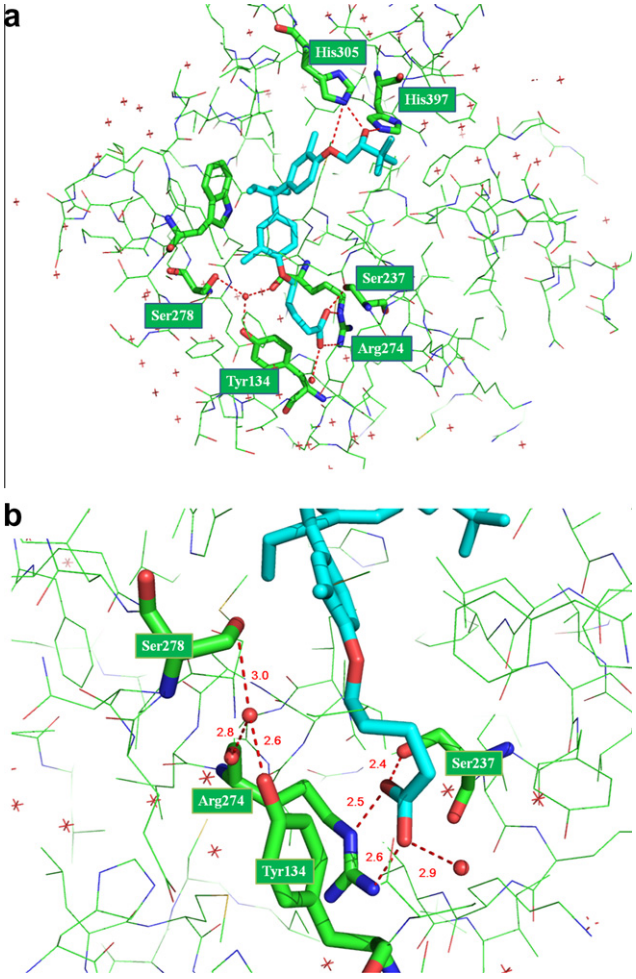


Figure 2. X-ray crystal structure of compound **4d** (blue) bound to VDR (green). Key hydrogen bonding interactions are shown by red dotted lines and each distance is indicated in Å. (a) Overall structure of compound **4d** bound to VDR. (b) Detailed hydrogen bonding network around A-part of compound **4d**. (PDB: 3AZ2).

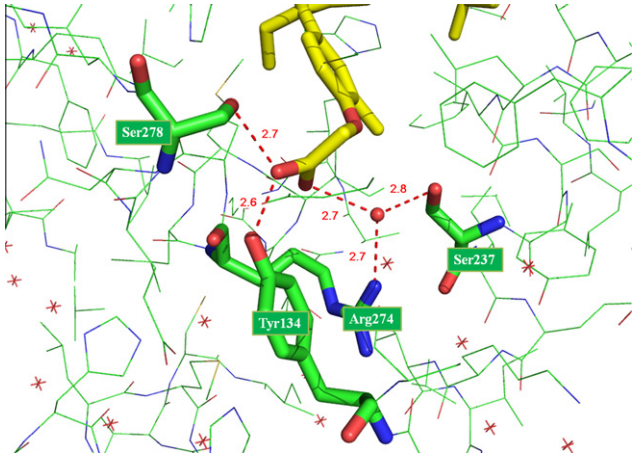


Figure 3. X-ray crystal structure of compound **4a** (yellow) bound to VDR (green). Key hydrogen bonding interactions are shown by red dotted lines and each distance is indicated in Å. (PDB: 3AZ1).

In an osteocalcin assay, **4a** showed 14-fold less potency than LG190178 but the potency of **4d** to it was similar. Although **6b**, which had the most potent VDRE reporter gene activity, showed only one-third the activity of LG190178, **6a** showed the most potent osteocalcin activity. This discrepancy between VDRE reporter

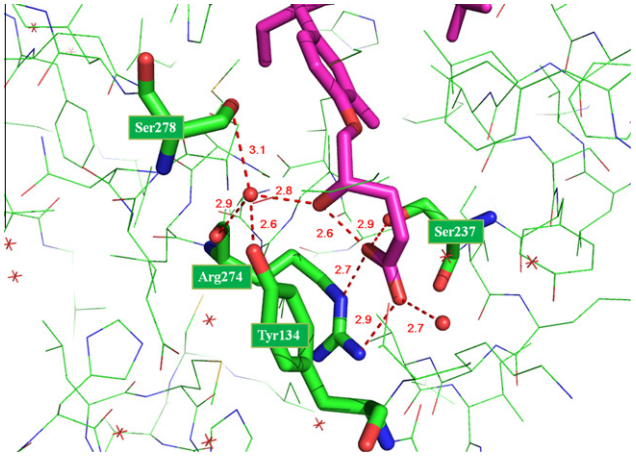


Figure 4. X-ray crystal structure of compound **6b** (magenta) bound to VDR (green). Key hydrogen bonding interactions are shown by red dotted lines and each distance is indicated in Å. (PDB: 3AZ3).

gene and osteocalcin activity could not be explained by the interactions between VDR and its ligand alone. It might be better to consider the interactions between the VDR ligand complex and other co-factors. For the transcriptional activation of VDR, it is required that the AF-2 transactivation motif of VDR interacts with several types of cofactor such as VDR interacting proteins (DRIPs).[27]

In the side chain part, LG190178 was originally reported as a racemic mixture.[11] However, Hakamata et al.[17] separated each isomer of the side chain and reported (*R*) isomer had stronger VDR agonistic activity than (*S*) isomer. The compounds reported here are all racemic mixtures of the side chain hydroxyl group because we used a comparison to LG190178 to evaluate the effect of specific modifications to the A-part. We expect (*R*) isomer of the side chain could exhibit stronger VDR agonistic activity and will discuss a detailed SAR analysis of these and other hydroxyl group configurations of the side chain part at another time.

Next, we assessed pharmacokinetic parameters in carboxylic acid derivatives (**4d** and **6a**) in rat at the oral and intravenous single dosage of 100 μg/kg (Table 2). 3-Hydroxypentanoic acid **6a** appears substantially better than pentanoic acid **4d**, with an especially dramatic improvement in total clearance in iv administration (3054 mL/h/kg for **4d** vs 88 mL/h/kg for **6a**). The significant improvement in clearance suggests that the gamma hydroxyl group of **6a** would stabilize the A-part from metabolic oxidation. This enhancement in pharmacokinetic properties, along with its excellent in vitro efficacy, led us to choose **6a** for an in vivo disease model study.

We evaluated the effect on the prevention of bone mineral density (BMD) loss of **6a** in osteoporosis model rats (Fig. 5). Female Sprague–Dawley rats, 8 weeks old, were either ovariectomized (OVX) to produce the estrogen-deficient condition which promotes BMD loss observed in postmenopausal women or were sham-operated. Compound **6a** was orally administered to three groups of OVX rats (0.22 μg/kg, 0.67 μg/kg and 2.0 μg/kg per day). Both the OVX-control and sham-operated groups were administered only vehicle. After daily administration for 4 weeks, BMD in the distal femur was measured by dual-energy X-ray absorptiometry. BMD in the OVX-control group was significantly reduced compared with the sham-operated group (Fig. 5). Compound **6a** dose-dependently prevented BMD loss caused by ovariectomy. The 0.67 μg/kg group of **6a** maintained the equivalent level of BMD to that of the sham-operated group without the body weight loss or hypercalcemia shown by the highest (2.0 μg/kg) administration group. These two in vivo results, the prevention of BMD loss and the elevation

Table 2
Pharmacokinetic parameters of **4d** and **6a**

	4d		6a	
	po	iv	po	iv
F (%)	33		13	
$T_{1/2}$ (h)	3.1	1.3	8.0	10.6
C_{max} (ng/mL)	3.6		11.6	
AUC_{inf} (ng/mL*h)	10.9	32.9	151.1	1151.6
MRT (h)	3.8	0.5	12.3	9.2
CL_t (mL/h/kg)	9350	3054	691	88

Oral and intravenous administration (100 μg/kg) in rat.

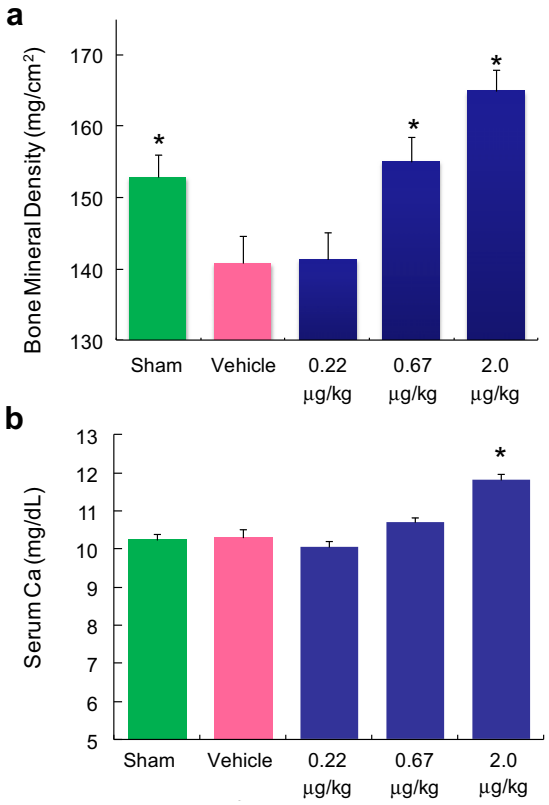


Figure 5. (a) Oral once-daily treatment of **6a** prevented BMD loss in osteoporosis model rats. (b) Serum calcium concentration in osteoporosis model rat. *P <0.05 versus vehicle.

of serum calcium concentration, were typical VDR agonistic actions which were reported in in vivo evaluations of secosteroidal vitamin D_3 analogs.[20,21]

4. Conclusion

Novel nonsecosteroidal vitamin D_3 agonists were explored. They have carboxylic acid instead of 1,3-diol of $1,25(OH)_2D_3$ and compounds **4d** and **5a** were identified as the optimized linker length analogs. Additional hydroxyl group in gamma position of the carboxyl group in **4d** and **5a** significantly improved metabolic stability. X-ray structure of **6b** exhibited the carboxyl group tightly interacting with Ser237 and Arg274 and the gamma hydroxyl group interacting with Ser278 and Tyr134 via crystal water. Through our SAR studies, compound **6a** was identified as the optimal compound showing excellent in vitro potency and pharmacokinetics. In a rat osteoporosis model, **6a** was confirmed to have significant prevention of bone loss without hypercalcemia via once-daily oral administration. Further modification from **6a** is ongoing and the SAR results will be published.

5. Experimental

5.1. Chemistry: General

Purchased reagents and solvents were used without further purification unless otherwise noted. ^1H and ^{13}C NMR spectra were carried out on VARIAN 400-MR spectrophotometers; chemical shifts are reported in parts per million (ppm) downfield from that of internal tetramethylsilane (TMS). Mass spectrophotometry was measured with a Waters ZQ2000 electrospray ionization (ESI) system. High-resolution mass spectra (HRMS) were recorded on Thermo Fisher Scientific LTQ Orbitrap XL (ESI) instruments. Chromatographic purification was carried out using Merck silica gel 60 (column) or Merck silica gel 60 PF_{254} (preparative TLC).

5.1.1. (4-{1-[4-(3,3-Dimethyl-2-oxo-butoxy)-3-methyl-phenyl]-1-ethyl-propyl}-2-methyl-phenoxy)-acetic acid ethyl ester (8a)

To a solution of **7** (32.3 mg, 0.084 mmol) in DMF (1.0 mL) were added K_2CO_3 (23 mg, 0.169 mmol) and ethyl bromoacetate (28 mg, 0.169 mmol). The mixture was stirred at 70 °C overnight. Then the mixture was diluted with AcOEt, washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated. The obtained residue was purified by silicagel chromatography (*n*-hexane/AcOEt = 100:0 to 20:80) to give **8a** (20.6 mg, 52%) as a colorless oil. ^1H NMR ($CDCl_3$) δ: 0.58 (6H, t, J = 7.3 Hz), 1.25 (9H, s), 1.29 (3H, t, J = 7.1 Hz), 2.01 (4H, q, J = 7.2 Hz), 2.22 (3H, s), 2.23 (3H, s), 4.26 (2H, q, J = 7.1 Hz), 4.60 (2H, s), 4.83 (2H, s), 6.49 (1H, d, J = 8.4 Hz), 6.57 (1H, d, J = 9.2 Hz), 6.87–6.92 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 210.0, 169.3, 153.9, 153.8, 141.7, 141.4, 130.8, 130.7, 126.1, 126.0, 126.0, 125.9, 110.2, 110.1, 69.6, 65.8, 61.1, 48.4, 43.2, 29.2, 26.3, 16.6, 16.5, 14.1, 8.4. MS (ESI positive): 486 $(M+NH_4)^+$.

5.1.2. 4-(4-{1-[4-(3,3-Dimethyl-2-oxo-butoxy)-3-methyl-phenyl]-1-ethyl-propyl}-2-methyl-phenoxy)-butyric acid ethyl ester (8c)

The yield was 46%. Colorless oil. ^1H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.3 Hz), 1.26 (9H, s), 1.26 (3H, t, J = 7.1 Hz), 2.01 (4H, q, J = 7.3 Hz), 2.09–2.15 (2H, m), 2.15 (3H, s), 2.24 (3H, s), 2.54 (2H, t, J = 7.3 Hz), 3.98 (2H, t, J = 6.1 Hz), 4.15 (2H, q, J = 7.2 Hz), 4.84 (2H, s), 6.50 (1H, d, J = 8.3 Hz), 6.67 (1H, d, J = 8.8 Hz), 6.89–6.94 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 210.1, 173.4, 154.5, 153.9, 141.5, 140.5, 130.7, 130.4, 126.0, 125.9, 125.4, 110.1, 109.6, 69.6, 66.4, 60.4, 48.3, 43.2, 30.9, 29.2, 26.3, 24.8, 16.7, 16.5, 14.2, 8.4. MS (ESI positive): 514 $(M+NH_4)^+$.

5.1.3. 5-(4-{1-[4-(3,3-Dimethyl-2-oxo-butoxy)-3-methyl-phenyl]-1-ethyl-propyl}-2-methyl-phenoxy)-pentanoic acid methyl ester (8d)

The yield was 31%. Colorless oil. ^1H NMR ($CDCl_3$) δ: 0.58 (6H, t, J = 7.3 Hz), 1.25 (9H, s), 1.81–1.86 (4H, m), 2.01 (4H, q, J = 7.3 Hz), 2.15 (3H, s), 2.24 (3H, s), 2.41 (2H, t, J = 7.1 Hz), 3.67 (3H, s), 3.94 (2H, t, J = 5.7 Hz), 4.83 (2H, s), 6.49 (1H, d, J = 8.4 Hz), 6.66 (1H, d, J = 8.4 Hz), 6.87–6.94 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 210.0, 174.0, 154.6, 153.9, 141.6, 140.4, 130.7, 130.4, 126.0, 126.0, 125.9, 125.4, 110.1, 109.6, 69.6, 67.0, 51.5, 48.3, 43.2, 33.7, 29.3, 28.8, 26.3, 21.8, 16.6, 16.5, 8.4. MS (ESI positive): 514 $(M+NH_4)^+$.

5.1.4. (4-{1-Ethyl-1-[4-(2-hydroxy-3,3-dimethyl-butoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-acetic acid (4a)

To a solution of **8a** (10.8 mg, 0.023 mmol) in MeOH (1.0 mL) was added 1 N NaOH (0.115 mL, 0.115 mmol). The mixture was

stirred at 60 °C for 30 min. After the mixture was poured into 1 N HCl, the products were extracted with CH_2Cl_2. The extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated. The obtained residue was dissolved in MeOH (0.5 mL) and THF (0.5 mL). To the solution was added $NaBH_4$ (2.6 mg, 0.069 mmol). The mixture was stirred at room temperature for 1 h. Then the mixture was poured into sat. NH_4Cl aq solution, and the products were extracted with CH_2Cl_2. The extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated. The obtained residue was purified preparative TLC (10% MeOH/$CHCl_3$) to give **4a** (8.5 mg, 84%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.58 (6H, t, J = 7.2 Hz), 1.01 (9H, s), 2.11 (4H, q, J = 7.2 Hz), 2.16 (3H, s), 2.19 (3H, s), 3.71 (1H, dd, J = 8.8, 2.5 Hz), 3.85 (1H, t, J = 8.9 Hz), 4.08 (1H, dd, J = 9.2, 2.5 Hz), 4.59 (2H, s), 6.59 (1H, d, J = 8.2 Hz), 6.69 (1H, d, J = 8.4 Hz), 6.86–6.96 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 173.2, 154.3, 153.3, 142.3, 140.9, 130.9, 130.6, 126.2, 126.1, 126.0, 125.5, 110.6, 110.0, 77.2, 69.1, 65.6, 48.4, 33.5, 29.2, 26.0, 16.6, 16.5, 8.4. HRMS (ESI negative): Calcd for $C_{27}H_{37}O_5$ 441.2646. Found: 441.2636 $(M-H)^-$.

5.1.5. 4-(4-{1-Ethyl-1-[4-(2-hydroxy-3,3-dimethyl-butoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-butyric acid (4c)

The yield was 74%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.2 Hz), 1.01 (9H, s), 2.02 (4H, q, J = 7.3 Hz), 2.09–2.15 (2H, m), 2.15 (3H, s), 2.17 (3H, s), 2.61 (2H, t, J = 7.2 Hz), 3.71 (1H, dd, J = 8.8, 2.5 Hz), 3.85 (1H, t, J = 9.0 Hz), 3.99 (2H, t, J = 5.9 Hz), 4.09 (1H, dd, J = 9.2, 2.5 Hz), 6.67 (2H, d, J = 8.6 Hz), 6.69 (2H, d, J = 8.6 Hz), 6.88–6.97 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 178.1, 154.4, 154.2, 141.2, 140.7, 130.6, 130.5, 126.1, 126.0, 125.5, 125.4, 110.0, 109.6, 77.2, 69.1, 66.2, 48.4, 33.5, 30.5, 29.3, 26.0, 24.6, 16.6, 16.5, 8.4. HRMS (ESI negative): Calcd for $C_{29}H_{41}O_5$ 469.2959. Found: 469.2965 $(M-H)^-$.

5.1.6. 5-(4-{1-Ethyl-1-[4-(2-hydroxy-3,3-dimethyl-butoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-pentanoic acid (4d)

The yield was 88%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.3 Hz), 1.01 (9H, s), 1.84–1.87 (4H, m), 2.01 (4H, q, J = 7.3 Hz), 2.15 (3H, s), 2.17 (3H, s), 2.45–2.47 (2H, m), 3.71 (1H, dd, J = 8.8, 2.5 Hz), 3.85 (1H, t, J = 8.9 Hz), 3.94–3.96 (2H, m), 4.09 (1H, dd, J = 9.2, 2.5 Hz), 6.66 (2H, d, J = 8.6 Hz), 6.69 (2H, d, J = 8.6 Hz), 6.88–6.98 (4H, m).^{13}C NMR ($CDCl_3$) δ: 179.1, 154.6, 154.2, 141.3, 140.4, 130.6, 130.4, 126.1, 126.0, 125.4, 125.4, 110.0, 109.6, 77.3, 69.1, 67.0, 48.3, 33.6, 33.5, 29.3, 28.7, 26.0, 21.5, 16.6, 16.5, 8.4. HRMS (ESI negative): Calcd for $C_{30}H_{43}O_5$ 483.3116. Found: 483.3111 $(M-H)^-$.

5.1.7. 1-(4-{1-Ethyl-1-[4-(3-hydroxy-propoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-3,3-dimethyl-butan-2-one (9)

To a solution of **7** (76 mg, 0.199 mmol) in DMF (2.0 mL) were added K_2CO_3 (54.9 mg, 0.397 mmol) and 3-bromo-1-propanol (55.2 mg, 0.397 mmol). The mixture was stirred at 50 °C overnight. Then the mixture was poured into sat. NH_4Cl aq solution and the products were extracted with AcOEt. The extracts were washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated. The obtained residue was purified by preparative TLC (n-hexane/AcOEt = 1:1) to give **9** (44.2 mg, 50%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.2 Hz), 1.25 (9H, s), 1.98–2.13 (6H, m), 2.16 (3H, s), 2.24 (3H, s), 3.89 (2H, t, J = 5.8 Hz), 4.11 (2H, t, J = 5.8 Hz), 4.83 (2H, s), 6.49 (1H, d, J = 8.4 Hz), 6.71 (1H, d, J = 8.4 Hz), 6.89–6.95 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 210.2, 154.4, 153.9, 141.5, 140.8, 130.7, 130.5, 126.1, 126.0, 125.9, 125.2, 110.1, 109.6, 69.5, 66.0, 61.1, 48.3, 43.2, 32.0, 29.2, 26.3, 16.6, 16.6, 8.4. MS (ESI positive): 463 $(M+Na)^+$.

5.1.8. 3-(4-{1-[4-(3,3-Dimethyl-2-oxo-butoxy)-3-methyl-phenyl]-1-ethyl-propyl}-2-methyl-phenoxy)-propionic acid (10)

To a solution of **9** (44 mg, 0.100 mmol) in DMF (1.0 mL) was added PDC (113 mg, 0.300 mmol). The mixture was stirred at room temperature for three days. Then the mixture was poured into 1 N HCl and the products were extracted with AcOEt. The extracts were washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated. The obtained residue was purified by preparative TLC (n-hexane/AcOEt = 1:1) to give **10** (4.3 mg, 9.5%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.58 (6H, t, J = 7.2 Hz), 1.25 (9H, s), 2.01 (4H, q, J = 7.3 Hz), 2.13 (3H, s), 2.24 (3H, s), 2.85 (2H, t, J = 6.1 Hz), 4.23 (2H, t, J = 6.1 Hz), 4.83 (2H, s), 6.49 (1H, d, J = 8.4 Hz), 6.70 (1H, d, J = 8.6 Hz), 6.89–6.96 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 210.1, 175.6, 154.0, 153.9, 141.5, 141.1, 130.7, 130.5, 126.1, 126.0, 125.9, 125.6, 110.1, 110.0, 69.6, 63.1, 48.4, 43.2, 34.4, 29.2, 26.3, 16.6, 16.4, 8.4. MS (ESI positive): 472 $(M+NH_4)^+$.

5.1.9. 3-(4-{1-Ethyl-1-[4-(2-hydroxy-3,3-dimethyl-butoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-propionic acid (4b)

The yield was 67%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.2 Hz), 1.01 (9H, s), 2.02 (4H, q, J = 7.2 Hz), 2.13 (3H, s), 2.17 (3H, s), 2.85 (2H, t, J = 6.3 Hz), 3.71 (1H, dd, J = 8.8, 2.5 Hz), 3.85 (1H, t, J = 8.9 Hz), 4.09 (1H, dd, J = 9.1, 2.4 Hz), 4.23 (2H, t, J = 6.2 Hz), 6.69 (1H, d, J = 8.4 Hz), 6.71 (1H, d, J = 8.2 Hz), 6.88–6.97 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 175.0, 154.2, 154.0, 141.1, 141.1, 130.6, 130.5, 126.1, 126.0, 125.7, 125.4, 110.0, 110.0, 77.2, 69.1, 63.1, 48.4, 34.3, 33.5, 29.2, 26.0, 16.6, 16.4, 8.4. HRMS (ESI negative): Calcd for $C_{28}H_{39}O_5$ 455.2803. Found: 455.2799 $(M-H)^-$.

5.1.10. (*S*)-5-(4-{1-[4-(3,3-Dimethyl-2-oxo-butoxy)-3-methyl-phenyl]-1-ethyl-propyl}-2-methyl-phenoxymethyl)-dihydro-furan-2-one (12)

The yield was 49%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.58 (6H, t, J = 7.3 Hz), 1.25 (9H, s), 2.01 (4H, q, J = 7.3 Hz), 2.15 (3H, s), 2.23 (3H, s), 2.28–2.36 (1H, m), 2.39–2.49 (1H, m), 2.52–2.61 (1H, m), 2.72–2.82 (1H, m), 4.06 (1H, dd, J = 10.3, 3.4 Hz), 4.17 (1H, dd, J = 10.4, 3.3 Hz), 4.83 (2H, s), 4.85–4.91 (1H, m), 6.49 (1H, d, J = 8.2 Hz), 6.66 (1H, d, J = 8.4 Hz), 6.86–6.97 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 210.0, 177.2, 154.0, 153.9, 141.5, 141.4, 130.7, 130.7, 126.2, 126.0, 125.9, 125.5, 110.1, 109.8, 77.9, 69.5, 69.3, 48.4, 43.2, 29.2, 28.3, 26.3, 24.1, 16.6, 8.4. MS (ESI positive): 498 $(M+NH_4)^+$.

5.1.11. (*S*)-5-(4-{1-Ethyl-1-[4-(2-hydroxy-3,3-dimethyl-butoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxymethyl)-dihydro-furan-2-one (13)

The yield was 41%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.2 Hz), 1.01 (9H, s), 2.02 (4H, q, J = 7.2 Hz), 2.15 (3H, s), 2.17 (3H, s), 2.27–2.36 (1H, m), 2.39–2.49 (1H, m), 2.51–2.61 (1H, m), 2.72–2.82 (1H, m), 3.70 (1H, dd, J = 8.7, 2.4 Hz), 3.85 (1H, t, J = 8.9 Hz), 4.04–4.11 (2H, m), 4.17 (1H, dd, J = 10.4, 3.3 Hz), 4.85–4.91 (1H, m), 6.66 (1H, d, J = 8.4 Hz), 6.69 (1H, d, J = 8.4 Hz), 6.88–6.97 (4H, m). ^{13}C NMR ($CDCl_3$) δ: 177.2, 154.3, 153.8, 141.6, 141.0, 130.7, 130.6, 126.1, 126.1, 125.5, 125.5, 110.0, 109.8, 77.9, 77.2, 69.3, 69.1, 48.4, 33.5, 29.2, 28.3, 26.0, 24.1, 16.6, 16.6, 8.4. MS (ESI positive): 505 $(M+Na)^+$.

5.1.12. (*S*)-5-(4-{1-Ethyl-1-[4-(2-hydroxy-3,3-dimethyl-butoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-4-hydroxy-pentanoic acid (6a)

The yield was 74%. Colorless oil. ^{1}H NMR (CD_3OD) δ: 0.58 (6H, t, J = 7.2 Hz), 1.00 (9H, s), 1.79–1.89 (1H, m), 1.93–2.00 (1H, m), 2.03 (4H, q, J = 7.2 Hz), 2.14 (3H, s), 2.15 (3H, s), 2.37 (2H, td, J = 7.3, 3.1 Hz), 3.61 (1H, dd, J = 7.8, 2.9 Hz), 3.84–3.91 (3H, m),

3.93–3.97 (1H, m), 4.11 (1H, dd, J = 10.0, 2.9 Hz), 6.75 (2H, dd, J = 8.4, 2.2 Hz), 6.84 (2H, dd, J = 5.5, 2.0 Hz), 6.95 (2H, dt, J = 8.4, 2.4 Hz). ^{13}C NMR (CD_3OD) δ: 181.1, 154.8, 154.7, 140.6, 140.6, 130.1, 130.1, 125.7, 125.7, 125.3, 109.7, 77.2, 71.7, 70.0, 69.4, 47.8, 34.0, 33.6, 30.0, 28.7, 25.2, 15.4, 15.3, 7.3. HRMS (ESI negative): Calcd for $C_{30}H_{43}O_6$ 499.3065. Found: 499.3060 $(M-H)^-$.

5.1.13. Trifluoromethanesulfonic acid 4-[1-ethyl-1-(4-hydroxy-3-methyl-phenyl)-propyl]-2-methyl-phenyl ester (15)

To a solution of **14** (9.0 g, 31.6 mmol) in CH_2Cl_2 (300 mL) were added pyridine (3.0 mL, 37.2 mmol) and trifluoromethanesulfonic anhydride (5.7 mL, 34.7 mmol) at 0 °C and the mixture was stirred for 1 h. The mixture was diluted with AcOEt, washed with sat. $NaHCO_3$ aq solution and brine, dried over anhydrous $MgSO_4$, and concentrated. The obtained residue was chromatographed on silica gel (*n*-hexane/AcOEt = 90:10 to 0:100) to afford **15** (4.9 g, 37%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.3 Hz), 2.03 (4H, q, J = 7.3 Hz), 2.20 (3H, s), 2.31 (3H, s), 4.78 (1H, br s), 6.67 (1H, d, J = 8.2 Hz), 6.82–6.86 (2H, m), 7.02–7.11 (3H, m). ^{13}C NMR ($CDCl_3$) δ: 151.6, 149.5, 146.2, 139.9, 131.6, 130.5, 129.5, 127.3, 126.6, 122.9, 120.1, 117.0, 114.2, 48.9, 29.2, 16.6, 16.0, 8.3. MS (ESI negative): 415 $(M-H)^-$.

5.1.14. 4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pent-1-ynyl)-3-methyl-phenyl]-propyl}-2-methyl-phenol (17)

To a solution of **15** (190 mg, 0.46 mmol) in MeCN (2.3 mL) were added 4,4-Dimethyl-pent-1-yn-3-ol (**16**) (103 mg, 0.92 mmol), $Pd(PPh_3)_4$ (53 mg, 0.046 mmol), CuI (9 mg, 0.046 mmol), and Et_3N (0.192 mL, 1.38 mmol). The mixture was stirred in a sealed tube at 80 °C for 3 h. The mixture was poured into $KHSO_4$ aq solution and the products were extracted with AcOEt. The extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated. The obtained residue was purified by silica gel chromatography (*n*-hexane/AcOEt = 100:0 to 84:16) to give **17** (59 mg, 34%) as a pale brown form. ^{1}H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.3 Hz), 1.07 (9H, s), 2.03 (4H, q, J = 7.2 Hz), 2.19 (3H, s), 2.38 (3H, s), 4.26 (1H, d, J = 6.1 Hz), 4.58 (1H, d, J = 1.6 Hz), 4.88 (1H, s), 6.65 (1H, d, J = 8.0 Hz), 6.83 (1H, d, J = 2.3 Hz), 6.94 (1H, dd, J = 8.0, 1.8 Hz), 7.00 (1H, s), 7.28 (1H, d, J = 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 151.6, 149.7, 140.2, 139.2, 131.3, 130.6, 129.1, 126.5, 125.5, 122.8, 119.3, 114.0, 92.1, 84.9, 72.0, 49.0, 36.1, 29.0, 25.4, 21.1, 16.1, 8.3. MS (ESI positive): 401 $(M+Na)^+$.

5.1.15. 4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenol (18)

To a solution of **17** (99 mg, 0.262 mmol) in AcOEt (2 mL) was added 10% Pd/C (10 mg) and the mixture was stirred at room temperature for 30 min under hydrogen atmosphere. The mixture was filtered through celite and concentrated to give **18** (98 mg, 98%) as a colorless form. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.3 Hz), 0.89 (9H, s), 1.46–1.57 (1H, m), 1.76–1.84 (1H, m), 2.03 (4H, q, J = 7.6 Hz), 2.19 (3H, s), 2.24 (3H, s), 2.52–2.59 (1H, m), 2.85–2.89 (1H, m), 3.27 (1H, dd, J = 10.6, 1.6 Hz), 5.26 (1H, br s), 6.64 (1H, d, J = 8.4 Hz), 6.85 (1H, dd, J = 8.3, 2.2 Hz), 6.90–6.94 (3H, m), 7.02 (1H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 151.4, 146.4, 140.8, 137.2, 134.8, 130.6, 129.8, 127.8, 126.6, 125.6, 122.6, 114.0, 80.1, 48.6, 35.0, 32.0, 30.4, 29.2, 25.7, 19.6, 16.1, 8.5. MS (ESI positive): 405 $(M+Na)^+$.

5.1.16. 5-(4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-pentanoic acid methyl ester (19a)

The yield was 78%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.2 Hz), 0.89 (9H, s), 1.45–1.55 (1H, m), 1.76–1.86 (5H, m), 2.04 (4H, q, J = 7.3 Hz), 2.16 (3H, s), 2.26 (3H, s), 2.41 (2H, t, J = 7.1 Hz), 2.54–2.58 (1H, m), 2.82–2.90 (1H, m), 3.25 (1H, d, J = 9.8 Hz), 3.67 (3H, s), 3.94 (2H, t, J = 5.6 Hz), 6.66 (1H, d, J = 8.4 Hz), 6.92–6.94 (4H, m), 7.02 (1H, d, J = 7.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 174.0, 154.6, 146.4, 140.3, 137.2, 134.8, 130.4, 129.8, 127.8, 126.1, 125.7, 125.4, 109.6, 80.0, 67.0, 51.5, 48.6, 35.0, 33.7, 32.0, 30.4, 29.2, 28.8, 25.6, 21.8, 19.7, 16.5, 8.5. MS (ESI positive): 519 $(M+Na)^+$.

5.1.17. 6-(4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-hexanoic acid ethyl ester (19b)

The yield was 78%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.2 Hz), 0.89 (9H, s), 1.25 (3H, t, J = 7.1 Hz), 1.39–1.56 (3H, m), 1.62–1.83 (4H, m), 1.84–1.92 (1H, m), 2.03 (4H, q, J = 7.4 Hz), 2.15 (3H, s), 2.25 (3H, s), 2.33 (2H, t, J = 7.4 Hz), 2.55–2.58 (1H, m), 2.82–2.90 (1H, m), 3.24 (1H, dd, J = 10.3, 1.3 Hz), 3.92 (2H, t, J = 6.3 Hz), 4.12 (2H, q, J = 7.2 Hz), 6.66 (1H, d, J = 8.4 Hz), 6.91–6.95 (4H, m), 7.02 (1H, d, J = 7.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 173.7, 154.7, 146.4, 140.2, 137.2, 134.7, 130.4, 129.8, 127.8, 126.1, 125.7, 125.4, 109.7, 79.9, 67.3, 60.2, 48.6, 35.0, 32.4, 32.0, 30.4, 29.2, 29.1, 25.8, 25.6, 24.7, 19.6, 16.5, 14.2, 8.5. MS (ESI positive): 547 $(M+Na)^+$.

5.1.18. 7-(4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-heptanoic acid ethyl ester (19c)

The yield was 83%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.3 Hz), 0.89 (9H, s), 1.25 (3H, t, J = 7.1 Hz), 1.46–1.54 (5H, m), 1.62–1.69 (2H, m), 1.76–1.83 (3H, m), 2.04 (4H, q, J = 7.3 Hz), 2.15 (3H, s), 2.25 (3H, s), 2.30 (2H, t, J = 7.5 Hz), 2.52–2.59 (1H, m), 2.82–2.90 (1H, m), 3.25 (1H, dd, J = 10.6, 1.4 Hz), 3.91 (2H, t, J = 6.4 Hz), 4.13 (2H, q, J = 7.2 Hz), 6.67 (1H, d, J = 8.4 Hz), 6.90–6.95 (4H, m), 7.02 (1H, d, J = 7.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 173.8, 154.7, 146.4, 140.1, 137.2, 134.7, 130.4, 129.8, 127.8, 126.0, 125.7, 125.4, 109.7, 79.9, 67.5, 60.2, 48.6, 35.0, 34.2, 32.0, 30.4, 29.2, 29.2, 28.9, 25.9, 25.6, 24.7, 19.6, 16.5, 14.2, 8.5. MS (ESI positive): 561 $(M+Na)^+$.

5.1.19. (*S*)-5-(4-{1-ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxymethyl)-dihydro-furan-2-one (20)

The yield was 88%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.59 (6H, t, J = 7.2 Hz), 0.89 (9H, s), 1.45–1.55 (1H, m), 1.75–1.83 (1H, m), 2.04 (4H, q, J = 7.2 Hz), 2.15 (3H, s), 2.25 (3H, s), 2.28–2.36 (1H, m), 2.39–2.49 (1H, m), 2.51–2.61 (1H, m), 2.65–2.80 (1H, m), 2.81–2.90 (1H, m), 3.24 (1H, dd, J = 10.4, 1.4 Hz), 4.06 (1H, dd, J = 10.3, 3.4 Hz), 4.16 (1H, dd, J = 10.3, 3.4 Hz), 4.87–4.90 (1H, m), 6.66 (1H, d, J = 8.6 Hz), 6.80–6.98 (4H, m), 7.02 (1H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 177.2, 153.8, 146.2, 141.5, 137.3, 134.8, 130.7, 129.8, 127.8, 126.2, 125.6, 125.5, 109.8, 79.9, 77.9, 69.3, 48.7, 35.0, 32.0, 30.4, 29.1, 28.3, 25.6, 24.1, 19.7, 16.6, 8.4. MS (ESI positive): 503 $(M+Na)^+$.

5.1.20. 5-(4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-pentanoic acid (5a)

The yield was 84%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.2 Hz), 0.89 (9H, s), 1.49–1.54 (1H, m), 1.76–1.82 (1H, m), 1.84–1.86 (4H, m), 2.04 (4H, q, J = 7.3 Hz), 2.16 (3H, s), 2.26 (3H, s), 2.45–2.47 (2H, m), 2.52–2.59 (1H, m), 2.83–2.90 (1H, m), 3.26 (1H, dd, J = 10.4, 1.4 Hz), 3.94–3.96 (2H, m), 6.67 (1H, d, J = 8.4 Hz), 6.90–6.96 (4H, m), 7.02 (1H, d, J = 7.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 179.0, 154.6, 146.4, 140.3, 137.2, 134.8, 130.4, 129.8, 127.8, 126.1, 125.7, 125.4, 109.6, 80.1, 67.0, 48.6, 35.0, 33.6, 32.0, 30.4, 29.2, 28.7, 25.6, 21.5, 19.7, 16.5, 8.5. HRMS (ESI negative): Calcd for $C_{31}H_{45}O_4$ 481.3323. Found: 481.3325 $(M-H)^-$.

5.1.21. 6-(4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-hexanoic acid (5b)

The yield was 76%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.2 Hz), 0.89 (9H, s), 1.46–1.59 (3H, m), 1.69–1.84 (5H, m), 2.04 (4H, q, J = 7.3 Hz), 2.15 (3H, s), 2.25 (3H, s), 2.39 (2H, t, J = 7.4 Hz), 2.52–2.59 (1H, m), 2.82–2.90 (1H, m), 3.26 (1H, d, J = 9.4 Hz), 3.93 (2H, t, J = 6.2 Hz), 6.67 (1H, d, J = 8.4 Hz), 6.90–6.96 (4H, m), 7.02 (1H, d, J = 7.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 179.2, 154.7, 146.4, 140.2, 137.2, 134.7, 130.4, 129.8, 127.8, 126.1, 125.7, 125.4, 109.7, 80.1, 67.3, 48.6, 35.0, 33.9, 32.0, 30.4, 29.2, 29.1, 25.7, 25.6, 24.4, 19.7, 16.5, 8.5. HRMS (ESI negative): Calcd for $C_{32}H_{47}O_4$ 495.3480. Found: 495.3480 $(M-H)^-$.

5.1.22. 7-(4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-heptanoic acid (5c)

The yield was 70%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.60 (6H, t, J = 7.2 Hz), 0.89 (9H, s), 1.39–1.56 (5H, m), 1.64–1.71 (2H, m), 1.75–1.84 (3H, m), 2.04 (4H, q, J = 7.3 Hz), 2.15 (3H, s), 2.25 (3H, s), 2.37 (2H, t, J = 7.5 Hz), 2.54–2.57 (1H, m), 2.82–2.90 (1H, m), 3.26 (1H, dd, J = 10.4, 1.6 Hz), 3.92 (2H, t, J = 6.4 Hz), 6.67 (1H, d, J = 8.4 Hz), 6.90–6.95 (4H, m), 7.02 (1H, d, J = 7.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 179.2, 154.7, 146.4, 140.2, 137.2, 134.7, 130.4, 129.8, 127.8, 126.0, 125.7, 125.4, 109.7, 80.0, 67.5, 48.6, 35.0, 33.8, 32.0, 30.4, 29.2, 29.2, 28.8, 25.9, 25.6, 24.6, 19.7, 16.5, 8.5. HRMS (ESI negative): Calcd for $C_{33}H_{49}O_4$ 509.3636. Found: 509.3627 $(M-H)^-$.

5.1.23. 5-(4-{1-Ethyl-1-[4-(3-hydroxy-4,4-dimethyl-pentyl)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-4(*S*)-hydroxy-pentanoic acid (6b)

The yield was 76%. Colorless oil. ^{1}H NMR (CD_3OD) δ: 0.58 (6H, t, J = 7.3 Hz), 0.87 (9H, s), 1.43–1.54 (1H, m), 1.69–1.89 (2H, m), 1.92–2.01 (1H, m), 2.05 (4H, q, J = 7.8 Hz), 2.14 (3H, s), 2.23 (3H, s), 2.34–2.40 (2H, m), 2.50–2.57 (1H, m), 2.82–2.91 (1H, m), 3.15 (1H, dd, J = 10.4, 1.4 Hz), 3.88–3.91 (2H, m), 3.92–4.00 (1H, m), 6.75 (1H, d, J = 8.6 Hz), 6.84 (1H, d, J = 2.0 Hz), 6.88–6.96 (3H, m), 7.01 (1H, d, J = 7.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 181.2, 154.7, 146.2, 140.5, 137.4, 134.4, 130.1, 129.5, 127.7, 125.7, 125.3, 125.2, 109.8, 78.9, 71.8, 70.0, 47.8, 34.5, 34.0, 31.9, 30.0, 29.9, 28.7, 24.9, 22.8, 18.3, 15.3, 7.3. HRMS (ESI negative): Calcd for $C_{31}H_{45}O_5$ 497.3272. Found: 497.3278 $(M-H)^-$.

5.2. VDRE reporter gene assay

MG-63 cells were plated at 2×10^3 cells/200 μL/well in a 96-well white cell-culture plate and were incubated at 37 °C in 5% CO_2 incubator for 24 h. Then, MG-63 cells were cotransfected with 0.05 μg of the pGV2–basic/VDRE–luciferase vector which contained three repeats of the VDRE sequence from mouse osteopontin promoter and 0.001 μg of pRL–SV40 vector (Promega Corporation, WI, USA) using Lipofectamine (Invitrogen). The cells were added to minimum essential medium (MEM) containing 5% fetal bovine serum treated with dextran-coated charcoal (DCC–FBS) and were incubated for 8 h. The cells were treated with the serial diluted compounds (final concentrations were 10^{-7} to 10^{-11} mol/L with 0.1% DMSO) and were incubated for an additional three days. After removing the supernatants, the cells were lysed in cell-lysis buffer and luciferase activity was measured by DLR™ Assay System (Promega Corporation, WI, USA) and the luminescence was detected by Wallac ALVO SX 1420 multi-label counter (Perkin-Elmer, Inc., MA, USA). The half maximal effective concentrations (EC_{50}) were determined and the inductive activity was calculated as the ratio of the EC_{50} value of the compounds to that of $1,25(OH)_2D_3$ (Solvay Pharmaceuticals, Weesp, The Netherlands) which was used as a positive control.

5.3. Osteocalcin induction assay

MG-63 cells were plated at 2×10^3 cells/well in 200 μL of serum-free MEM in a 96-well plate and were incubated at 37 °C in 5% CO_2 incubator for 24 h. After washing cells with 5% DCC–FBS/MEM (culture medium), the cells were added to culture medium and treated with the serial diluted compounds (final concentrations were 10^{-7} to 10^{-11} mol/L with 0.1% DMSO), and incubated for 8 h. After changing culture medium to fresh one, the cells were incubated for an additional four days, and then supernatant was collected and stored at −80 °C.

The frozen supernatant was thawed slowly at room temperature and Gla-type osteocalcin EIA kit (Takara Bio. Inc., Tokyo, Japan) was used to measure osteocalcin. Absorbance at 450 nm was measured on a plate reader (Model 3550, Bio–Rad Laboratories, CA, USA) and each concentration was calculated by comparison with standards using μplate Manager III software (Bio–Rad Laboratories). The EC_{50} values were determined and the inductive activity was calculated as the ratio of the EC_{50} value of the compounds to that of $1,25(OH)_2D_3$.

5.4. Bone mineral density and serum calcium evaluation in the ovariectomized rats

Sham-operated (n = 8) and OVX eight-week-old female Sprague–Dawley (Crl:CD(SD)) rats (Charles River Japan Inc.) were used. One day after the surgery, OVX rats were divided into four groups (n = 8), and were orally administered compound **6a** (0.22, 0.67, 2.0 μg/kg, five times per week) or vehicle (medium-chain triglyceride (MCT), 1 mL/kg, five times per week) for 4 weeks. Vehicle-administered OVX rats and sham-operated rats served as controls. After 24 h from the last administration, blood was drawn from the abdominal aorta under ether anesthesia. Serum was collected after the centrifugation of the blood samples and was stored at −20 °C. The right femur was stored in 70% ethanol at 4 °C. Serum calcium concentration was measured by an autoanalyzer (Hitachi 7170, Tokyo, Japan). The BMD in distal femur was measured by dual-energy X-ray absorptiometry (DCS-600-EX, Aloka, Japan).

5.5. Crystallographic structure analysis

Protein sample preparation and crystallization were based on the methods by Moras et al.[24,25] X-ray diffraction data collection and data processing were carried out by generally accepted methods. After brief soaking in the buffer containing the 30% glycerol, 0.6 M ammonium sulfate and 0.1 M MES (pH6.0), crystals were trapped in the fiber loops and flash-cooled with liquid nitrogen. Crystals were stored and transported in the dry shipper. Data

Table 3
Crystal and diffraction data of VDR with compound **4a**, **4d**, and **6b**

	4a	4d	6b
Wavelength (Å)	1.0	1.0	1.0
Cell (Å)			
(a)	44.6	44.5	44.3
(b)	51.7	51.8	52.2
(c)	131.6	132.4	132.0
Resolution (Å)	2.1	2.0	1.9
Completeness (last shell) (%)	99.3 (98.9)	93.7 (88.6)	89.6 (80.0)
Rsym (last shell) (%)	7.4 (25.3)	7.7 (17.4)	6.9 (18.2)
I/Sigma (last shell)	6.1 (2.7)	6.0 (3.8)	6.7 (3.6)
Rcryst	19.5	18.8	22.6
rmsd bond length (Å)	0.010	0.011	0.010
rmsd bond angles (deg)	0.96	1.18	0.99
No. of non-hydrogen protein atoms	1993	1993	1993
No. of water molecules	235	298	148

collections were carried out at the synchrotron beamline BL32B2 of the Spring-8 in Hyogo, Japan, operated by Pharmaceutical Consortium for Protein Structure Analysis. Crystals were kept frozen during the data collection with vapor stream from the liquid nitrogen. X-ray diffraction data was collected with the R-AXIS V imaging plate area detector. The crystals were isomorphous with the space group P212121 reported by Moras et al.[24,25] for the $1,25(OH)_2D_3$-VDR complex. Data were processed with CCP4 and MOSFLM. The initial structure model for the refinement was constructed by removing all the water and the ligand atoms in the $1,25(OH)_2D_3$-VDR complex structure in the Protein Databank (PDB ID: 1db1). A rigid body refinement was followed by simulated annealing using the CNX. Electron density in the ligand-binding pocket clearly showed the ligand conformation unambiguously. After fitting the ligand model into the electron density, the structure refinement was continued with the software autoBUSTER and the water molecules were placed automatically. The data collection and the refinement statistics are summarized in Table 3.

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