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Synthesis of C-2 substituted vitamin D derivatives having ringed side chains and their biological evaluation, especially biological effect on bone by modification at the C-2 position†

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In order to obtain vitamin D derivatives, which have strong activity for enhancing bone growth, we designed vitamin D derivatives with various substitutions at the C-2 position. Novel 2 α-substituted vitamin D derivatives were synthesized starting from D-glucose as a chiral template of the A-ring with a CD-ring bromoolefin unit using the Trost coupling method. We evaluated these compounds by two *in vitro* assays, affinity to VDR and transactivation assays, using human osteosarcoma (Hos) cells, and demonstrated the SAR of the C-2 position of VD_3. Furthermore, by using the OVX model, we found that compound **5c**, which has a hydroxypropoxy side chain at C-2 and 2,2-dimethyl cyclopentanone in the CD-ring side chain, has a strong activity for enhancing bone growth, same as the reported compound, 2α-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 **1d**, and this derivative shows a possibility that calcemic activity is less than **1d** *in vivo*.

1. Introduction

1α,25-Dihydroxyvitamin D_3 (Fig. 1, **1a**) shows biological activities through the interaction with a vitamin D receptor (VDR), which is a member of the nuclear hormone receptor superfamily, and acts as a ligand-dependent gene transcription factor with co-activators.[1,2] Although the biological activities of **1a** vary, such as the proliferation and differentiation of various types in tumor cells, and the regulation of immune reactions,[3,4] the most important physiological role of **1a** is the regulation of calcium and phosphorus metabolism as well as bone remodeling *via* its action in the bone, intestine and kidney; therefore, **1a** is used as a therapeutic drug for osteoporosis, a systemic skeletal disease characterized by low bone mineral density (BMD) leading to reduced bone strength and fracture.

However, the use of vitamin D as a therapeutic agent is limited because of its calcemic and phosphatemic activities; therefore, in order to find new vitamin D analogs, which are more efficacious, safer and more selective than natural **1a**, numerous analogs of **1a** have been developed.[4,5] Most of the analogs have a modified side chain. As an example, 1α,25-dihydroxyvitamin D_3-26,23-lactones **2**, which was reported by Ishizuka *et al.*, has a unique structure

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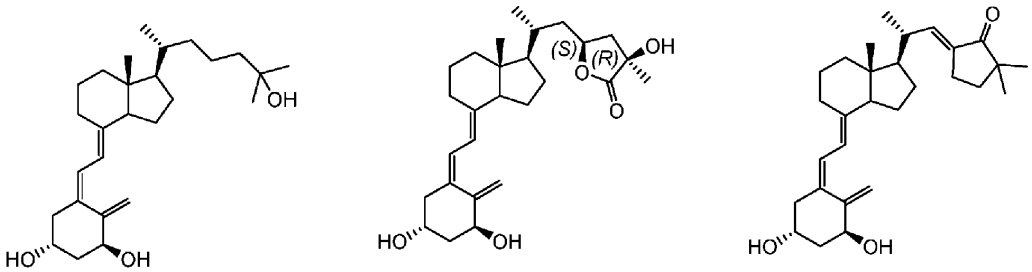
† Electronic supplementary information (ESI) available: Detailed experimental procedures including spectral data of compounds **14**, **15** and **5b–5h**. See DOI: 10.1039/c1ob05142c



1a: 1α,25-$(OH)_2$-vitamin D_3 **2**: 1α-(OH)-**23*S*-26*R***-D_3-lactone **3**

Fig. 1 Structures of 1α,25-dihydroxyvitamin D_3 (**1**) and its derivatives.

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with a lactone ring on the side chain.[6] We therefore synthesized and evaluated vitamin D derivatives with a ringed side chain in their structures. In a series of derivatives, we report that compound **3**, which has a cyclopentanone in the side chain, showed strong affinity with VDR.[7]

On the other hand, Kittaka *et al.* developed the first systematic synthesis of novel analogs of vitamin D_3 based on the structural modification of the A-ring in order to investigate A-ring structure–activity relationships.[8–13] During the course of their studies, they found that the introduction of methyl (**1b**),[8] 3-hydroxypropyl (**1c**),[9] and 3-hydroxypropoxy (**1d**) [10] groups at the C-2α position showed 2- to 4-fold higher binding affinity relative to the natural hormone **1a** (Fig. 2). In addition, Saito *et al.* reported that 2α modification of TEI-9647 (**4a**), which is the first vitamin D_3 antagonist, increases antagonistic activity significantly.[14]

Against this background, we modified **3** with 2α substitution in order to obtain VD_3 derivatives, which have strong activity for enhancing bone growth. As mentioned above, the 2α-methyl group (**1b**), 2α-3-hydroxypropyl group (**1c**), and 2α-3-hydroxypropoxy group (**1d**) were appropriate substituted groups for the higher affinity to VDR. Recent research about co-crystals of these ligands and the ligand-binding domain (LBD) of VDR clarified the reason for the higher activity of these compounds.[15] Namely, the C-2α methyl group of **1b** interacts with amino acid residues Ser237, Leu233, and Phe150 in the LBD, and each C-2α terminal hydroxy group of **1c** and **1d** forms a new hydrogen bond between the hydroxy group and Arg274; therefore, we were interested in the effect of these substitutions for enhancing bone growth and we designed **5a–5c** (Fig. 3). In addition, we designed **5d–5h** as new 2α substituted groups. As stated above, the co-crystals of **1c** and **1d** only showed an interaction between the terminal hydroxy group and Arg274 of VDR, but there remained a cavity around the alkyl side chain of **1c** and **1d**. We therefore designed **5d** and **5e** because we expected that more occupation of the cavity around the C-2 position of the A-ring would increase the affinity to VDR and the biological activities. Furthermore, we designed **5f–5h** in order to investigate the effects for the affinity to VDR and the biological activities by the difference of the terminal group in 2α-substituted groups.

2. Results

Our synthesis plan of 2α-substituted vitamin D_3 derivatives is shown in Scheme 1. The triene skeleton of the desired vitamin D_3 derivatives (**5a–5h**) was constructed by Trost's Pd-catalyzed alkenylative cyclization [16] of A-ring precursor enynes (**7a–7h**) with the CD-ring bromoolefin counterpart (**6**).[7] The A-ring precursor enynes (**7a–7h**) could be synthesized from D-glucose derivatives on the basis of the reported scheme.[10]

2.1. Synthesis

2.1.1. Preparation of A-ring precursors 7a–7h. We synthesized the A-ring precursors **7d** and **7g**, in which the cyclopropyl group is introduced into the center of the 2α side chain from the D-glucose derivative (Scheme 2).

Treatment of the known epoxide **8** [17] with 2,2-cyclopropane-1,3-diol and KO^{t}Bu under heat conditions gave 3-(3-hydroxy-2,2-ethanopropyl)oxyaltropyranoside **9**. Protection of the primary alcohol using pivaloyl chloride, followed by *O*-silylation, gave

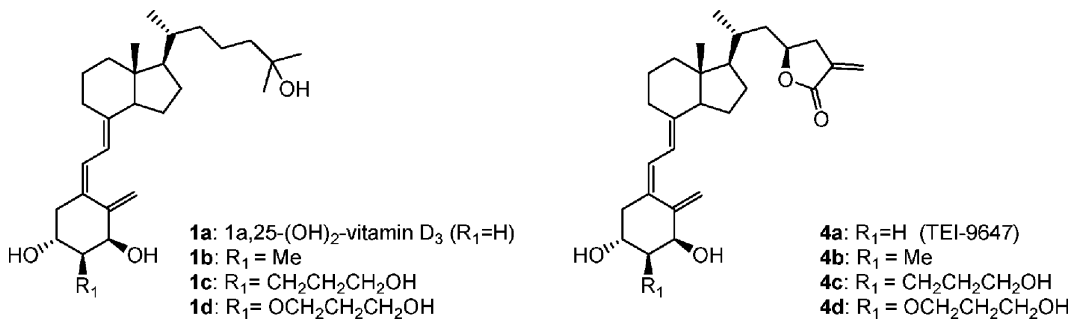

Fig. 2 Structures of 1α,25-dihydroxyvitamin D_3 (**1a**), 1α-hydroxyvitamin D_3-26,23-lactones (TEI-9647:**4a**) and their representative C-2α-modified analogs (**1b–d**, **4b–d**).

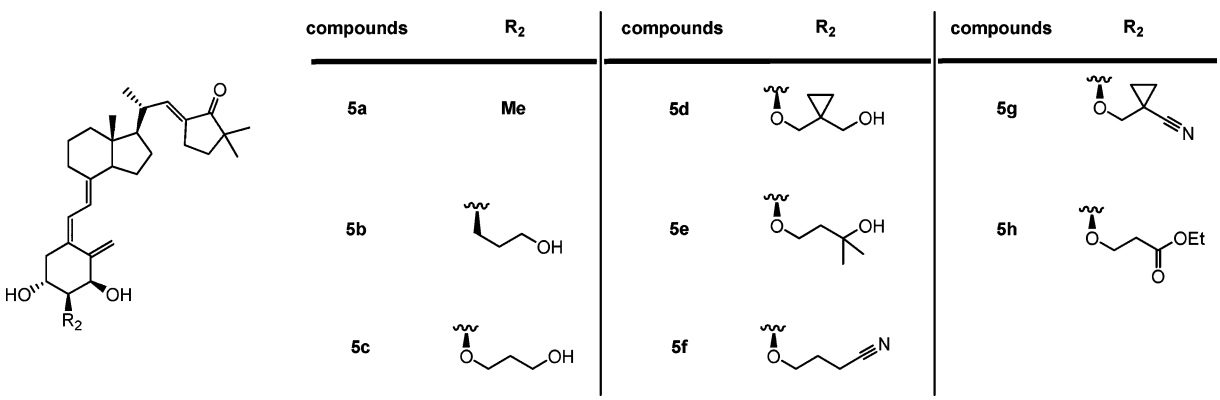

Fig. 3 Design of derivatives of **3** (R_2 = H).

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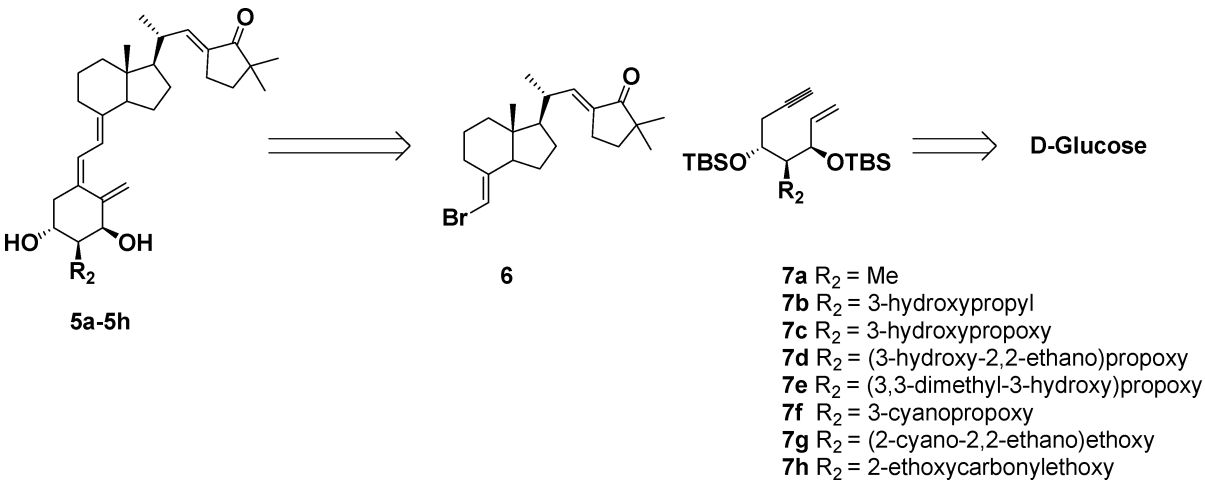
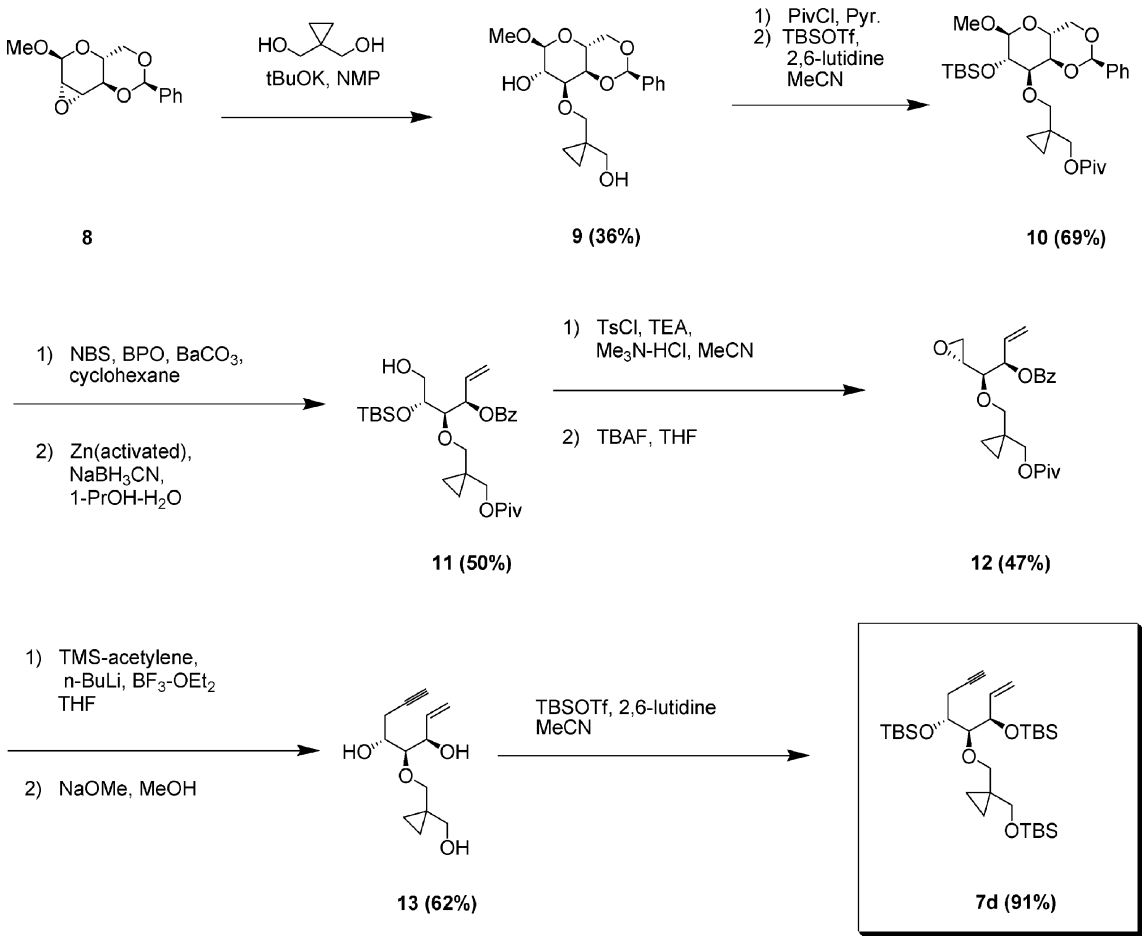


Scheme 1 Retrosynthesis.



Scheme 2 Synthetic route of **7d**.

protected altropyranoside **10**. Treatment with NBS, followed by reduction using activated zinc powder and $NaBH_3CN$ gave alcohol **11**. Tosylation and subsequent deprotection with TBAF provided epoxide **12**. Ethynylation using lithium TMS acetylide in the presence of BF_3-Et_2O in THF and methanolysis gave triol **13**. Finally, protection of triol by TBS groups gave the desired enyne A-ring precursor **7d**.

For the A-ring precursor **7g**, the end of 2α side chain of **7d** was substituted with a cyano group and synthesized as follows (Scheme 3). Protection of the primary alcohol of **13** with a pivaloyl group, followed by silylation and deprotection of the pivaloyl group gave alcohol **14**. Oxidation of alcohol followed by conversion to oxime and dehydration provided nitrile **7g**.

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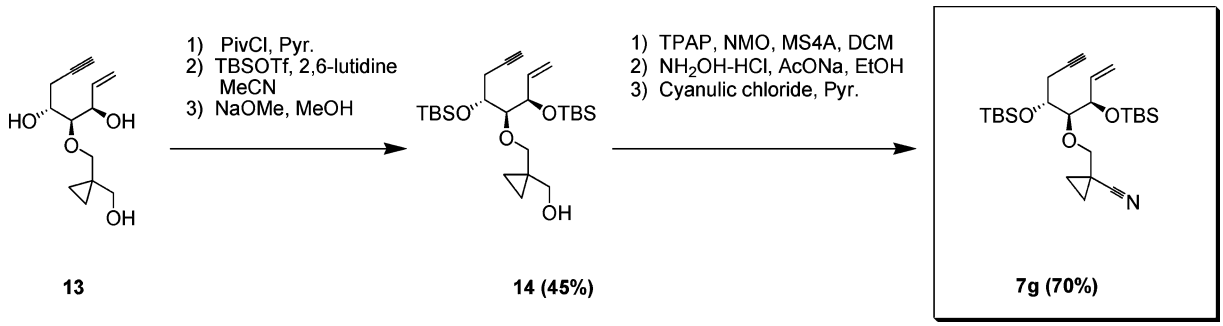
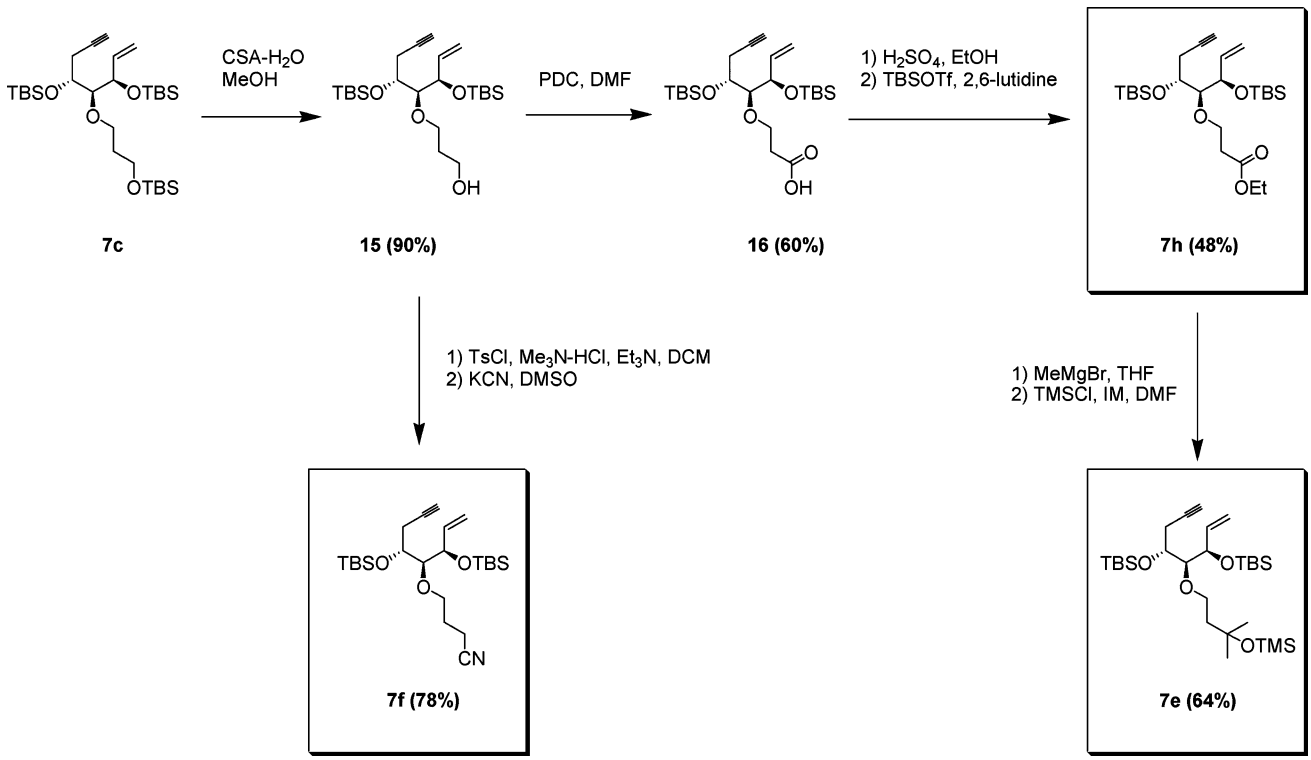


Scheme 3 Synthetic route of **7g**.



Scheme 4 Synthetic route of **7e**, **7f** and **7h**.

For the A-ring precursor **7e**, the primary alcohol of the 2α side chain of **7c** was changed to the tertiary alcohol, was synthesized as follows (Scheme 4). The known enyne A-ring precursor **7c** [10] was treated with camphorsulfonic acid to give alcohol **15**. Oxidation of **15** with excess PDC (5 mol. equiv.) provided carboxylic acid **16**. Esterification followed by re-protection gave the A-ring precursor **7h**. Moreover, the desired A-ring precursor **7e** was provided by treatment of **7h** with methyl magnesium bromide, followed by protection. Furthermore, the A-ring precursor **7f** was obtained by tosylation of **15** and substitution to the cyano group.

2.1.2. Synthesis of 2-substituted vitamin D_3 derivatives. The palladium-catalyzed coupling reaction of the above enynes **7a–7h** and bromoolefin **6**, followed by deprotection of silyl groups and purification by reversed-phase HPLC provided the desired compounds **5a–5h**, respectively (Scheme 5).

2.2. Biological evaluation

2.2.1. *In vitro* evaluation (affinity to VDR and transactivation assays on Hos cells). The newly synthesized derivatives were evaluated *in vitro*. We evaluated new derivatives by two *in vitro* assays, affinity to VDR and transactivation assays, using human osteosarcoma (Hos) cells. The evaluation results are shown in Table 1.

As described above, it was reported that the methyl group, hydroxypropyl group, and 3-hydroxypropoxy group at C-2 contributed to increased affinity to VDR, but when the side chain in the CD-ring was the ring, there was a difference in activity between the above appropriate substitution groups. Namely, modification by the 3-hydroxypropoxy group at C-2, compound **5c**, increased the affinity to VDR, but 2α-Me modification, **5a**, and 3-hydroxypropyl modification, **5b**, did not contribute to improve the affinity to VDR markedly. Additionally, new derivatives **5d** and **5e**, which have branched 3-hydroxypropoxy side chains at

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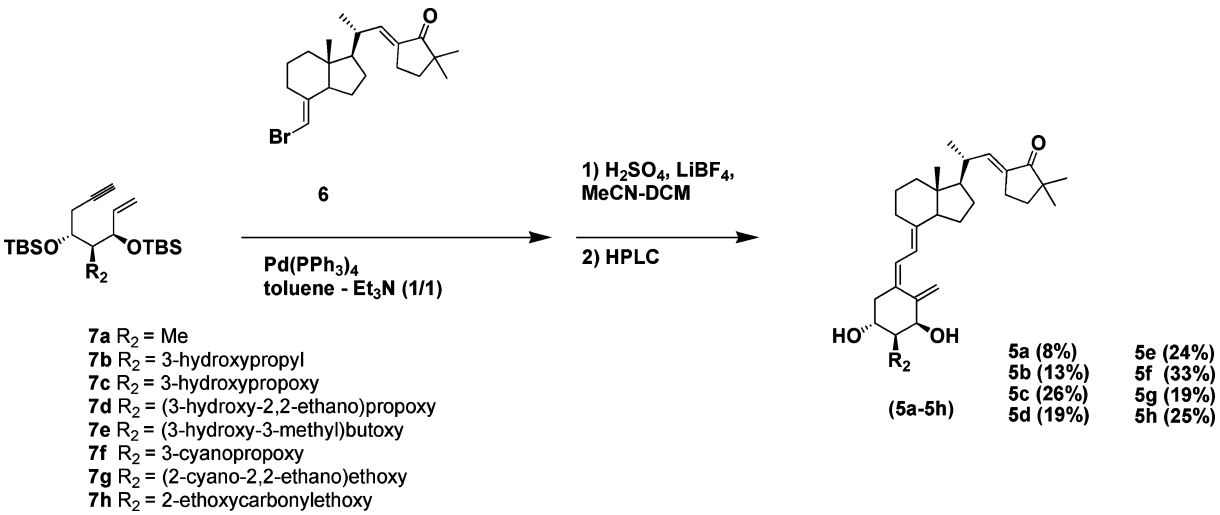

Scheme 5 Coupling and deprotection of A-ring precursor and CD-bromoolefin.

Table 1 VDR binding affinity and osteocalcin promoter transactivation in Hos cells

compd	VDR affinity (%)[a]	Transactivation assay (EC_{50}, pM)	compd	VDR affinity (%)	Transactivation assay (EC_{50}, pM)
1	100	30	**5d**	5.5	36.9
3	22.7	16.2	**5e**	2	630
5a	11.6	20	**5f**	10	59.0
5b	32.3	11.2	**5g**	18	105
5c	58.8	6.7	**5h**	1	7460

[a] The potency of **1** is normalized to 100.

C-2, also decreased the affinity to VDR. In particular, **5e** led to a significantly decreased affinity. In the case of compounds **5f–5h**, in which terminal groups in the side chain are substituted from the hydroxy group to other groups, the affinity to VDR of compounds **5f** and **5g** was moderate, but that of **5h** was decreased.

With respect to *trans* activity assay on Hos cells, it was found that there was a moderate correlation between activity of *trans* activity assay and the affinity to VDR (Fig. 4). Especially, compound **5c** showed a stronger activity (6.9 pM) than that of natural hormone **1** and compound **5c**.

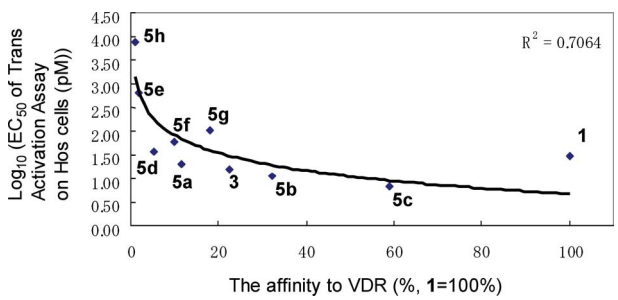

Fig. 4 Relationship between the affinity to VDR and the transactivation assay on Hos cells of **1**, **3** and **5a–5h**.

2.2.2. ***In vivo*** **evaluation: Evaluation of effects of derivatives on bone mineral loss in ovariectomized rats.** As described above, we found that compound **5c** had strong activity on Hos cells; therefore, we evaluated the *in vivo* therapeutic effect of compound **5c** using an ovariectomized rat therapeutic model (OVX). Twelve-week-old Sprague-Dawley female rats (Charles River, Japan) were ovariectomized and fed a normal diet containing 1.0% Ca *ad libitum* for 4 weeks. The rats were then administrated vitamin D_3 derivatives at doses from 0.006 μg kg^{-1} to 1 μg kg^{-1}, 5 times a week for 4 weeks. The sham and OVX groups were administrated MCT alone. Twenty-four hours after the final administration, blood was collected under ether anesthesia, the rats were then euthanized, and BMD of the spine (L4–L5) bone mass was measured by dual X-ray absorptiometry. The results of BMD and serum Ca density of 3 vitamin D_3 derivatives are shown in Fig. 5, 6 and 7, respectively.

As shown in Fig. 5a and 5b, the starting compound **3** did not show such a strong increase of BMD, although it showed strong activity *in vitro*, and the high dose of **3** especially showed hypercalcemia. In the case of 2α-hydroxypropoxy-1,25-$(OH)_2$-vitamin D_3 **1d** (Fig. 6a and 6b), it showed an increase of BMD, which was not linear. Furthermore, the calcemic activity was observed from the lowest dose. It was found that by stepping up the dose, the calcemic activity was stronger than the increase of BMD.

On the other hand, newly synthesized derivative **5c** (Fig. 7a and 7b) showed a significant increase of BMD at 0.025 μg kg^{-1}, and the significant difference of the calcemic activity was not observed at this dose.

3. Discussion

In this research, we designed novel vitamin D derivatives with the reported and new 2α side chains. We designed and synthesized compounds **5d** and **5e** because we expected that the terminal

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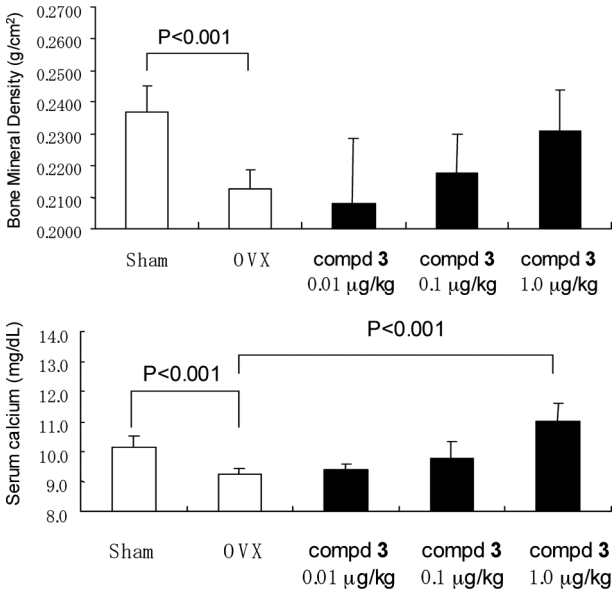

Fig. 5 **(a)** Spiral bone mineral density (BMD) in the OVX rat therapeutic model administrated compound **3** orally **(b)** Serum calcium level in the OVX rat therapeutic model administrated compound **3** orally.

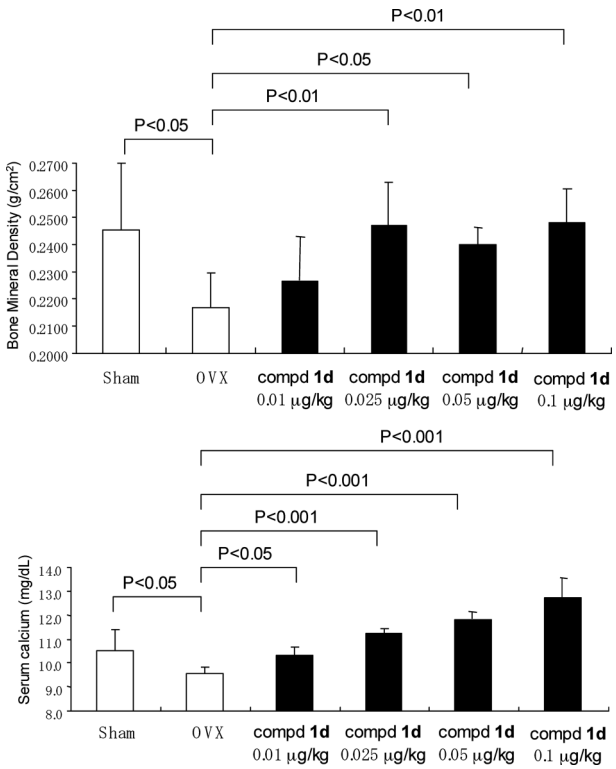

Fig. 6 **(a)** Spiral bone mineral density (BMD) in the OVX rat therapeutic model administrated compound **1d** orally **(b)** Serum calcium level in the OVX rat therapeutic model administrated compound **1d** orally.

hydroxy group of these compounds would form hydrogen bonds with Arg274 as reported with compound **1d**; however, the affinity to VDR of compounds **5d** and **5e** was decreased more than that of compound **5c**. From these results, we consider that the terminal hydroxy group of 2α side chains in compounds **5d** and **5e** might not be able to form a hydrogen bond with Arg274 because the

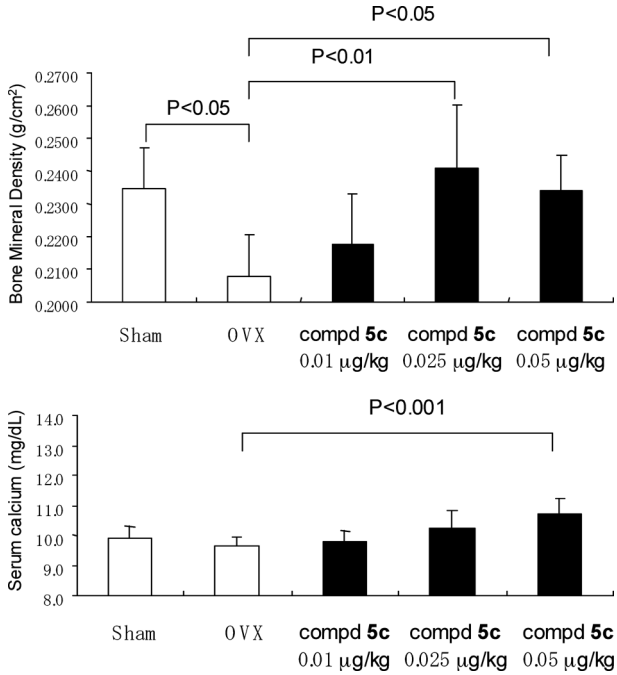

Fig. 7 **(a)** Spiral bone mineral density (BMD) in the OVX rat therapeutic model administrated compound **5c** orally **(b)** Serum calcium level in the OVX rat therapeutic model administrated compound **5c** orally.

terminal hydroxy groups of these compounds might move by insertion of the substituted groups into the alkyl side chains. In addition, we designed compounds **5f–5h**, in which the terminal groups were substituted. In these compounds, the affinity to VDR of compounds **5f** and **5g** did not markedly decrease. In the previous paper,[9b,10c] it was reported that VD_3 derivatives, in which alkyl side chains were introduced at C-2, markedly decreased the affinity to VDR except for the 2α-methyl substituted VD_3 derivative.[9a] From the difference between the activity of the cyano side chains and alkyl side chains, it was supposed that compounds **5f** and **5g** might interact with amino acids in VDR.

About the activity for enhancing bone growth, we evaluated newly synthesized derivatives by transactivation assays on Hos cells. The activity showed a moderate correlation with the affinity to VDR. So, we consider the activity for enhancing bone growth was based on the affinity to VDR.

Furthermore, we evaluated 2α-substituted vitamin D_3 derivatives *in vivo*. It was confirmed that compound **1d** has strong activity for enhancing bone growth (Fig. 6a), but the calcemic activity was also observed from the lowest dose (Fig. 6b), so we consider that compound **1d** has a strong calcemic activity. On the other hand, compound **5c** showed a strong activity for enhancing bone growth, same as **1d** (Fig. 7a), but the significant difference of the calcemic activity was not observed at 0.025 μg kg^{-1} (Fig.7b). From these results, we consider that compound **5c** possibly has a calcemic activity less than that of **1d**. It was supposed that the structure of the side chain in the CD-ring would contribute to the separate activity for enhancing bone growth from calcemic activity.

4. Conclusion

We designed and synthesized novel vitamin D_3 derivatives with various substituted side chains at the C-2 position in order to

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obtain vitamin D_3 derivatives with strong activity for enhancing bone growth. As the result of *in vitro* experiments, we demonstrated the SAR of the C-2 position of VD_3. Furthermore, by using the OVX model, we found that compound **5c**, which has a hydroxypropoxy side chain at C-2 and 2,2-dimethyl cyclopentanone in the CD-ring side chain, has a strong activity for enhancing bone growth, same as the reported compound, 2α-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 **1d**, and this derivative shows a possibility that the calcemic activity is less than that of **1d** *in vivo*.

5. Experimental section

5.1. Synthesis

5.1.1. General. NMR spectra were measured using a JEOL AL-400 magnetic resonance spectrometer. Infrared spectra data were recorded on a JASCO FTIR-5300 spectrometer. Mass spectra were measured on a Shimadzu LC-MS-IT-TOF. Specific optical rotations were measured using a JASCO P-1030 polarimeter. Purification by flash column chromatography on silica gel was carried out using a Biotage FLASH system. Preparative thin layer chromatography was performed using Merck Kieselgel F^{254} plates. Reversed-phase HPLC was carried out on a Shimadzu LC-2010 system.

5.1.2. Methyl 4,6-*O*-benzylidene-3-[(3-hydroxy-2,2-ethano)-propyl]oxy-α-D-altoropyranoside (9). To a stirred solution of epoxide **8** (6.0 g, 22.7 mmol) in *N*-methyl-pyrolidone (60 mL) was added 2,2-cyclopropane-1,3-propanediol (15 g, 146.8 mmol) and KO^{t}Bu (10.19 g, 90.8 mmol) at room temperature. The solution was stirred at 130 °C for 8 h. The reaction mixture was cooled to room temperature, the solution was diluted with H_2O (240 mL), and Dia-ion HP-20SS (Mitsubishi Chemical Industries, 30 g) was added to the solution and stirred at room temperature overnight. Filtration was followed by washing with saturated aq. NH_4Cl, H_2O, and elution by acetone. The elution was evaporated, the residue was diluted with EtOAc, and the solution was washed with brine. The organic layer was dried over $MgSO_4$, followed by filtration and concentration. The residue was purified by flash column chromatography on silica gel (*n*-hexane–EtOAc = 20/80) to provide 2.97 g of **9** as a colorless syrup (8.1 mmol, 36%). $[\alpha]_D^{25}$ +71.1 (*c* 1.0, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1217, 1099, 1045 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.51–7.36 (5H, m), 5.54 (1H, s), 4.61 (1H, s), 4.40–4.29 (2H, m), 4.08 (1H, t, *J* = 4.3 Hz), 4.01 (1H, dd, *J* = 9.3, 2.7 Hz), 3.93 (1H, br s), 3.83–3.75 (3H, m), 3.60–3.50 (3H, m), 3.41 (3H, s), 0.59–0.41 (3H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 137.6, 129.8, 129.2, 128.4, 126.3, 126.2, 102.6, 101.2, 80.1, 76.0, 70.4, 69.8, 69.3, 59.1, 55.5, 22.8, 9.2, 9.1; EI-LRMS *m/z* 389.1 (M+Na); EI-HRMS calcd for $C_{19}H_{26}O_7Na$ 389.1571 (M+Na), found 389.1576.

5.1.3. Methyl 4,6-*O*-Benzylidene-2-(*tert*-butyldimethylsilyloxy-3-[3-(2,2-dimethylpropionyl)oxy-2,2-ethanopropyl]oxy-α-D-altoropyranoside (10). To a solution of **9** (2.97 g, 8.10 mmol) in pyridine (30 mL), pivaloyl chloride (1.15 mL, 9.32 mmol) was added at 0 °C and stirred at the same temperature for 1 h. Dry MeOH (3 mL) was added to the reaction mixture and stirred at room temperature for 5 min. The reaction mixture was evaporated and diluted with toluene. The solution was washed with brine, and the organic layer was dried over $MgSO_4$, followed by filtration and concentration. The residue was dissolved in dry CH_2Cl_2 (20 mL) and cooled to 0 °C. To the solution were added 2,6-lutidine (1.3 mL, 11.6 mmol) and *t*-butyldimethylsilyl trifluoromethanesulfonate (2.14 mL, 9.32 mmol), and the reaction mixture was stirred at room temperature for 1 h. Dry MeOH (5 mL) was added to the reaction mixture and stirred at room temperature for 5 min. The reaction mixture was evaporated and diluted with toluene. The solution was washed with brine, and the organic layer was dried over $MgSO_4$, followed by filtration and concentration. Purification by flash silica gel chromatography (*n*-hexane–EtOAc = 95/5, then 90/10) gave **10** (3.19 g, 5.61 mmol) in 69% as a colorless syrup. $[\alpha]_D^{25}$ +36.71 (*c* 1.0, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1716, 1217, 1107, 1047 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.49–7.34 (5H, m), 5.56 (1H, s), 4.45 (1H, s), 4.29–4.25 (2H, m), 4.18 (1H, d, *J* = 11.2 Hz), 3.98–3.92 (3H, m), 3.75 (1H, t, *J* = 12.0 Hz), 3.65 (1H, t, *J* = 2.7 Hz), 3.60 (1H, d, *J* = 9.5 Hz), 3.52 (1H, d, *J* = 9.5 Hz), 3.35 (3H, s), 1.19 (9H, s), 0.91 (9H, s), 0.61–0.51 (4H, m), 0.10 (3H, s), 0.10 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 178.5, 137.8, 129.0, 128.2, 126.2, 102.3, 102.2, 73.5, 70.0, 69.4, 67.6, 58.4, 55.3, 38.8, 27.2, 25.7, 25.5, 20.4, 18.0, 8.2, 8.1, −5.0; EI-LRMS *m/z* 587.2 (M+Na), 582.2 (M+H_2O); EI-HRMS calcd for $C_{30}H_{48}O_8SiNa$ 587.3011 (M+Na), found 587.3012.

5.1.4. (2*R*,3*R*,4*R*)-4-(Benzoyloxy)-2-(*tert*-butyldimethylsiloxy)-3-[3-(2,2-dimethylpropionyl)oxy-2,2-ethanopropyl]oxyhex-5-en-1-ol (11). To a solution of **10** (3.17 g, 5.61 mmol) in cyclohexane (63 mL) were added $BaCO_3$ (775 mg, 3.92 mmol), benzoyl peroxide (136 mg, 0.56 mmol), and *N*-bromo succinimide (1.21 g, 6.73 mmol) at room temperature, and the reaction mixture was refluxed for 1 h. The reaction mixture was cooled to room temperature followed by filtration with celite. The residue was washed with cylcohexane. The combined organic layer was washed with brine and then dried over $MgSO_4$, followed by filtration and concentration. The residue (4.0 g) was dissolved in a mixture of 1-PrOH (36 mL) and H_2O (4 mL). To the solution were added activated zinc powder (7.38 g, 112.2 mmol) and $NaBH_3CN$ (1.42 g, 22.4 mmol), and the mixture was stirred vigorously at 110 °C for 1 h. The reaction mixture was cooled to room temperature, followed by filtration. Almost all 1-PrOH was removed by evaporation and the residue was diluted with EtOAc, washed with brine, and dried over $MgSO_4$. After filtration and concentration, purification by flash chromatography on silica gel (*n*-hexane–EtOAc = 90/10 then 80/20) provided alcohol **11** (1.50 g, 2.80 mmol) in 50% yield (2 steps). $[\alpha]_D^{25}$ +31.21 (*c* 1.0, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1716, 1215 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 8.05–8.02 (2H, m), 7.59–7.43 (3H, m), 6.11 (1H, ddd, *J* = 11.0, 17.3, 6.0 Hz), 5.78–5.75 (1H, m), 5.41 (1H, dt, *J* = 17.3, 1.3 Hz), 5.30 (1H, dt, *J* = 10.4, 1.2 Hz), 4.17 (1H, d, *J* = 11.4 Hz), 3.96–3.93 (2H, m), 3.81 (1H, dd, *J* = 11.4, 5.1 Hz), 3.73–3.68 (2H, m), 3.64 (1H, d, *J* = 9.7 Hz), 3.50 (1H, d, *J* = 9.7 Hz), 1.18 (9H, s), 0.90 (9H, s), 0.55 (4H, t, *J* = 1.9 Hz), 0.09 (3H, s), 0.07 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 178.5, 165.3, 133.1, 133.0, 130.2, 129.5, 128.4, 118.9, 82.4, 75.1, 74.9, 72.6, 67.4, 63.9, 38.8, 27.1, 25.8, 20.7, 18.0, 8.6, −4.6; EI-LRMS *m/z* 557.2 (M+Na); EI-HRMS calcd for $C_{29}H_{46}O_7SiNa$ 557.2905, found 557.2914.

5.1.5. (3*R*,4*R*,5*R*)-3-(Benzoyloxy)-4-[3-(2,2-dimethylpropionyl)oxy-2,2-ethanopropyl]oxy-5,6-epoxyhex-1-ene (12). To a solution of **11** (2.41 g, 4.5 mmol) in dry MeCN (25 mL) were added

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triethylamine (1.26 mL, 9 mmol), trimethylamine hydrochloride salt (86 mg, 0.9 mmol), and *p*-toluenesulfonyl chloride (1.30 g, 6.8 mmol) at room temperature and stirred at the same temperature for 1 h. The reaction mixture was quenched with saturated aq.$NaHCO_3$ followed by evaporation. The residue was diluted with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, followed by filtration and concentration. The residue (3.31 g) was diluted with THF (18 mL) and tetrabutyl ammonium fluoride (1 M in THF, 13.5 mL, 13.5 mmol) was added to the solution and refluxed for 1.5 h. The reaction mixture was cooled to room temperature and diluted with EtOAc, washed with brine. The organic layer was dried over $MgSO_4$, filtrated and concentrated. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 90/10) to give **12** (851 mg, 2.1 mmol) in 47% yield. $[\alpha]_D^{25}$ +31.21 (*c* 1.0, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1718, 1271, 1217, 1165, 1111 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 8.05–8.02 (2H, m), 7.59–7.43 (3H, m), 6.11 (1H, ddd, J = 11.0, 17.3, 6.0 Hz), 5.78–5.75 (1H, m), 5.41 (1H, dt, J = 17.3, 1.3 Hz), 5.30 (1H, dt, J = 10.4, 1.2 Hz), 4.17 (1H, d, J = 11.4 Hz), 3.96–3.93 (2H, m), 3.81 (1H, dd, J = 11.4, 5.1 Hz), 3.73–3.68 (2H, m), 3.64 (1H, d, J = 9.7 Hz), 3.50 (1H, d, J = 9.7 Hz), 1.18 (9H, s), 0.90 (9H, s), 0.55 (4H, t, J = 1.9 Hz), 0.09 (3H, s), 0.07 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 178.5, 165.3, 133.1, 133.0, 130.2, 129.5, 128.4, 118.9, 82.4, 75.1, 74.9, 72.6, 67.4, 63.9, 38.8, 27.1, 25.8, 20.7, 18.0, 8.6, –4.6; EI-LRMS m/z 425.1 (M+Na), 420.2 (M+H_2O), 403.1 (M+H); EI-HRMS calcd for $C_{23}H_{30}O_6Na$ 425.1935, found 425.1929

5.1.6. (3*R*,4*S*,5*R*)-4-[3-Hydroxy-2,2-ethanopropyl]oxyoct-1en-7yne-3,5-diol (13). A solution of trimethylsilyl acetylene (1.62 mL, 11.5 mmol) in THF (3 mL) under N_2 gas atmosphere was cooled in dry ice–acetone. To the solution was added *n*-butyl lithium in hexane (2.64 M, 3.97 mL, 10.5 mmol) and the solution was stirred at the same temperature for 45 min. To the solution were added a solution of **12** (846 mg, 2.1 mmol) in THF (6 mL) and BF_3-diethylether complex (0.343 mL, 2.73 mmol) successively and the reaction mixture was stirred at the same temperature for 2 h, and then at 0 °C for 1 h. Saturated aq. NH_4Cl was added to the solution and the reaction mixture was warmed to room temperature and extracted with EtOAc. The organic layer was washed with saturated aq. $NaHCO_3$, brine, dried over $MgSO_4$, and evaporated. The residue was dissolved in dry MeOH (10 mL) and sodium methoxide (870 mg, 6.3 mmol) was added to the solution. The solution was stirred at 50 °C for 1 h. The reaction mixture was cooled to room temperature and evaporated. The residue was diluted with EtOAc and the solution was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 60/40, then 50/50 then 35/65) to give **13** (311.5 mg, 1.30 mmol) in 62% yield.

$[\alpha]_D^{25}$ –15.06 (*c* 0.5, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1217, 1041 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ: 5.57 (1H, ddd, J = 17.0, 11.0, 6.0 Hz), 4.88 (1H, dt, J = 17.0, 1.7 Hz), 4.73 (1H, dt, J = 11.0, 1.7 Hz), 3.85–3.81 (1H, m), 3.51 (1H, ddd, J = 8.4, 5.7, 2.1 Hz), 3.16 (1H, d, J = 9.5 Hz), 3.05 (1H, d, J = 9.5 Hz), 2.85 (2H, dd, J = 4.6, 2.2 Hz), 2.12–1.92 (2H, m), 1.85 (1H, t, J = 2.7 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ: 139.95, 116.22, 83.51, 81.92, 77.77, 71.46, 71.07, 67.23, 24.31, 23.77, 9.14, 9.02; EI-LRMS m/z 263.1 (M+Na); EI-HRMS calcd for $C_{13}H_{20}O_4Na$ 263.1254, found 263.1244.

5.1.7. (3*R*,4*S*,5*R*)-3,5-Bis-[(*tert*-butyldimethylsilyl)oxy]-4-[3-(*tert*-butyldimethylsilyl)oxy-2,2-ethanopropyl]oxyoct-1-en-7-yne (7d). To a solution of **13** (257.0 mg, 1.07 mmol) in CH_2Cl_2 (5 mL) were added 2,6-lutidine (0.748 mL, 0.42 mmol) and *t*-butyldimethylsilyl trifluoromethanesulfonate (1.11 mL, 4.82 mmol) at 0 °C and they were stirred at room temperature for 1 h. Dry MeOH (2 mL) was added to the reaction mixture at room temperature and stirred at the same temperature for 10 min. The reaction mixture was diluted with hexane, washed with H_2O and dried over $MgSO_4$, followed by filtration and evaporation. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 96/4) to give **7d** (571 mg, 0.979 mmol) in 91% yield. $[\alpha]_D^{25}$ +4.62 (*c* 0.5, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1253, 1217, 1086 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ: 6.01–5.92 (1H, m), 5.20 (1H, d, J = 17.3 Hz), 5.11 (1H, d, J = 12.2 Hz), 4.32 (1H, dd, J = 7.2, 3.8 Hz), 3.96 (1H, dd, J = 11.4, 5.4 Hz), 3.62 (2H, dd, J = 14.4, 10.0 Hz), 3.49 (2H, dd, J = 9.9, 7.4 Hz), 3.36 (1H, t, J = 4.3 Hz), 2.53 (1H, ddd, J = 16.8, 6.6, 2.7 Hz), 2.36 (1H, ddd, J = 16.8, 6.6, 2.7 Hz), 1.94 (1H, t, J = 2.7 Hz), 0.90 (9H, s), 0.88 (9H, s), 0.88 (9H, s), 0.40 (4H, d, J = 7.3 Hz), 0.12 (3H, s), 0.09 (3H, s), 0.06 (3H, s), 0.03 (3H, s), 0.02 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 139.1, 116.0, 84.9, 82.5, 75.7, 75.5, 74.5, 71.7, 69.6, 65.5, 26.0, 25.8, 25.7, 23.8, 22.8, 18.4, 18.2, 18.1, 7.8, 7.4, –4.0, –4.1, –4.2, –4.7, –5.3, –5.3; EI-LRMS m/z 605.7 (M+Na); EI-HRMS calcd for $C_{31}H_{62}O_4Si_3Na$ 605.3848, found 605.3862.

5.1.8. (3*R*,4*S*,5*R*)-3,5-Bis-[(*tert*-butyldimethylsilyl)oxy]-4-[2-(cyano)-2,2-ethanopropyl]oxyoct-1-en-7-yne (7g). To a solution of **14** (155 mg, 0.33 mmol) in CH_2Cl_2 (3 mL) were added Dess–Martin reagent (212 mg, 0.5 mmol) at 0 °C and it was stirred at the same temperature for 1.5 h. The reaction mixture was diluted with EtOAc and washed with aq. $Na_2S_2O_3$, aq. $NaHCO_3$ successively. The organic layer was dried over $MgSO_4$, filtered and evaporated. The residue (251.3 mg) was dissolved in EtOH (4 mL), and hydroxylamine hydrochloride (46 mg, 0.66 mmol) and sodium acetate (82 mg, 1.0 mmol) were added to the solution. The reaction mixture was stirred at room temperature for 1.5 h. The reaction mixture was diluted with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, followed by filtration and evaporation. The residue (290 mg) was dissolved in pyridine (6 mL), and cyanuric chloride (243 mg, 1.32 mmol) was added to the solution. The reaction mixture was stirred at 60 °C overnight. The reaction mixture was cooled to room temperature and diluted with toluene. The solution was washed with H_2O, brine successively. The organic layer was dried over $MgSO_4$, filtered and evaporated. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 95/5) to give **7g** (107.2 mg, 0.23 mmol) in 70% yield. $[\alpha]_D^{25}$ –3.3 (*c* 1.0, $CHCl_3$); IR (film, $CHCl_3$) 3314, 2930, 1637, 1253, 1095 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$) δ: 5.98 (1H, ddd, J = 17.0, 10.0, 6.0 Hz), 5.24 (1H, ddd, J = 17.2, 1.5, 1.0 Hz), 5.18 (1H, ddd, J = 10.0, 1.5, 1.0 Hz), 4.35–4.32 (1H, m), 3.86 (1H, q, J = 5.5 Hz), 3.74 (1H, d, J = 10. 0 Hz), 3.65 (1H, d, J = 10.0 Hz), 3.48 (1H, dd, J = 5.7, 3.3 Hz), 2.51 (1H, dq, J = 16.9, 2.8 Hz), 2.37 (1H, dq, J = 17.1, 2.6 Hz), 1.98 (1H, t, J = 2.7 Hz), 1.23–1.20 (2H, m), 1.02–0.98 (2H, m), 0.91 (9H, s), 0.89 (9H, s), 0.12 (3H, s), 0.12 (3H, s), 0.08 (3H, s), 0.04 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 137.9,

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122.6, 116.7, 85.1, 81.2, 74.8, 74.2, 71.1, 70.3, 31.5, 25.9, 25.8, 24.1, 22,7, 18.1, 18.0, 14.1, 12.3, 11.8, 10.6, –4.2, –4.4, –4.8; EI-LRMS *m/z* 557.2 (M+Na); EI-HRMS calcd for $C_{25}H_{45}NO_3Si_2$ 464.3011, found 464.3021.

5.1.9. (3*R*,4*S*,5*R*)-3,5-Bis-[(*tert*-butyldimethylsilyl)oxy]-5-(3-cyanopropyl)oxyoct-1-en-7-yne (7f). To a solution of **15** (1.64 g, 3.70 mmol) in CH_2Cl_2 (15 mL) were added triethylamine (1.03 mL, 7.40 mmol) and trimethylamine hydrochloride (35.4 mg, 0.370 mmol) and it was stirred under an argon atmosphere at 0 °C. *p*-Toluenesulfonyl chloride (1.06 g, 5.56 mmol) was added to the reaction mixture, which was warmed to room temperature overnight. H_2O was added to the reaction mixture at room temperature, it was stirred for 30 min, and CH_2Cl_2 was removed by evaporation. The residue was diluted with EtOAc, and washed with H_2O, saturated aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, followed by filtration and evaporation. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 95/5) to give a tosylate (1.85 g, 3.10 mmol). To the tosylate (1.85 g, 3.10 mmol) in *N*,*N*-dimethylformamide (15 mL) were added potassium cyanide (404 mg, 6.20 mmol) and 18-crown-6-ether (78.7 mg, 0.31 mmol), and it was stirred at 50 °C overnight. The reaction mixture was cooled to room temperature and diluted with H_2O. The mixture was extracted with EtOAc, and the organic layer was washed with brine and dried over Na_2SO_4, followed by filtration and evaporation. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 95/5 then 90/10) to give **7f** (1.31 g, 2.88 mmol) in 78%. $[\alpha]_D^{25}$ –5.37 (*c* 1.0, $CHCl_3$); IR (film)) 3314, 2930, 1095 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.97–5.89 (1H, m), 5.23–5.18 (2H, m), 4.30 (1H, dd, *J* = 7.0, 3.0 Hz), 3.84–3.68 (3H, m), 3.44 (1H, dd, *J* = 5.6, 3.2 Hz), 2.53–2.39 (3H, m), 2.35 (1H, dq, *J* = 16.9, 2.6 Hz), 1.99 (1H, t, *J* = 2.7 Hz), 1.92–1.85 (2H, m), 0.91 (9H, s), 0.90 (9H, s), 0.10 (3H, s), 0.08 (6H, s), 0.05 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ:138.0, 119.9, 116.6, 85.4, 81.2, 74.6, 71.3, 70.3, 70.1, 26.5, 25.9, 25.8, 24.3, 18.2, 18.1, 14.2, –4.1, –4.3, –4.4, –4.7; EI-LRMS *m/z* 474.2 (M+Na), EI-HRMS calcd for $C_{24}H_{45}O_3Si_2Na$ 474.2830, found 474.2832.

5.1.10. 3-{[(3*R*,4*S*,5*R*)-3,5-Bis-(*t*-butyldimethylsilyl)oxyoct-1-en-7-yn-4-yl]oxy}propanoic acid (16). To a solution of **15** (540.5 mg, 1.22 mmol) in DMF (5 mL) was added PDC (2.25 g, 6.10 mmol) at room temperature and it was stirred at the same temperature overnight. Water was added to the reaction mixture and extracted twice with EtOAc. The combined organic layer was washed with brine and dried over $MgSO_4$, followed by filtration and evaporation. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 91/9 then 85/15 then 67/33) to give **16** (335.3 mg, 0.73 mmol) in 60% yield. $[\alpha]_D^{25}$ +0.58 (*c* 0.5, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1215 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.91 (1H, ddd, *J* = 11.0, 17.0, 6.0 Hz), 5.30–5.18 (2H, m), 4.32 (1H, dd, *J* = 6.8, 3.4 Hz), 3.98–3.93 (2H, m), 3.82 (1H, q, *J* = 5.5 Hz), 3.52 (1H, dd, *J* = 6.1, 3.7 Hz), 2.64–2.61 (2H, m), 2.46 (1H, ddd, *J* = 17.1, 2.8, 6.0 Hz), 2.36 (1H, ddd, *J* = 17.2, 2.5, 6.0 Hz), 2.01 (1H, t, *J* = 2.7 Hz), 0.91 (18H, s), 0.10 (3H, s), 0.09 (3H, s), 0.08 (3H, s), 0.07 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 174.4, 137.1, 117.4, 85.7, 80.7, 74.3, 70.9, 70.7, 68.0, 35.3, 29.7, 25.9, 25.8, 24.3, 18.2, 18.1, 13.0, –4.2, –4.4, –4.5, –4.8; EI-LRMS *m/z* 479.1 (M+Na). EI-HRMS calcd for $C_{25}H_{45}NO_3Si_2Na$ 479.2644, found 479.2640.

5.1.11. (3*R*,4*S*,5*R*)-3,5-Bis-[(*tert*-butyldimethylsilyl)oxy]-4-[2-(ethoxycarbonyl)ethyl]oxyoct-1-en-7-yne (7h). To a solution of **16** (180 mg, 0.394 mmol) in EtOH (2 mL) was added sulfonic acid (44 μL, 0.79 mmol) at 0 °C and it was refluxed for overnight. The reaction mixture was cooled to room temperature and quenched with saturated aq. $NaHCO_3$. After evaporation of the reaction mixture, the residue was diluted with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, filtered and evaporated. The residue (88 mg) was dissolved in dry CH_2Cl_2 (2 mL) and cooled to 0 °C. 2,6-Lutidine (177 μL, 1.576 mmol) and *t*-butyldimethylsilyl trifluoromethanesulfonate (272 μL, 1.182 mmol) were added to the solution and stirred at room temperature for 1 h. The reaction mixture was quenched with dry MeOH (2 mL), diluted with EtOAc and washed with H_2O. The organic layer was dried over $MgSO_4$, filtrated and evaporated. The residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 95/5) to give **7h** (92.7 mg, 0.191 mmol) in 48% yield. $[\alpha]_D^{25}$ +0.58 (*c* 0.5, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1215 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.99–5.91 (1H, m), 5.21 (1H, ddd, *J* = 17.3, 1.1, 2.0 Hz), 5.14 (1H, ddd, *J* = 10.4, 1.0, 2.0 Hz), 4.32–4.29 (1H, m), 4.13 (2H, q, *J* = 7.2 Hz), 4.02–3.95 (1H, m), 3.89–3.81 (2H, m), 3.40 (1H, dd, *J* = 5.5, 3.5 Hz), 2.56 (2H, t, *J* = 6.7 Hz), 2.48 (1H, ddd, *J* = 17.0, 3.0, 6.0 Hz), 2.35 (1H, ddd, *J* = 17.0, 3.0, 6.0 Hz), 1.96 (1H, t, *J* = 2.7 Hz), 1.26 (3H, t, *J* = 7.1 Hz), 0.90 (9H, s), 0.89 (9H, s), 0.09 (3H, s), 0.08 (3H, s), 0.07 (3H, s), 0.03 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 171.7, 138.6, 116.1, 85.4, 81.9, 74.7, 71.4, 69.9, 67.9, 60.3, 35.6, 25.9, 25.8, 24.0, 18.2, 18.1, 14.2, –4.1, –4.2, –4.4, –4.8; EI-LRMS *m/z* 507.2 (M+Na). EI-HRMS calcd for $C_{25}H_{48}O_5Si_2Na$ 507.2933, found 507.2919.

5.1.12. (3*R*,4*S*,5*R*)-3,5-Bis-[(*tert*-butyldimethylsilyl)oxy]-5-[[3-methyl-3-(trimethylsilyl)oxy]butyloxy]oct-1-en-7-yne (7e). To a solution of **7h** (68 mg, 0.14 mmol) in THF (2.1 mL) was added MeMgBr (1 M in THF, 0.58 mL, 0.58 mmol) at room temperature and it was stirred at the same temperature for 2h. The reaction mixture was quenched with saturated aq. NH_4Cl, diluted with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, followed by filtration and evaporation. To a solution of crude product in DMF (1 mL) were added imidazole (29 mg, 0.42 mmol) and trimethylsilyl chloride (26.6 μL, 0.21 mmol) at 0 °C and it was stirred at room temperature for 1 h. The reaction was quenched with dry MeOH (1 mL). The reaction mixture was diluted with EtOAc, washed with brine and dried over $MgSO_4$. After filtration and evaporation, the residue was purified by flash column chromatography on silica gel (hexane–EtOAc = 98/2) to give **7e** (49 mg, 0.09 mmol) in 64% yield. $[\alpha]_D^{25}$ +4.62 (*c* 0.5, $CHCl_3$); IR (film, $CHCl_3$) 3020, 1215 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.00–5.92 (1H, m), 5.23–5.11 (2H, m), 4.33–4.29 (1H, m), 3.87–3.60 (3H, m), 3.37 (1H, dd, *J* = 5.6, 3.4 Hz), 2.52–2.32 (2H, m), 1.96 (1H, t, *J* = 2.3 Hz), 1.75 (2H, t, *J* = 7.6 Hz), 1.23 (3H, s), 1.22 (3H, s), 0.91 (9H, s), 0.89 (9H, s), 0.13 (3H, s), 0.10 (3H, s), 0.10 (9H, d, *J* = 1.7 Hz), 0.07 (3H, s), 0.03 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 138.8, 115.9, 85.3, 82.1, 74.7, 73.0, 71.6, 69.8, 69.4, 30.4, 30.2, 25.9, 25.8, 14.1, 18.2, 18.1, 2.6, –4.1, –4.2, –4.4, –4.7; EI-LRMS *m/z* 507.2 (M-TMS+2H_2O), 493.2 (M-TMS+Na); EI-HRMS calcd for $C_{25}H_{50}O_4Si_2Na$ (M-TMS+Na) 493.3140, found 493.3136.

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5.1.13. (5*Z*,7*E*)-(1*S*,2*S*,3*R*)-2-Methyl-20-[(2,2-dimethylcyclopentanone-(5*E*)-ylidene)] methyl-9,10-seco-5,7,10(19)-pregnatriene-1,3-diol (5a). Under N_2 atmosphere, a solution of **6** (40 mg, 0.105 mmol), **7a** (52 mg, 0.148 mmol) and $Pd(PPh_3)_4$ (20 mg, 0.019 mmol) in toluene (1 mL) and Et_3N (1 mL) was stirred at 110 °C for 2 h. The reaction mixture was evaporated and purified with PTLC (Merck Kiseigel plate Art. 113794 1 mm, the eluent was hexane–EtOAc = 95/5) to give a crude product (32.4 mg), which was dissolved in dry MeCN (1 mL) and CH_2Cl_2 (1 mL). To the solution was added $LiBF_4$ (20 mg, 0.21 mmol) and 1 M H_2SO_4 in MeCN (20 µL, 0.02 mmol) at 0 °C and it was stirred at the same temperature for 1.5 h. After the work up, the crude product (6.0 mg) was obtained with preparative TLC (Merck Kiseigel plate Art. 113794 1 mm, the eluent was hexane–EtOAc = 50/50). Further purification with reversed-phase HPLC (YMC-Pack ODS column, 30–250 mm, 10 mL min^{-1}, eluent A: MeCN–H_2O = 5/95, eluent B: MeCN–MeOH–H_2O = 59.5/40/0.5, eluent A/B = 15/85) gave **5a** (4.0 mg, 0.008 mmol, 8% yield). $[\alpha]_D^{25}$ +128.8 (*c* 0.1, $CHCl_3$); IR (film, $CHCl_3$) 3348, 2974, 2885, 1454, 1381, 1089, 1051 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.43–6.37 (2H, m), 6.00 (1H, d, J = 11.2 Hz), 5.27 (1H, s), 4.99 (1H, d, J = 1.9 Hz), 4.30 (1H, br s), 3.84 (1H, br s), 2.84 (1H, dd, J = 12.2, 3.9 Hz), 2.67 (1H, dd, J = 13.4, 4.2 Hz), 2.53–2.33 (3H, m), 2.23 (1H, dd, J = 13.4, 8.1 Hz), 2.03–1.90 (3H, m), 1.76–1.65 (5H, m), 1.54–1.34 (7H, m), 1.17–1.11 (1H, m), 1.08 (3H, d, J = 6.8 Hz), 1.07 (3H, s), 1.05 (3H, s), 1.04 (3H, d, J = 6.0 Hz), 0.57 (3.0H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 211.2, 146.5, 142.6, 152.5, 133.7, 133.2, 124.7, 117.1, 113.2, 75,4, 71.7, 56.5, 55.9, 46.0, 45.6, 44.2, 43.5, 40.3, 37.2, 35.4, 29.0, 26.8, 23.8, 23.5, 23.3, 22.2, 19.2, 12.5, 12.4; EI-LRMS *m/z* 475.2 (M+Na) 435.2 (M-H_2O+H), 417.2 (M-2H_2O+H); EI-HRMS calcd for $C_{30}H_{44}O_3Na$ (M+Na) 475.3183, found 475.3171.

5.2. Biology

5.2.1. Evaluation of affinity to vitamin D receptor (VDR). The binding affinity to VDR was evaluated using a 1α,25-$(OH)_2VD_3$ assay kit (POLARSCREEN VITAMIN D RECEPTOR COMPETITOR ASSAY, RED, Cat.No.PV4569; Invitrogen). The solution of test compound (1 mM in EtOH) was diluted 10 times with DMSO. The solution was diluted 50 times with the assay buffer included in the kit. The solution was defined as the compound solution. On the other hand, VDR/Fluoromone and VDR RED, both of which are included in the kit, were diluted with the assay buffer included in the kit so that the concentration of VDR/Fluoromone was 2.8 nM, and that of VDR RED was 2 nM in the mixture. The solution was defined as the VDR/Fluoromone and VDR RED complex. To a 384-well Black plate (Coring, #3677) was added the compound solution (10 µL), and the VDR/Fluoromone and VDR RED complex (10 µL) was added to each well. The mixture was incubated at 20–25 °C for 2 h. The polarized fluorescence in each well was measured (384 nm, emission: 595 nm, excitation: 535 nm, time: 250 ms/well). All compounds were evaluated with N = 2 within the range from 10^{-6} M to 10^{-10} M. IC_{50} values were calculated using the average measured value. The activities of each compound were shown as a relative value in which the activity of natural hormone **1** was normalized to 100%.

5.2.2. General procedure for transactivation assay of human osteocalcin promoter. The human osteocalcin gene promoter fragment −838/+10 was cloned into the reporter plasmid pGL3 (Promega) as reported. Human VDR and RXR genes were cloned into expression vector pcDNA3 (Invitrogen). Hos cells were maintained in phenol red-free DMEM (Invitrogen) containing 10% FCS (Invitrogen). Prior to transfection, the cells were plated in a 96-well plate at a density of 400,000 cells per well in Opti-MEM (Invitrogen). The cells were transfected with human osteocalcin reporter vector (pGL3-hOc: 100 ng/well), human VDR and RXR expression vector (pcDNA-hVDR, pcDNA-hRXR: 10 ng/well) and phRL-TK (Promega: 25 ng/well) using 0.45 µL Lipofectamine 2000 reagent (Invitrogen). After incubation at 37 °C for 3 h, the culture media were replaced with phenol red-free DMEM containing 10% FCS. The cells were treated with ethanol vehicle or various concentrations of compounds (from 0.1 pM to 100 nM). After incubation at 37 °C for 24 h, the luciferase activity of the cells was quantitated by a luminometer (Berthold) using the Dual-Glo luciferase assay system (Promega).

5.2.3. Evaluation of derivatives on bone mineral loss in ovariectomized rats. Twelve-week-old Sprague-Dawley female rats (Charles River, Japan) were ovariectomized and fed *ad libitum* with a normal diet containing 1.0% Ca for 4 weeks. The rats were orally administrated vitamin D derivatives at various doses in MCT as the vehicle 5 times a week for 4 weeks. The sham and OVX groups were administrated MCT alone. Twenty-four hours after the final administration, blood was collected under ether anesthesia, and the rats were euthanized. The BMD of spine (L4–L5) bone mass was measured by a dual X-ray absorptiometer (QDR-2000; HOLOGIC, USA). The results are expressed as the mean ± standard error of the mean. The statistical significance of differences between the OVX and experimental groups was analyzed by Student's t-test.

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