Synthesis of 24,24-Ethanovitamin D_3 Lactones Using Ruthenium-Catalyzed Intermolecular Enyne Metathesis: Potent Vitamin D Receptor Antagonists

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Abstract: Novel vitamin D receptor antagonists, 24,24-ethanovitamin D_3-26,23-lactones **6** and **7** and their 2α-functionalized analogues **6a–c** and **7a–c** were synthesized and their biological activities were evaluated. The triene structure of vitamin D_3 was constructed using Pd-catalyzed alkenylative cyclization of A-ring precursor enynes **12** and **12a–c** with the CD-ring bromo-olefin counterpart having 24,24-ethano-α-methylene-γ-lactone on the side chain (**21** or **22**). The CD-ring precursors **21** and **22** were efficiently synthesized via Ru-catalyzed intermolecular enyne metathesis of **15** with ethylene to give dienone **17** followed by cyclopropanation. The VDR antagonistic activity of the newly designed vitamin D_3 lactones **6** and **7** increased to 2.8 times that of TEI-9647 (**2**) in a HL-60 cell differentiation evaluating system. Moreover, introduction of three substituents, that is, a methyl (**6a** and **7a**), a 3-hydroxypropyl (**6b** and **7b**), or a 3-hydroxypropoxyl group (**6c** and **7c**) into the C2α position of **6** and **7**, resulted in marked enhancement, up to 19 times, of the antagonistic activity toward VDR.

Key words: vitamins , antagonist, lactones, ruthenium, metathesis, enynes

1α,25-Dihydroxyvitamin D_3 (**1**) (Figure 1) is the most physiologically active metabolite of vitamin D_3 and regulates various biological events, including bone metabolism as well as the proliferation and differentiation of various types of tumor cells.[1,2] In most cases, the biological responses of **1** are mediated via interaction with its specific nuclear receptor, vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily and acts as a ligand-dependent gene transcription factor with co-activators.[3,4] Recently, we have synthesized several 1α,25-dihydroxyvitamin D_3 analogues, which systematically introduced an alkyl, ω-hydroxyalkyl, and ω-hydroxyalkoxyl group into the C2α position of **1**.[5–9] Some of these C2α-modified vitamin D_3 analogues showed unique biological profiles. In particular, introduction of the 2α-methyl (**1a**),[5] 2α-(3-hydroxypropyl) (**1b**),[6] and 2α-(3-hydroxypropoxy) (**1c**)[7] groups led to a 2- to 4-fold higher binding affinity for the bovine thymus VDR relative to the natural hormone **1** with potent agonistic activity.

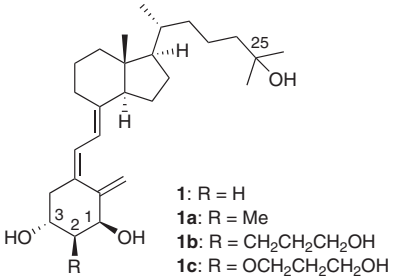

Figure 1 Structures of 1α,25-dihydroxyvitamin D_3 (**1**) and its C2α-modified analogues **1a–c**

In 1999, 25-dehydro-1α-hydroxyvitamin D_3-26,23-lactones, TEI-9647 (**2**) and TEI-9648 (**3**) in Figure 2,[10] were identified as the first VDR antagonists[11] during the course of experiments on the side chain modification of 1α,25-dihydroxyvitamin D_3-26,23-lactone, which is the natural metabolite of **1**.[12] Both **2** and **3** specifically antagonize the VDR-mediated genomic action.[13] Namely, **2** and **3** inhibit the differentiation of human leukemia cells (HL-60 cells) induced by **1**.[10a] Moreover, **2** suppresses the gene expression of 25-hydroxyvitamin D_3-24-hydroxylase in human osteosarcoma cells[10b] and in HL-60 cells.[10d] The unprecedented biological profiles of **2** and **3** prompted us to investigate the structure–activity relationships of the vitamin D_3 lactone derivatives, and we found some pertinent modifications of **2** and **3** that resulted in a marked enhancement of their biological activities.[14] That is, introduction of the above three motifs, i.e., the methyl, the 3-hydroxypropyl or the 3-hydroxypropoxyl group, into the C2α position of **2** and **3**, increased the antagonistic activity up to 30-fold in the case of **2b**.[14a] On the other hand, it was found that both the VDR-binding affinity and antagonistic activity of **2** and **3** were affected by the orientation of the substituents on the lactone ring.[14b] Interestingly, vitamin D_3 lactones with dimethyl groups at position C24 on the lactone ring (especially **4**) had 12 times the antagonistic activity of **2**.[14c] Furthermore, we demonstrated that double functionalization of the C2α and C24 positions of **2** and **3** remarkably increased the antagonistic activity, that is, **4a** showed 89-fold stronger antagonism than **2**, and **5a** was 19-fold more potent than **3**.

On the basis of the above results, we newly designed 24,24-ethanovitamin D_3-26,23-lactones **6** and **7** to investigate further the structure–activity relationships on the

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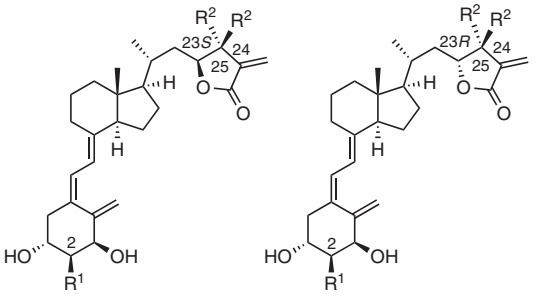
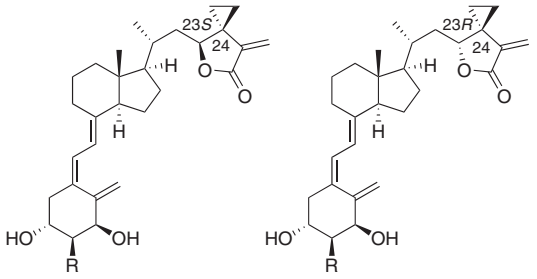


2: $R^1 = R^2 = H$ (TEI-9647) **3**: $R^1 = R^2 = H$ (TEI-9648)
2a: R^1 = Me, R^2 = H **3a**: R^1 = Me, R^2 = H
2b: $R^1 = (CH_2)_3OH$, R^2 = H **3b**: $R^1 = (CH_2)_3OH$, R^2 = H
2c: $R^1 = O(CH_2)_3OH$, R^2 = H **3c**: $R^1 = O(CH_2)_3OH$, R^2 = H
4: R^1 = H, R^2 = Me **5**: R^1 = H, R^2 = Me
4a: $R^1 = R^2$ = Me **5a**: $R^1 = R^2$ = Me

Figure 2 Structures of 25-dehydro-1α-hydroxyvitamin D_3-26,23-lactones (TEI-9647: **2** and TEI-9648: **3**), and their C2α-modified (**2a–c** and **3a–c**), 24-modified (**4** and **5**), and 2,24-double modified analogues (**4a** and **5a**)

lactone ring core structure, and particularly, the effect of the rigid spiro[2.4]ring system on the biological activities (Figure 3). Furthermore, we expected the biological activities of the ethanolactone derivatives (**6** and **7**) to be enhanced by introducing three motifs, that is, the methyl (**6a** and **7a**), the 3-hydroxypropyl (**6b** and **7b**) and the 3-hydroxypropoxyl group (**6c** and **7c**) as in our previous studies.[8,9,14] Here we report the synthesis and biological evaluation of the novel potent VDR antagonists, 24,24-ethano-1α-hydroxyvitmin D_3 lactones and their C2α-modified analogues.

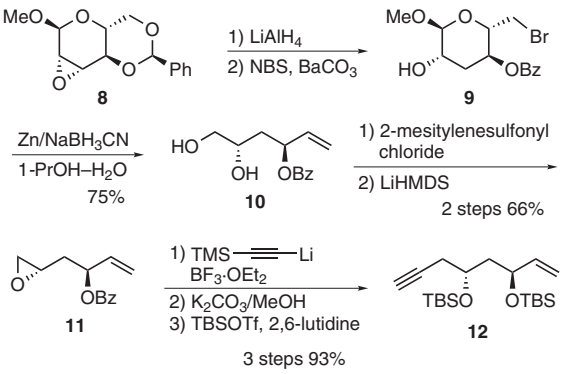


6: R = H **7**: R = H
6a: R = Me **7a**: R = Me
6b: R = $CH_2CH_2CH_2OH$ **7b**: R = $CH_2CH_2CH_2OH$
6c: R = $OCH_2CH_2CH_2OH$ **7c**: R = $OCH_2CH_2CH_2OH$

Figure 3 Newly designed 24,24-ethanovitamin D_3 lactones **6** and **7** and their 2α-modified analogues (**6a–c** and **7a–c**)

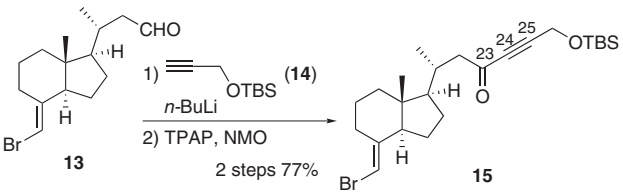
Synthesis and Biological Evaluation of 24,24-Ethanovitamin D_3-26,23-Lactones

First of all, A-ring precursor **12** was synthesized in an improved manner from epoxide **8**.[15] That is, according to the reported procedure,[16] **8** was reacted with $LiAlH_4$ followed by oxidative cleavage of the benzylidene acetal by NBS to give the known compound **9**. Pyranose ring-opening of **9** with activated zinc in the presence of $NaBH_3CN$ provided **10** in 75% yield. The primary hydroxyl group of the diol **10** was sulfonated, and the resulting monosufonate was treated with lithium hexamethyldisilazide to give the epoxide **11**. Introduction of the TMS-ethynyl group into **11** followed by deprotection under basic conditions gave a diol. The resulting diol was protected by the TBS groups to provide the desired A-ring precursor **12** (Scheme 1).[17]



Scheme 1 Improved synthesis of A-ring precursor **12**

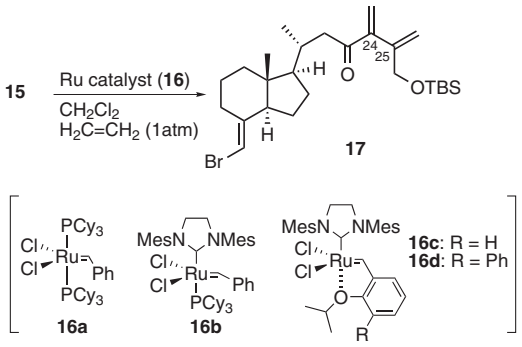
Next, we synthesized the CD-ring counterpart having the ethano-α-methylene-γ-lactone unit on the side chain via Mori's Ru-catalyzed intermolecular enyne metathesis with ethylene.[18] The metathesis precursor **15** was prepared by the addition of lithium siloxymethylacetylide generated from **14** and BuLi to the aldehyde **13**[14a] and the following TPAP oxidation (Scheme 2).



Scheme 2 Synthesis of alkynone **15**

The Ru-catalyzed intermolecular enyne metathesis of **15** with ethylene was investigated for the introduction of a methylene unit into positions C24 and C25 (based on the steroidal numbering), respectively (Table 1). First of all, according to Mori's procedure, the alkyne **15** was treated with the first generation Grubbs catalyst **16a** under ethylene gas (run 1). However, no metathesis product was obtained. The second generation Grubbs catalyst **16b**[19a] was not effective for the intermolecular enyne metathesis of **15**, either, and only decomposition of **15** was observed (runs 2 and 3). On the other hand, when the Hoveyda–Grubbs catalyst **16c**[19b] was used, the dienone **17** was obtained in 29% (run 4). The same type of Ru catalyst **16d** reported by Blechert[19c] was found to be more effective for the metathesis of **15** with ethylene, and **17** was produced in 55% yield.[19d] Finally, we disclose that the alkynone–ethylene metathesis proceeded smoothly in the presence of 10 mol% of **16d** at 0 °C to provide the desired dienone derivative **17** in high yield (run 6).

Table 1 Ruthenium-Catalyzed Enyne Metathesis of **15** with Ethylene

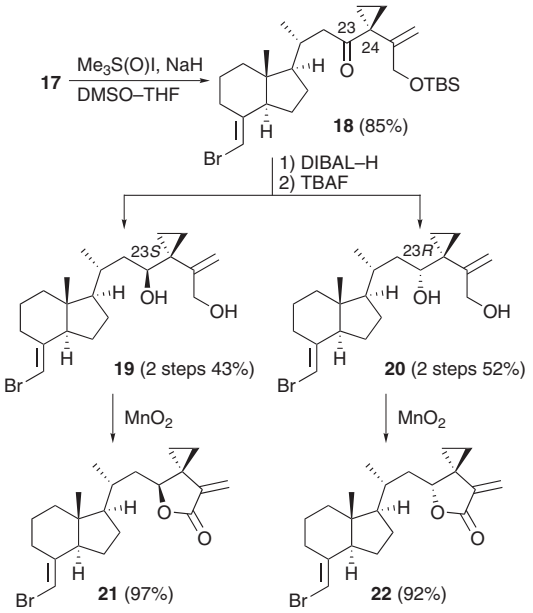


Run	'Ru' (mol%)	Temp (°C)	Time (h)	Yield[a] (%)
1	**16a** (5)	r.t.	16	0 (80)
2	**16b** (5)	r.t.	24	0 (88)
3	**16b** (10)[b]	reflux	4	0 (44)
4	**16c** (20)[c]	r.t.	24	29 (32)
5	**16d** (20)[d]	r.t.	53	55 (6)
6	**16d** (10)	0 °C	1.5	92

[a] The yield in parentheses is that of recovered **15**.
[b] 5 mol% of **16b** was used. After 2 h, 5 mol% of **16b** was added.
[c] 5 mol% of **16c** was used. Additional amounts of **16c** were successively added (5 mol%, 5 h, 10 mol%, 19 h).
[d] 5 mol% of **16d** was used. Additional amounts of **16d** were successively added (5 mol%, 24 h, 10 mol%, 48 h).

Transformation of the dienone **17** into the CD-ring bromo-olefin precursors **21** and **22** is shown in Scheme 3. Cyclopropanation by the 1,4-addition of trimethylsulfoxonium ylide to **17** provided the desired cyclopropyl ketone derivative **18** in good yield. Treatment of **18** with DIBAL-H followed by desilylation of the TBS group using TBAF gave two diol derivatives **19** and **20**, which were stereoisomers with respect to the position C23 on the side chain, in 43% yield (2 steps) and in 53% yield (2 steps), respectively. The crystal structure of **20** revealed the absolute configuration of position C23 of **20** to be *R* (Figure 4).[20] From this result, the absolute configuration of position C23 of its stereoisomer **19** was determined to be *S*. Finally, the diol **19** was converted into the desired CD-ring precursor **21** by oxidation using MnO_2 in 97% yield. Similarly, the lactone **22** was obtained from **20** by MnO_2 oxidation in 92% yield.

The construction of a vitamin D triene skeleton was achieved by Trost's alkenylative cyclization[7,21] of the A-ring precursor **12** with the CD-ring counterpart **21** or **22** (Scheme 4). That is, each CD-ring precursor **21** or **22** reacted with enyne **12** in the presence of Pd(0) catalyst, and the protected vitamin D_3 derivative was produced. Acid-mediated deprotection gave the desired 24,24-ethanovitamin D_3 lactone derivatives **6** and **7**, respectively.



Scheme 3 Synthesis of CD-ring precursors **21** and **22**

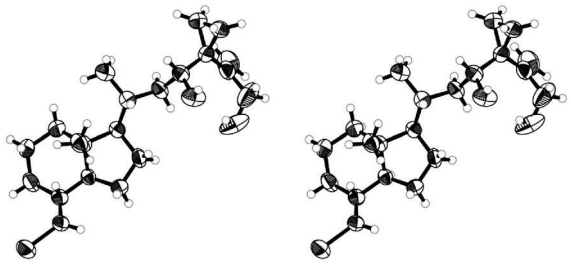
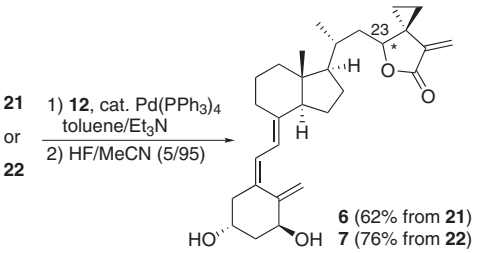


Figure 4 Stereoscopic view of the crystal structure of compound **20**. The thermal displacement parameters are drawn at 50%



Scheme 4 Synthesis of 24,24-ethanovitamin D_3-26,23-lactones **6** and **7**

Biological activities of the synthesized 24,24-ethanovitamin D_3 lactones **6** and **7** were evaluated and the data are shown in Table 2. We also show the data of 24,24-dimethylvitamin D_3 lactones **4** and **5** for comparison. The binding affinity of **6** and **7** for the chick intestinal VDR was examined as described previously.[22] The binding affinity of (23*S*)-24,24-ethanovitamin D_3 lactone (**6**) for the VDR increased remarkably to 13.9 times that of **2** (1.7 times more potent than the natural hormone **1**). In the case of TEI-9648 type analogue **7**, the binding affinity for the VDR decreased to one tenth that of **3**. The antagonistic activities of **6** and **7** were assessed by the NBT-reduction

method[23] in terms of inhibition of HL-60 cell differentiation induced by 10 nM of the natural hormone **1**. Although the antagonistic activity of (23*S*)-24,24-ethanovitmain D_3 lactone **6** was weaker than that of the corresponding 24,24-dimethly analogue **4**, **6** was 2.8-fold more active than the original **2**. On the other hand, the (23*R*)-isomer **7** showed little antagonistic activity in contrast to (23*R*)-24,24-dimethylvitamin D_3 lactone **5** which was highly active compared to the original **3**.

Table 2 Biological Activities of 24,24-Ethanovitamin D_3 Lactones **6** and **7**

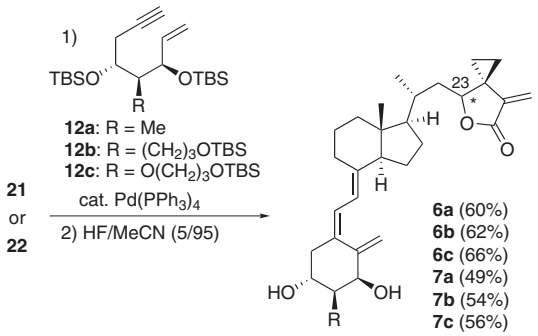
Compounds	Binding affinity for VDR[a]	Antagonistic activity (IC_{50}, nM)[b]
1	100	–
TEI-9647 (**2**)	12	9.4
4[c]	37	0.71
6	167	3.3
TEI-9648 (**3**)	7	134.4
5[c]	18	51.5
7	0.7	>3000

[a] Chick intestinal VDR. The potency of **1** is normalized to 100.
[b] Antagonistic activity was assessed in terms of IC_{50} for the differentiation of HL-60 cells induced by 10 nM of **1**.
[c] See ref.[14c]

Effect of C2α Modification of 24,24-Ethanovitamin D_3 Lactones

Next, we turned our attention to the C2α-funtionalization of the 24,24-ethanovitmain D_3 lactones **6** and **7**. We demonstrated that the C2α-modification of 24-methylvitamin D_3 lactones[14b] as well as 24,24-dimethyl derivatives[14c] markedly enhanced their biological activities. From our previous results, we expected a high increase in VDR-binding affinity and marked enhancement of antagonistic activity through the C2α-functionalization of **6** and **7**. The C2α-modified analogues were similarly synthesized by the coupling reaction of the CD-ring precursors **21** and **22** with the A-ring precursor enynes **12a**,[24] **12b**[14c] and **12c**,[7] respectively (Scheme 5).

The evaluation of the biological activities of **6a–c** and **7a–c** demonstrated that the C2α-modification was effective in improving the biological activities of 24,24-ethanovitamin D_3 lactones **6** and **7** (Table 3). Namely, the VDR-binding affinity of (23*S*)-isomers **6a–c** remained high. In particular, the 2α-methyl analogue **6a** showed 9.3 times stronger VDR-binding affinity than the original TEI-9647 (**2**). On the other hand, the C2α-modification of TEI-9648 (**3**) type analogue **7** increased the low VDR affinity of **7** to 1.9–4.1 times higher than TEI-9648 (**3**) (**7a–c**). The antagonistic activity was also enhanced by the C2α-functionalization. In the case of TEI-9647 type analogues **6a–c**, the



Scheme 5 Synthesis of C2α-modified 24,24-ethanovitamin D_3-26,23-lactones **6a–c** and **7a–c**

VDR antagonistic activity increased to 13–19 times that of **2** (4.5–6.7 times stronger than **6**). The antagonistic activity of (23*R*)-lactone analogue **7** was strongly affected by the substituents at position C2α (**7a–c**). That is, (23*R*)-24,24-ethanovitamin D_3 lactone having the 2α-methyl group (**7a**) showed 2.4-times higher VDR antagonistic activity than TEI-9648 (**3**). On the other hand, the antagonistic activity of the vitamin D_3 lactone derivatives having the longer side-chain at the C2α position (**7b** and **7c**) decreased to about half that of **8**.

Table 3 Biological Activities of 2α-Modified 24,24-Ethanovitamin D_3 Lactones **6a–c** and **7a–c**

Compounds	Binding affinity for VDR[a]	Antagonistic activity (IC_{50}, nM)[b]
TEI-9647 (**2**)	12	9.4
6a	111	0.49
6b	83	0.74
6c	59	0.55
TEI-9648 (**3**)	7	134.4
7a	15	55
7b	29	240
7c	13	220

[a] Chick intestinal VDR. The potency of **1** is normalized to 100.
[b] Antagonistic activity was assessed in terms of IC_{50} for the differentiation of HL-60 cells induced by 10 nM of **1**.

It is generally accepted that the first step in VDR-mediated transactivation is a ligand-binding process to the ligand-binding domain (LBD) of the apo form of VDR. Next, the ligand-VDR complex changes conformation into a transcriptionally active holo form, which binds to the co-activators to activate the target gene.[25] During conformational change, helix 12, which is the most C-terminal α-helix of VDR and has the site for interaction with other proteins such as co-activators, is important and controls whether the function of a ligand is agonism or antagonism.[26] When the VDR antagonist TEI-9647 (**2**) binds to the LBD of a VDR, the complex would change into an un-

usual transcriptionally inactive form.[27] We speculate that some amino acid residues in the LBD participate in the conformational change of the VDR through the interaction of the *exo*-methylene moiety on the lactone ring of **2**. Namely, there are two cysteine residues, Cys403 on helix 11 and Cys410 in the hinge region between helix 11 and helix 12 in the LBD of the hVDR. Recently, it was revealed that the two cysteines, Cys403 and Cys410, play an important role in the VDR antagonism of TEI-9647 (**2**).[28,29] Furthermore, the *exo*-methylene lactone structure is dispensable for the antagonistic action of the vitamin D_3 lactones.[30] Based on the results, we consider that the nucleophilic thiol groups of the cysteines could attack the α-methylene-γ-lactone of **2** via 1,4-addition to give the corresponding cysteine adduct.[31] Such interaction between the ligand and the LBD might prevent the positioning of helix 12. As a result, the complex of VDR and antagonist **2** could not adopt the transcriptionally active conformation. Therefore, it is thought that the VDR antagonists whose *exo*-methylene moiety is located at a more favorable position to interact with Cys403 and/or Cys410 show stronger VDR antagonistic activity.

The novel synthesized vitamin D_3 lactones **6**, **6a–c** and **7a–c**, which showed more potent antagonistic activity, might be situated in a preferable position to interact with the cysteine residues after the binding to the LBD of the VDR. On the other hand, the *exo*-methylene moiety of the weaker VDR antagonist (23*R*)-ethanovitamin D_3 lactone (**7**) is possibly located in an unfavorable position for interaction with the cysteines. Further investigation of the mechanism of antagonistic action is currently in progress.

We have succeeded in the development of novel potent vitamin D receptor antagonists, 24,24-ethano-1α-hydroxyvitamin D_3-26,23-lactones **6** and **7** and their C2α-functionalized analogues **6a–c** and **7a–c**. The VDR antagonists are expected to be potent therapeutic agents for some diseases caused by the hypersensitivity of the VDR to 1α,25-dihydroxyvitamin D_3 such as Paget's disease of bone.[32] We expect these analogues with potent anti-vitamin D activity to contribute to our understanding of the mechanisms involved in the expression of antagonistic activity toward VDR as well as to finding new medicines for treating Paget's disease of bone.

All manipulations were performed under an argon atmosphere unless otherwise mentioned. All solvents and reagents were purified when necessary using standard procedures. Ethylene gas was used without purification. Column chromatography was performed on silica gel 60 N (Kanto Chemical Co., Inc., 100–210 μm), and flash column chromatography was performed on silica gel 60 (Merck, 40–63 μm). NMR spectra were measured on a JEOL AL-400 magnetic resonance spectrometer. IR spectra were recorded on a JASCO FTIR-8000 spectrometer. MS were measured on a JEOL JMX-SX 102 mass spectrometer. Specific optical rotations were measured on JASCO DIP-370 digital polarimeter.

(2*S*,4*S*)-4-Benzoyloxy-5-hexene-1,2-diol (10)

To a solution of **9** (1.5 g, 4.4 mmol) in 1-propanol–H_2O (9:1, 44 mL) were added activated Zn dust (13 g, 199 mmol) and $NaBH_3CN$ (1.8 g, 29 mmol) at 95 °C, and the mixture was stirred at the same temperature for 40 min. After the mixture was filtered through a Celite pad, the filtrate was concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 1:1) to give **10**.

Yield: 785 mg (75%); colorless oil; $[\alpha]_D^{23}$ +10.9 (*c* 1.54, $CHCl_3$).

IR (neat): 3381, 1716, 1604, 1275, 1026 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 1.75–1.90 (m, 2 H), 2.09 (dd, *J* = 7.3, 4.2 Hz, 1 H), 3.26 (d, *J* = 3.9 Hz, 1 H), 3.51 (ddd, *J* = 11.1, 6.9, 4.2 Hz, 1 H), 3.64 (ddd, *J* = 11.1, 7.3, 3.4 Hz, 1 H), 3.75 (m, 1 H), 5.24 (d, *J* = 10.4 Hz, 1 H), 5.79 (d, *J* = 17.3 Hz, 1 H), 5.78 (m, 1 H), 5.98 (ddd, *J* = 17.3, 10.4, 5.9 Hz, 1 H), 7.46 (dd, *J* = 7.8, 7.8 Hz, 2 H), 7.59 (dd, *J* = 7.8, 7.8 Hz, 1 H), 8.07 (d, *J* = 7.8 Hz, 2 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 38.1, 66.4, 68.1, 72.0, 116.3, 128.2 (2 C), 129.4 (2 C), 129.7, 133.0, 136.1, 166.3.

EI-LRMS: *m*/*z* = 236 (M^+), 219, 105, 77.

EI-HRMS: *m*/*z* calcd for $C_{13}H_{16}O_4$: 236.1049; found: 236.1054.

(3*S*)-3-Benzoyloxy-4-(*S*)-oxyranylbut-1-ene (11)

To a solution of **10** (560 mg, 2.4 mmol) in pyridine (2.4 mL) was added 2-mesitylenesulfonyl chloride (596 mg, 2.7 mmol) at 0 °C, and the mixture was stirred at r.t. for 16 h. To the mixture was added H_2O, and this was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was roughly purified by flash column chromatography (silica gel; hexane–AcOEt, 3:1) to give a crude sulfonate compound (827 mg). To a solution of the crude sulfonate (827 mg) in THF (20 mL) was added a solution of LiHMDS in THF (1.0 M, 3.0 mL, 3.0 mmol) at –78 °C, and the mixture was warmed to 0 °C over 1 h. To the mixture was added sat. aq NH_4Cl, and the aq layer was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 20:1) to give **11**

Yield: 342 mg (2 steps, 66%); colorless oil; $[\alpha]_D^{23}$ +14.3 (*c* 1.12, $CHCl_3$).

IR (neat): 1720, 1649, 1603, 1026 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 1.94–2.05 (m, 2 H), 2.52 (dd, *J* = 4.8, 2.8 Hz, 1 H), 2.77 (dd, *J* = 4.8, 4.8 Hz, 1 H), 3.07 (m, 1 H), 5.25 (ddd, *J* = 10.6, 1.2, 1.2 Hz, 1 H), 5.39 (ddd, *J* = 17.1, 1.2, 1.2 Hz, 1 H), 5.73 (ddt, *J* = 1.2, 6.1, 6.1 Hz, 1 H), 5.97 (ddd, *J* = 17.1, 10.6, 6.1 Hz, 1 H), 7.45 (dd, *J* = 7.1, 7.1 Hz, 2 H), 7.57 (dd, *J* = 7.1, 7.1 Hz, 1 H), 8.07 (d, *J* = 7.1 Hz, 2 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 37.9, 47.0, 48.9, 72.6, 116.9, 128.2 (2 C), 129.4 (2 C), 130.0, 132.9, 135.6, 165.3.

EI-LRMS: *m*/*z* = 218 (M^+), 193, 105, 77.

EI-HRMS: *m*/*z* calcd for $C_{13}H_{14}O_3$: 218.0943; found: 218.0948.

(3*S*,5*R*)-Bis(*tert*-butyldimethylsilyloxy)oct-1-en-7-yne (12)

To a solution of TMS acetylene (0.59 mL, 4.2 mmol) in THF (1.5 mL) was added a solution of BuLi in hexane (1.6 M, 2.2 mL, 3.5 mmol) at –78 °C, and the mixture was stirred at the same temperature for 30 min. To the mixture were added a solution of **11** (335 mg, 1.5 mmol) and $BF_3{\cdot}OEt_2$ (0.23 mL, 1.8 mmol) at –78 °C, and the mixture was warmed to r.t. over 2 h. To the mixture was added sat. aq NH_4Cl, and the aqueous layer was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was dissolved in MeOH (5.1 mL). To the solution was added K_2CO_3 (635 mg, 4.6 mmol) at 0 °C, and the mixture was stirred at r.t. for 1 h. The mixture was partitioned between H_2O and Et_2O, and this was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 3:1–2:1) to give (3*S*,5*R*)-oct-1-en-7-yne-3,5-diol.

Yield: 208 mg (97%); colorless oil; $[\alpha]_D^{23}$ –0.003 (*c* 1.16, $CHCl_3$).

IR (neat): 3374, 3301, 2120, 1645, 1219, 1072 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 1.74 (ddd, *J* = 14.5, 7.5, 2.9 Hz, 1 H), 1.83 (ddd, *J* = 14.5, 8.8, 3.3 Hz, 1 H), 2.05 (t, *J* = 2.5 Hz, 1 H), 2.35–2.50 (m, 3 H), 2.80 (br d, *J* = 3.2 Hz, 1 H), 4.11 (m, 1 H), 4.49 (m, 1 H), 5.16 (ddd, *J* = 10.4, 1.5, 1.5 Hz, 1 H), 5.29 (ddd, *J* = 17.0, 1.5, 1.5 Hz, 1 H), 5.92 (ddd, *J* = 17.0, 10.4, 5.8 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 27.2, 41.2, 67.0, 69.8, 70.7, 80.7, 114.4, 140.2.

EI-LRMS: *m*/*z* = 122 $[(M - H_2O)]^+$, 101, 83, 66.

EI-HRMS: *m*/*z* calcd for $C_8H_{10}O$ (M – H_2O): 122.0732; found: 122.0739.

To a solution of the above diol (416 mg, 3.0 mmol) in CH_2Cl_2 (15 mL) were added TBSOTf (1.7 mL, 7.4 mmol) and 2,6-lutidine (1.0 mL, 8.6 mmol) at 0 °C, and the mixture was stirred at the same temperature for 1 h. To the mixture was added sat. aq NH_4Cl, and the aq layer was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (silica gel; hexane–EtOAc, 50:1) to give **12**, whose spectral data were identical with those reported previously by us.[17]

Yield:1.1 g (96%); colorless oil.

(*R*)-6-[(1*R*,4*E*,3a*R*,7a*R*)-4-Bromomethylene-7a-methyloctahydroinden-1-yl)]-1-(*tert*-butyldimethylsilyloxy)-hept-2-yn-4-one (15)

To a solution of **14**[33] (692 mg, 4.1 mmol) in THF (3.8 mL) was added a solution of BuLi in hexane (1.5 M, 2.7 mL, 4.1 mmol) at –78 °C, and the mixture was stirred at the same temperature for 1 h. To the mixture was added a solution of **13** (607 mg, 2.0 mmol) in THF (3 mL) at –78 °C, and the mixture was stirred at the same temperature for 1 h. To the mixture was added sat. aq NH_4Cl at –78 °C, and the resulting mixture was warmed to r.t. The mixture was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was dissolved in CH_2Cl_2 (6.8 mL). To the solution was added tetrapropylammonium perruthenate (214 mg, 0.61 mmol) and *N*-methylmorpholine *N*-oxide (357 mg, 3.0 mmol) at 0 °C, and the mixture was stirred at r.t. for 30 min. After the mixture was diluted with Et_2O and filtered through a short column (silica gel), the filtrate was concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 50:1) to give **15**.

Yield: 733 mg (2 steps, 77%); colorless oil; $[\alpha]_D^{27}$ +55.8 (*c* 1.49, $CHCl_3$).

IR (neat): 2212, 1676, 1632, 1254, 1103 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.13 (s, 6 H), 0.61 (s, 3 H), 0.91 (s, 9 H), 0.99 (d, *J* = 6.3 Hz, 3 H), 1.25–1.38 (m, 3 H), 1.40–1.72 (m, 5 H), 1.89 (m, 1 H), 1.95–2.03 (m, 2 H), 2.10 (m, 1 H), 2.29 (dd, *J* = 15.4, 10.1 Hz, 1 H), 2.64 (dd, *J* = 15.4, 3.4 Hz, 1 H), 2.88 (m, 1 H), 4.46 (s, 2 H), 5.65 (m, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = –5.2 (2 C), 11.9, 18.2, 19.7, 22.0, 22.4, 25.7 (3 C), 27.7, 30.9, 33.3, 39.6, 45.5, 51.5, 52.3, 55.5, 55.7, 84.1, 90.1, 97.6, 144.5, 187.2.

EI-LRMS: *m*/*z* = 466 (M^+), 409, 255, 175, 147.

EI-HRMS: *m*/*z* calcd for $C_{24}H_{39}O_2{}^{79}BrSi$: 466.1902; found: 466.1889.

(*R*)-6-[(1*R*,4*E*,3a*R*,7a*R*)-4-Bromomethylene-7a-methyloctahydroinden-1-yl]-2-[(*tert*-butyldimethylsilyloxy)methyl]-3-methylenehept-1-en-4-one (17) (Table 1, Run 6)

To a solution of **15** (98 mg, 0.21 mmol) in CH_2Cl_2 (2.1 mL) was added Ru catalyst **16d** (15 mg, 0.021 mmol) at 0 °C, and the mixture was stirred under ethylene (1 atm) at the same temperature for 1.5 h. After the mixture was concentrated, the residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 50:1) to give **17**.

Yield: 96 mg (92%); colorless oil; $[\alpha]_D^{28}$ +64.3 (*c* 1.21, $CHCl_3$).

IR (neat): 1686, 1632, 1095 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.061 (s, 6 H), 0.60 (s, 3 H), 0.90 (s, 9 H), 0.94 (d, *J* = 6.6 Hz, 3 H), 1.23–1.39 (m, 3 H), 1.41–1.73 (m, 5 H), 1.80–2.13 (m, 4 H), 2.44 (dd, *J* = 16.1, 10.0 Hz, 1 H), 2.70 (dd, *J* = 16.1, 2.9 Hz, 1 H), 2.88 (m, 1 H), 4.26 (dd, *J* = 1.4, 1.4 Hz, 2 H), 5.11 (d, *J* = 1.4 Hz, 1 H), 5.33 (d, *J* = 1.4 Hz, 1 H), 5.65 (s, 1 H), 5.68 (s, 1 H), 5.76 (s, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = –5.3 (2 C), 11.9, 18.3, 19.9, 22.0, 22.5, 25.9 (3 C), 27.7, 31.0, 33.1, 39.8, 45.6, 46.4, 55.76, 55.84, 65.5, 97.5, 114.2, 122.2, 144.6, 145.4, 149.2, 202.4.

EI-LRMS: *m*/*z* = 494 (M^+), 479, 437, 415, 345, 253, 211, 183.

EI-HRMS: *m*/*z* calcd for $C_{26}H_{43}O_2{}^{79}BrSi$: 494.2216; found: 494.2208.

(*R*)-6-[(1*R*,4*E*,3a*R*,7a*R*)-4-Bromomethylene-7a-methyloctahydroinden-1-yl)]-2-[(*tert*-butyldimethylsilyloxy)methyl]-3,3-ethanohept-1-en-4-one (18)

Trimethylsulfoxonium iodide (49 mg, 0.22 mmol) and NaH (60% in mineral oil, 10.5 mg, 0.26 mmol) were weighed in a dry flask. DMSO (1 mL) was poured into the flask at r.t. and the resulting heterogeneous mixture was stirred at the same temperature for 20 min. To the mixture was added a solution of **17** (104 mg, 0.20 mmol) in THF–DMSO (7:1; 2.3 mL) at 10 °C, and the resulting mixture was stirred at r.t. for 30 min. To the mixture was added sat. aq NH_4Cl at 10 °C and the mixture was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 100:1) to give **18**.

Yield: 86 mg (85%); colorless oil; $[\alpha]_D^{28}$ +70.1 (*c* 1.15, $CHCl_3$).

IR (neat): 1695, 1651, 1632, 1103 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.066 (s, 6 H), 0.58 (s, 3 H), 0.85–0.93 (m, 2 H), 0.87 (d, *J* = 6.4 Hz, 3 H), 0.91 (s, 9 H), 1.20–1.33 (m, 5 H), 1.38–1.70 (m, 5 H), 1.82 (m, 1 H), 1.90–2.05 (m, 3 H), 2.35 (dd, *J* = 16.5, 10.0 Hz, 1 H), 2.51 (dd, *J* = 16.5, 3.1 Hz, 1 H), 2.86 (m, 1 H), 4.13 (dd, *J* = 1.6, 1.6 Hz, 2 H), 5.13 (d, *J* = 1.6 Hz, 1 H), 5.41 (d, *J* = 1.6 Hz, 1 H), 5.63 (br s, 1 H)

^{13}C NMR (100 MHz, $CDCl_3$): δ = –5.40, –5.38, 11.9, 16.0, 16.2, 18.4, 19.9, 22.0, 22.5, 25.9 (3 C), 27.5, 31.0, 32.7, 35.6, 39.8, 45.5, 46.4, 55.7, 55.9, 65.3, 97.5, 114.5, 144.7, 147.5, 209.1.

EI-LRMS: *m*/*z* = 508 (M^+), 451, 377, 297, 227.

EI-HRMS: *m*/*z* calcd for $C_{27}H_{45}O_2{}^{79}BrSi$: 508.2372; found: 508.2366.

(4*S*,6*R*)-6-[(1*R*,4*E*,3a*R*,7a*R*)-4-Bromomethylene-7a-methyloctahydroinden-1-yl]-3,3-ethano-2-methyleneheptane-1,4-diol (19)

To a solution of **18** (86 mg, 0.17 mmol) in toluene (1.7 mL) was added a solution of DIBAL-H in toluene (1.0 M, 0.25 mL, 0.25 mmol) at –78 °C, and the mixture was stirred at the same temperature for 1 h. To the mixture were added a few drops of MeOH and sat. aq potassium sodium tartrate at –78 °C, and the mixture was stirred at r.t. for 30 min. The aq layer was extracted with Et_2O, and the organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 30:1) to give 23*S*-alcohol (39 mg, 45%) and 23*R*-alcohol (48 mg, 55%), respectively.

23*S*-Alcohol

$[\alpha]_D^{27}$ +29.4 (*c* 0.83, $CHCl_3$).

IR (neat): 3424, 1641, 1255, 1042 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.11 (s, 6 H), 0.41 (ddd, *J* = 9.3, 5.4, 4.1 Hz, 1 H), 0.56 (m, 1 H), 0.57 (s, 3 H), 0.69 (ddd, *J* = 9.3, 5.4, 4.1 Hz, 1 H), 0.78 (ddd, *J* = 9.3, 5.4, 4.1 Hz, 1 H), 0.93 (s, 9 H), 0.97 (d, *J* = 6.6 Hz, 3 H), 1.18–2.05 (m, 15 H), 2.82–2.93 (m, 2 H), 4.13 (d, *J* = 12.3 Hz, 1 H), 4.23 (d, *J* = 12.3 Hz, 1 H), 5.01 (d, *J* = 1.9 Hz, 1 H), 5.19 (s, 1 H), 5.63 (s, 1 H)

^{13}C NMR (100 MHz, $CDCl_3$): δ = –5.2 (2 C), 10.4, 11.5, 11.9, 18.4, 19.8, 22.0, 22.6, 25.9 (3 C), 27.5, 30.3, 31.1, 33.8, 39.9, 42.5, 45.5, 55.8, 56.7, 67.0, 76.9, 97.3, 116.7, 145.0, 147.8.

EI-LRMS: *m*/*z* = 510 (M^+), 492, 453, 435, 361, 281, 241, 227, 147.

EI-HRMS: *m*/*z* calcd for $C_{27}H_{47}O_2{}^{79}BrSi$: 510.2528; found: 510.2528.

23*R*-Alcohol

$[\alpha]_D^{25}$ +113.7 (*c* 1.04, $CHCl_3$).

IR (neat): 3382, 1634, 1256, 1026 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.11 (s, 6 H), 0.43–0.53 (m, 2 H), 0.57 (s, 3 H), 0.64–0.78 (m, 2 H), 0.91 (d, *J* = 6.6 Hz, 3 H), 0.92 (s, 9 H), 1.04 (ddd, *J* = 13.8, 10.9, 1.7 Hz, 1 H), 1.18–1.72 (m, 11 H), 1.91 (m, 1 H), 1.96 (ddd, *J* = 12.5, 7.1, 1.5 Hz, 1 H), 2.01 (ddd, *J* = 12.5, 2.3, 2.3 Hz, 1 H), 2.80–2.92 (m, 2 H), 4.11 (d, *J* = 12.5 Hz, 1 H), 4.21 (d, *J* = 12.5 Hz, 1 H), 4.99 (d, *J* = 1.7 Hz, 1 H), 5.18 (s, 1 H), 5.63 (s, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = –5.2 (2 C), 11.3, 11.9, 18.4, 18.7, 22.0, 22.6, 25.7, 26.0 (3 C), 27.8, 31.0, 31.1, 32.5, 39.9, 41.8, 45.6, 56.0, 56.5, 66.8, 74.8, 97.3, 116.7, 145.0, 147.9.

EI-LRMS: *m*/*z* = 492 ($M - H_2O)^+$, 453, 435, 361, 281, 241, 227, 147.

EI-HRMS: *m*/*z* calcd for $C_{27}H_{45}O^{79}BrSi$ ($M - H_2O$): 492.2423; found: 492.2423.

To the 23*S*-alcohol (197 mg, 0.39 mmol) in THF (3.9 mL) was added a solution of TBAF in THF (1.0 M, 0.77 mL, 0.77 mmol) at 0 °C, and the mixture was stirred at r.t. for 1.5 h. To the mixture was added sat. aq NH_4Cl at 0 °C, and the mixture was extracted with Et_2O. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4) and concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 3:1) to give **19**.

Yield: 146 mg (96%); colorless solid; mp 167 °C (recrystallized from THF); $[\alpha]_D^{24}$ +50.4 (*c* 0.80, THF).

IR (KBr): 3148, 1637, 1256, 1022 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.47 (ddd, *J* = 9.3, 5.2, 4.0 Hz, 1 H), 0.58 (ddd, *J* = 9.3, 5.2, 4.0 Hz, 1 H), 0.59 (s, 3 H), 0.66 (ddd, *J* = 9.3, 5.2, 4.0 Hz, 1 H), 0.85 (ddd, *J* = 9.3, 5.2, 4.0 Hz, 1 H), 0.98 (d, *J* = 6.6 Hz, 3 H), 1.20–1.70 (m, 11 H), 1.80 (ddd, *J* = 13.9, 7.1, 2.9 Hz, 1 H), 1.85–2.04 (m, 3 H), 2.88 (m, 1 H), 2.94 (br s, 1 H), 2.57 (t, *J* = 6.6 Hz, 1 H), 4.13 (s, 2 H), 5.01 (d, *J* = 1.7 Hz, 1 H), 5.22 (s, 1 H), 5.64 (s, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 10.6, 11.4, 11.9, 19.8, 22.1, 22.6, 27.6, 30.4, 31.1, 33.9, 39.9, 41.9, 45.6, 55.8, 56.6, 66.7, 77.8, 97.4, 117.5, 145.0, 148.1.

EI-LRMS: *m*/*z* = 396 (M^+), 378, 299, 254, 225, 175, 127.

EI-HRMS: *m*/*z* calcd for $C_{21}H_{33}O_2{}^{79}Br$: 396.1664; found: 396.1679.

Anal. Calcd for $C_{21}H_{33}O_2Br$: C, 63.47; H, 8.37. Found: C, 63.55; H, 8.67.

(4*R*,6*R*)-6-[(1*R*,4*E*,3a*R*,7a*R*)-4-Bromomethylene-7a-methyloctahydroinden-1-yl]-3,3-ethano-2-methyleneheptane-1,4-diol (20)

Similar to the synthesis of **19** from the 23*S*-alcohol, the crude product, which was obtained from the above 23*R*-alcohol (269 mg, 0.53 mmol) and TBAF (1.0 M THF solution, 1.1 mL, 1.1 mmol) in THF (5.3 mL) at r.t. for 15 min, was purified by flash column chromatography (silica gel; hexane–EtOAc, 4:1) to give **20**.

Yield: 195 mg (93%); colorless solid; mp 167–170 °C (recrystallized from THF–hexane); $[\alpha]_D^{25}$ +142.9 (*c* 1.19, THF).

IR (KBr): 3318, 1634, 1219, 1014 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.45–0.55 (m, 2 H), 0.55 (s, 3 H), 0.65–0.78 (m, 2 H), 0.92 (d, *J* = 6.6 Hz, 3 H), 1.09 (ddd, *J* = 14.1, 10.8, 1.5 Hz, 1 H), 1.20–1.75 (m, 10 H), 1.85–2.06 (m, 3 H), 2.83–2.95 (m, 2 H), 3.13 (br s, 2 H), 4.08 (d, *J* = 12.2 Hz, 1 H), 4.12 (d, *J* = 12.2 Hz, 1 H), 5.00 (d, *J* = 1.7 Hz, 1 H), 5.20 (s, 1 H), 5.64 (s, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.4, 11.5, 12.0, 18.8, 22.1, 22.6, 27.9, 31.1, 31.2, 32.6, 39.9, 41.2, 45.6, 56.0, 56.3, 66.5, 75.8, 97.4, 117.7, 144.9, 148.2.

EI-LRMS: *m*/*z* = 396 (M^+), 378, 299, 254, 225, 175, 127.

EI-HRMS: *m*/*z* calcd for $C_{21}H_{33}O_2{}^{79}Br$: 396.1663; found: 396.1656.

Anal. Calcd for $C_{21}H_{33}O_2Br$: C, 63.47; H, 8.37. Found: C, 63.73; H, 8.74.

(*S*)-4-{(*R*)-2-[(1*R*,4*E*,3a*R*,7a*R*)-4-Bromomethylene-7a-methyloctahydroinden-1-yl]propyl}-7-methylene-5-oxaspiro[2.4]heptan-6-one (21)

To a solution of **19** (61 mg, 0.15 mmol) in CH_2Cl_2 (3.1 mL) was added MnO_2 (346 mg, 4.0 mmol) at r.t., and the mixture was stirred at the same temperature for 30 h. After the mixture was filtered through a short column (silica gel; Et_2O), the filtrate was concentrated. The residue was purified by flash column chromatography (silica gel; hexane–EtOAc, 30:1) to give **21** (59 mg, 97%) as an amorphous solid.

$[\alpha]_D^{27}$ +27.5 (*c* 0.88, $CHCl_3$).

IR (film, $CHCl_3$): 1759, 1660, 1342, 1118 cm^{-1}.

^1H NMR (400 MHz, $CDCl_3$): δ = 0.56 (s, 3 H), 0.85 (ddd, *J* = 9.8, 7.2, 4.9 Hz, 1 H), 0.95 (ddd, *J* = 10.0, 7.2, 4.9 Hz, 1 H), 1.01 (ddd, *J* = 9.8, 6.5, 4.9 Hz, 1 H), 1.06 (d, *J* = 6.6 Hz, 3 H), 1.21 (ddd, *J* = 10.0, 6.5, 4.9 Hz, 1 H), 1.25–1.73 (m, 11 H), 1.88–2.05 (m, 3 H), 2.88 (m, 1 H), 4.49 (dd, *J* = 8.7, 4.0 Hz, 1 H), 5.02 (s, 1 H), 5.64 (s, 1 H), 5.93 (s, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.8, 14.8, 16.5, 19.9, 22.1, 22.5, 27.0, 27.8, 31.0, 34.5, 39.4, 39.7, 45.6, 55.6, 55.8, 81.4, 97.5, 113.5, 140.9, 144.7, 169.7.

EI-LRMS: *m*/*z* = 392 (M^+), 377, 313, 255, 227, 147.

EI-HRMS: *m*/*z* calcd for $C_{21}H_{29}O_2{}^{79}BrSi$: 392.1351; found: 392.1368.

(*R*)-4-{(*R*)-2-[(1*R*,4*E*,3a*R*,7a*R*)-4-Bromomethylene-7a-methyloctahydroinden-1-yl]propyl}-7-methylene-5-oxaspiro[2.4]heptan-6-one (22)

Similar to the synthesis of **21** from **19**, a crude product, which was obtained from **20** (42 mg, 0.10 mmol) and MnO_2 (272 mg, 3.1 mmol) in CH_2Cl_2 (3.1 mL) at r.t. for 45 h, was purified by flash column chromatography (silica gel; hexane–EtOAc, 20:1) to give **22**.

Yield: 38 mg (92%); amorphous solid; $[\alpha]_D^{26}$ +192.5 (*c* 0.68, $CHCl_3$).

IR (film, $CHCl_3$): 1765, 1657, 1630, 1379, 1117 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.59 (s, 3 H), 0.87 (m, 1 H), 0.91–1.06 (m, 3 H), 0.98 (d, *J* = 6.6 Hz, 3 H), 1.15 (ddd, *J* = 10.1, 6.4, 4.8 Hz, 1 H), 1.20–1.38 (m, 3 H), 1.40–1.73 (m, 6 H), 1.74–1.92 (m, 2 H), 1.97 (ddd, *J* = 12.8, 6.8, 1.6 Hz, 1 H), 2.01 (ddd, *J* = 12.8, 2.7, 2.7 Hz, 1 H), 2.88 (m, 1 H), 4.50 (dd, *J* = 11.7, 2.0 Hz, 1 H), 5.02 (s, 1 H), 5.65 (br s, 1 H), 5.94 (s, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.9, 14.8, 16.9, 18.4, 22.0, 22.5, 26.1, 27.6, 31.0, 32.5, 39.8, 39.9, 45.6, 55.8, 56.2, 79.4, 97.5, 113.6, 141.0, 144.6, 169.6.

EI-LRMS: *m*/*z* = 392 (M^+), 377, 313, 255, 227, 147.

EI-HRMS: *m*/*z* calcd for $C_{21}H_{29}O_2{}^{79}BrSi$: 392.1351; found: 392.1341.

Vitamin D_3 Lactones; General Procedure

To a solution of an A-ring precursor (1.5 equiv to a CD-ring precursor), and the CD-ring precursor in toluene were added Et_3N and $Pd(PPh_3)_4$ (30 mol% to the CD-ring precursor) and the mixture was stirred at 110 °C for 1–1.5 h. After the mixture was filtered through a silica gel pad, the filtrate was concentrated. The crude product was dissolved in MeCN (2 mL). To the solution was added a solution of concd HF (10%) in MeCN (2 mL) at 0 °C, and the mixture was stirred at r.t. Sat. aq $NaHCO_3$ was added, and the mixture was extracted with EtOAc. The organic layer was washed with sat. aq NaCl, dried (Na_2SO_4), and concentrated. The residue was purified by TLC (silica gel) to give the vitamin D_3 derivative. Further purification for biological assays was conducted by reversed-phase recycle HPLC (YMC-Pack ODS column, 20 × 150 mm, 9.9 mL/min, eluent: MeCN–H_2O, 90:10).

(23*S*)-25-Dehydro-24,24-ethano-1α-hydroxyvitamin D_3-26,23-Lactone (6)

According to the General Procedure, a crude product, which was obtained from **21** (25 mg, 64 μmol), **12** (35 mg, 95 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (22 mg, 19 μmol) in toluene (1.5 mL) at 110 °C for 1.5 h, was treated with concd HF in MeCN for 1 h. After the usual work-up, the crude product was purified by preparative TLC (silica gel; hexane–EtOAc, 2:3) to give **6**.

Yield: 19 mg (2 steps, 62%); amorphous solid; $[\alpha]_D{}^{27}$ –17.0 (*c* 1.18, $CHCl_3$).

IR (film, $CHCl_3$): 3387, 1759, 1657, 1611, 1342, 1055 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.55 (s, 3 H), 0.83 (ddd, *J* = 9.9, 7.1, 5.1 Hz, 1 H), 0.95 (ddd, *J* = 9.9, 7.1, 4.7 Hz, 1 H), 1.01 (ddd, *J* = 9.9, 6.5, 4.7 Hz, 1 H), 1.05 (d, *J* = 6.6 Hz, 3 H), 1.16–1.75 (m, 14 H), 1.85–2.08 (m, 5 H), 2.31 (dd, *J* = 13.5, 6.5 Hz, 1 H), 2.59 (dd, *J* = 13.5, 3.4 Hz, 1 H), 2.82 (m, 1 H), 4.90 (m, 1 H), 4.43 (m, 1 H), 4.49 (dd, *J* = 8.8, 3.9 Hz, 1 H), 4.99 (s, 1 H), 5.01 (s, 1 H), 5.33 (s, 1 H), 5.93 (s, 1 H), 6.01 (d, *J* = 11.2 Hz, 1 H), 6.37 (d, *J* = 11.2 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.0, 14.8, 16.5, 20.0, 22.3, 23.6, 27.1, 27.8, 29.1, 34.6, 39.5, 40.4, 42.9, 45.3, 46.0, 56.1, 56.7, 66.8, 70.8, 81.6, 111.7, 113.5, 117.1, 124.8, 133.0, 141.0, 142.7, 147.6, 170.0.

EI-LRMS: *m*/*z* = 452 (M^+), 434, 416, 311, 285, 134, 105.

EI-HRMS: *m*/*z* calcd for $C_{29}H_{40}O_4$: 452.2926; found: 452.2925.

(23*R*)-25-Dehydro-24,24-ethano-1α-hydroxyvitamin D_3-26,23-Lactone (7)

According to the General Procedure, a crude product, which was obtained from **22** (28 mg, 71 μmol), **12** (39 mg, 107 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (25 mg, 21 μmol) in toluene (1.5 mL) at 110 °C for 1.5 h, was treated with concd HF in MeCN for 1 h. After the usual work up, the crude product was purified by preparative TLC (silica gel; hexane–EtOAc, 2:3) to give **7**.

Yield: 25 mg (2 steps, 76%); amorphous solid; $[\alpha]_D{}^{27}$ +92.0 (*c* 1.76, $CHCl_3$).

IR (film, $CHCl_3$): 3428, 1755, 1657, 1607, 1342, 1215, 1051 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.57 (s, 3 H), 0.82–1.05 (m, 4 H), 0.97 (d, *J* = 6.6 Hz, 3 H), 1.15 (ddd, *J* = 10.1, 6.4, 4.9 Hz, 1 H), 1.20–2.08 (m, 17 H), 2.31 (dd, *J* = 13.4, 6.6 Hz, 1 H), 2.60 (dd, *J* = 13.4, 3.2 Hz, 1 H), 2.82 (m, 1 H), 4.23 (br s, 1 H), 4.42 (br s, 1 H), 4.51 (dd, *J* = 11.6, 1.8 Hz, 1 H), 5.00 (s, 1 H), 5.02 (s, 1 H), 5.33 (s, 1 H), 5.94 (s, 1 H), 6.01 (d, *J* = 11.2 Hz, 1 H), 6.46 (d, *J* = 11.2 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.0, 14.8, 16.8, 18.4, 22.2, 23.5, 26.4, 27.6, 29.0, 32.5, 39.8, 40.5, 42.8, 45.2, 46.0, 56.3, 57.0, 66.8, 70.8, 79.6, 111.8, 113.7, 117.2, 124.8, 133.1, 141.2, 142.6, 147.6, 169.9.

EI-LRMS: *m*/*z* = 452 (M^+), 434, 416, 311, 285, 134, 105.

EI-HRMS: *m*/*z* calcd for $C_{29}H_{40}O_4$: 452.2927; found: 452.2930.

(23*S*)-25-Dehydro-24,24-ethano-2α-methyl-1α-hydroxyvitamin D_3-26,23-Lactone (6a)

According to the General Procedure, a crude product, which was obtained from **21** (25 mg, 62 μmol), **12a** (36 mg, 94 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (22 mg, 19 μmol) in toluene (1.5 mL) at 110 °C for 1.5 h, was treated with concd HF in MeCN for 2.5 h. After the usual work up, the crude product was purified by preparative TLC (silica gel; hexane–EtOAc, 1:1) to give **6a**.

Yield: 17 mg (2 steps, 60%); amorphous solid; $[\alpha]_D{}^{24}$ +0.30 (*c* 1.34, $CHCl_3$).

IR (film, $CHCl_3$): 3422, 1757, 1651, 1628, 1608, 1342, 1198, 1028 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.54 (s, 3 H), 0.84 (ddd, *J* = 9.9, 7.1, 5.0 Hz, 1 H), 0.91–1.05 (m, 2 H), 1.05 (d, *J* = 6.6 Hz, 3 H), 1.08 (d, *J* = 6.8 Hz, 3 H), 1.15–1.75 (m, 14 H), 1.85–2.05 (m, 4 H), 2.23 (dd, *J* = 13.6, 7.7 Hz, 1 H), 2.67 (dd, *J* = 13.6, 4.2 Hz, 1 H), 2.82 (m, 1 H), 3.84 (ddd, *J* = 7.7, 7.7, 4.2 Hz, 1 H), 4.31 (br d, *J* = 2.4 Hz, 1 H), 4.49 (dd, *J* = 8.7, 3.8 Hz, 1 H), 5.00 (br d, *J* = 2.0 Hz, 1 H), 5.01 (br s, 1 H), 5.28 (s, 1 H), 5.93 (s, 1 H), 6.00 (d, *J* = 11.2 Hz, 1 H), 6.38 (d, *J* = 11.2 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.0, 12.6, 14.8, 16.5, 20.0, 22.3, 23.5, 27.1, 27.8, 29.0, 34.6, 39.5, 40.4, 43.4, 44.2, 46.0, 56.1, 56.6, 71.6, 75.3, 81.9, 113.1, 113.4, 117.0, 124.6, 133.1, 141.0, 142.7, 146.4, 169.8.

EI-LRMS: *m*/*z* = 466 (M^+), 448, 430, 265, 166.

EI-HRMS: *m*/*z* calcd for $C_{30}H_{42}O_4$: 466.3083; found: 466.3087.

(23*R*)-25-Dehydro-24,24-ethano-2α-methyl-1α-hydroxyvitamin D_3-26,23-Lactone (7a)

According to the General Procedure, a crude product, which was obtained from **22** (21 mg, 52 μmol), **12a** (30 mg, 79 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (18 mg, 16 μmol) in toluene (1.5 mL) at 110 °C for 1 h, was treated with concd HF in MeCN for 2.5 h. After the usual work up, the crude product was purified by preparative TLC (silica gel; hexane–EtOAc, 1:1) to give **7a**.

Yield: 12 mg (2 steps, 49%); amorphous solid; $[\alpha]_D{}^{25}$ +118.9 (*c* 0.85, $CHCl_3$).

IR (film, $CHCl_3$): 3441, 1754, 1657, 1604, 1344, 1215, 1055 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.56 (s, 3 H), 0.83–1.05 (m, 4 H), 0.97 (d, *J* = 6.3 Hz, 3 H), 1.08 (d, *J* = 6.8 Hz, 3 H), 1.15 (ddd, *J* = 10.3, 6.4, 4.8 Hz, 1 H), 1.20–1.33 (m, 4 H), 1.40–2.05 (m, 12 H), 2.23 (dd, *J* = 13.5, 7.8 Hz, 1 H), 2.67 (dd, *J* = 13.5, 4.1 Hz, 1 H), 2.82 (m, 1 H), 3.85 (ddd, *J* = 7.8, 7.8, 4.1 Hz, 1 H), 4.31 (br d, *J* = 3.4 Hz, 1 H), 4.51 (dd, *J* = 11.5, 1.7 Hz, 1 H), 5.00 (br d, *J* = 2.0

Hz, 1 H), 5.02 (s, 1 H), 5.28 (s, 1 H), 5.94 (s, 1 H), 6.00 (d, *J* = 11.2 Hz, 1 H), 6.38 (d, *J* = 11.2 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.2, 12.6, 14.9, 16.9, 18.4, 22.3, 23.5, 26.5, 27.6, 29.1, 32.6, 39.8, 40.6, 43.5, 44.2, 46.0, 56.4, 57.0, 71.7, 75.3, 79.5, 113.1, 113.6, 117.1, 124.6, 133.2, 141.1, 142.6, 146.5, 169.7.

EI-LRMS: *m*/*z* = 466 (M^+), 448, 430, 265, 166.

EI-HRMS: *m*/*z* calcd for $C_{30}H_{42}O_4$: 466.3083; found: 466.3075.

(23*S*)-25-Dehydro-24,24-ethano-2α-(3-hydroxypropyl)-1α-hydroxyvitamin D_3-26,23-Lactone (6b)

According to the General Procedure, a crude product, which was obtained from **21** (29 mg, 74 μmol), **12b** (60 mg, 111 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (26 mg, 22 μmol) in toluene (1.5 mL) at 110 °C for 1.5 h, was treated with concd HF in MeCN for 2.5 h. After the usual work-up, the crude product was purified by preparative TLC (silica gel; EtOAc) to give **6b**.

Yield: 23 mg (2 steps 62%); amorphous solid; $[\alpha]_D^{24}$ +20.1 (*c* 1.78, $CHCl_3$).

IR (film, $CHCl_3$): 3351, 1759, 1655, 1343, 1198, 1055 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.53 (s, 3 H), 0.84 (ddd, *J* = 9.9, 7.0, 5.0 Hz, 1 H), 0.95 (ddd, *J* = 9.9, 7.0, 4.6 Hz, 1 H), 1.01 (ddd, *J* = 9.9, 6.4, 4.6 Hz, 1 H), 1.05 (d, *J* = 6.6 Hz, 3 H), 1.15–1.75 (m, 17 H), 1.85–2.05 (m, 3 H), 2.13 (br s 1 H), 2.24 (dd, *J* = 13.2, 8.3 Hz, 1 H), 2.60 (br s, 2 H), 2.65 (dd, *J* = 13.2, 4.3 Hz, 1 H), 2.82 (m, 1 H), 3.60–3.73 (m, 2 H), 3.87 (ddd, *J* = 8.3, 8.3, 4.3 Hz, 1 H), 4.36 (br d, *J* = 2.9 Hz, 1 H), 4.49 (dd, *J* = 8.7, 3.8 Hz, 1 H), 4.98 (d, *J* = 2.0 Hz, 1 H), 5.01 (s, 1 H), 5.26 (br s, 1 H), 5.93 (s, 1 H), 5.99 (d, *J* = 11.2 Hz, 1 H), 6.38 (d, *J* = 11.2 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.0, 14.8, 16.5, 20.0, 22.3, 22.8, 23.5, 27.1, 27.8, 29.0, 30.1, 34.6, 39.5, 40.4, 44.1, 46.0, 49.0, 56.1, 56.6, 62.6, 70.2, 73.5, 81.6, 113.4, 113.5, 117.1, 124.5, 133.0, 141.0, 142.7, 146.4, 169.8.

EI-LRMS: *m*/*z* = 510 (M^+), 492, 474, 415, 327, 309.

EI-HRMS: *m*/*z* calcd for $C_{32}H_{46}O_5$: 510.3345; found: 510.3342.

(23*R*)-25-Dehydro-24,24-ethano-2α-(3-hydroxypropyl)-1α-hydroxyvitamin D_3-26,23-Lactone (7b)

According to the General Procedure, a crude product, which was obtained from **22** (29 mg, 74 μmol), **12b** (60 mg, 111 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (26 mg, 22 μmol) in toluene (1.5 mL) at 110 °C for 1.5 h, was treated with concd HF in MeCN for 2.5 h. After the usual work-up, the crude product was purified by preparative TLC (silica gel; EtOAc) to give **7b**.

Yield: 20 mg (2 steps 54%); amorphous solid; $[\alpha]_D^{24}$ +123.4 (*c* 1.57, $CHCl_3$).

IR (film, $CHCl_3$): 3358, 1757, 1651, 1343, 1196, 1051 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.56 (s, 3 H), 0.82–1.05 (m, 4 H), 0.97 (d, *J* = 6.6 Hz, 3 H), 1.15 (m, 1 H), 1.20–2.05 (m, 18 H), 2.19 (br s, 1 H), 2.24 (dd, *J* = 13.2, 8.6 Hz, 1 H), 2.60 (br s, 2 H), 2.65 (dd, *J* = 13.2, 4.3 Hz, 1 H), 2.83 (m, 1 H), 3.60–3.75 (m, 2 H), 3.88 (ddd, *J* = 8.6, 8.6, 4.3 Hz, 1 H), 4.36 (d, *J* = 2.7 Hz, 1 H), 4.50 (dd, *J* = 11.5, 1.5 Hz, 1 H), 4.97 (d, *J* = 1.7 Hz, 1 H), 5.02 (s, 1 H), 5.27 (d, *J* = 1.7 Hz, 1 H), 5.94 (s, 1 H), 6.00 (d, *J* = 11.4 Hz, 1 H), 6.37 (d, *J* = 11.4 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.2, 14.9, 16.9, 18.4, 22.3, 22.8, 23.5, 26.4, 27.7, 29.0, 30.1, 32.6, 39.8, 40.6, 44.3, 46.0, 49.0, 56.3, 56.9, 62.6, 70.2, 73.5, 79.5, 113.5, 113.7, 117.2, 124.4, 133.1, 141.1, 142.4, 146.4, 169.8.

EI-LRMS: *m*/*z* = 510 (M^+), 492, 474, 415, 327, 309.

EI-HRMS: *m*/*z* calcd for $C_{32}H_{46}O_5$: 510.3345; found: 510.3344.

(23*S*)-25-Dehydro-24,24-ethano-2α-(3-hydroxypropoxyl)-1α-hydroxyvitamin D_3-26,23-Lactone (6c)

According to the General Procedure, a crude product, which was obtained from **21** (24 mg, 62 μmol), **12c** (52 mg, 93 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (21 mg, 19 μmol) in toluene (1.5 mL) at 110 °C for 1.5 h, was treated with concd HF in MeCN for 2.5 h. After the usual work-up, the crude product was purified by preparative TLC (silica gel; EtOAc) to give **6c**.

Yield: 21 mg (2 steps, 66%); amorphous solid; $[\alpha]_D^{23}$ +17.2 (*c* 1.65, $CHCl_3$).

IR (film, $CHCl_3$): 3395, 1759, 1659, 1342, 1059 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.54 (s, 3 H), 0.84 (ddd, *J* = 9.8, 7.1, 4.7 Hz, 1 H), 0.95 (ddd, *J* = 9.8, 6.7, 4.7 Hz, 1 H), 1.01 (ddd, *J* = 9.8, 6.7, 4.7 Hz, 1 H), 1.05 (d, *J* = 6.6 Hz, 3 H), 1.15–2.05 (m, 17 H), 2.24 (dd, *J* = 13.5, 8.7 Hz, 1 H), 2.67 (dd, *J* = 13.5, 4.5 Hz, 1 H), 2.74 (br s, 3 H), 2.82 (m, 1 H), 3.37 (dd, *J* = 7.6, 3.2 Hz, 1 H), 3.70–3.93 (m, 4 H), 4.05 (ddd, *J* = 8.7, 7.6, 4.5 Hz, 1 H), 4.44 (d, *J* = 3.2 Hz, 1 H), 4.49 (dd, *J* = 8.7, 3.8 Hz, 1 H), 5.01 (s, 1 H), 5.08 (d, *J* = 2.0 Hz, 1 H), 5.38 (d, *J* = 1.0 Hz, 1 H), 5.93 (s, 1 H), 6.00 (d, *J* = 11.1 Hz, 1 H), 6.40 (d, *J* = 11.1 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.0, 14.8, 16.5, 20.0, 22.3, 23.5, 27.1, 27.8, 29.1, 31.9, 34.6, 39.4, 40.3, 41.0, 46.0, 56.1, 56.6, 61.1, 68.3, 68.5, 71.9, 81.6, 84.5, 113.4, 116.1, 117.2, 125.3, 131.7, 141.0, 143.0, 144.1, 169.8.

EI-LRMS: *m*/*z* = 526 (M^+), 508, 490, 464, 432, 265, 171.

EI-HRMS: *m*/*z* calcd for $C_{32}H_{46}O_6$: 526.3295; found: 526.3297.

(23*R*)-25-Dehydro-24,24-ethano-2α-(3-hydroxypropoxyl)-1α-hydroxyvitamin D_3-26,23-Lactone (7c)

According to the General Procedure, a crude product, which was obtained from **22** (30 mg, 77 μmol), **12c** (64 mg, 115 μmol), Et_3N (1.5 mL) and $Pd(PPh_3)_4$ (27 mg, 23 μmol) in toluene (1.5 mL) at 110 °C for 1.5 h, was treated with concd HF in MeCN for 2.5 h. After the usual work-up, the crude product was purified by preparative TLC (silica gel; EtOAc) to give **7c**.

Yield: 22 mg (2 steps, 56%); amorphous solid; $[\alpha]_D^{23}$ +107.1 (*c* 1.72, $CHCl_3$).

IR (film, $CHCl_3$): 3382, 1759, 1651, 1628, 1343, 1057 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.56 (s, 3 H), 0.82–1.05 (m, 4 H), 0.97 (d, *J* = 6.4 Hz, 3 H), 1.15 (m, 1 H), 1.20–1.35 (m, 3 H), 1.40–2.06 (m, 12 H), 2.23 (dd, *J* = 13.2, 8.7 Hz, 1 H), 2.67 (dd, *J* = 13.2, 4.5 Hz, 1 H), 2.81 (br s, 3 H), 2.84 (m, 1 H), 3.37 (dd, *J* = 7.5, 2.9 Hz, 1 H), 3.73–3.92 (m, 4 H), 4.06 (ddd, *J* = 8.7, 7.5, 4.5 Hz, 1 H), 4.45 (d, *J* = 2.9 Hz, 1 H), 4.51 (br d, *J* = 10.7 Hz, 1 H), 5.02 (s, 1 H), 5.08 (s, 1 H), 5.38 (s, 1 H), 5.94 (s, 1 H), 6.01 (d, *J* = 11.2 Hz, 1 H), 6.40 (d, *J* = 11.2 Hz, 1 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.2, 14.9, 16.9, 18.4, 22.2, 23.5, 26.5, 27.6, 29.0, 31.9, 32.6, 39.8, 40.5, 41.0, 46.0, 56.3, 57.0, 61.0, 68.3, 68.4, 71.8, 79.5, 84.4, 113.7, 116.1, 117.3, 125.2, 131.8, 141.1, 142.8, 144.2, 169.8.

EI-LRMS: *m*/*z* = 526 (M^+), 508, 490, 464, 432, 265, 171.

EI-HRMS: *m*/*z* calcd for $C_{32}H_{46}O_6$: 526.3294; found: 526.3291.

Vitamin D Receptor (VDR) Binding Assay

[26,27-*Methyl*-^{3}H]-1α,25-dihydroxyvitamin D_3 (specific activity 6.623 TBq/mmol, 15,000 dpm, 15.7 pg) and various amounts of 1α,25-dihydroxyvitamin D_3 and the analogue to be tested were dissolved in absolute EtOH (50 μL) in 12 × 75-mm polypropylene tubes. The chick intestinal VDR (0.2 mg) and gelatin (1 mg) in aq phosphate buffer solution (1 mL; 25 nM KH_2PO_4, 0.1 M KCl, and 1 mM dithiothreitol, pH 7.4) were added to each tube in an ice bath. The assay tubes were incubated in a shaking water bath for 1 h at 25 °C and then chilled in an ice bath. Polypropylene glycol 6000

(40%; 1 mL) in distilled H_2O was added to each tube, which was mixed vigorously and centrifuged (2260 × *g*) for 60 min at 4 °C. After the supernatant was decanted, the bottom of the tube containing the pellet was cut off into a scintillation vial containing 10 mL of dioxane-based scintillation fluid and the radioactivity was measured with a Beckman liquid scintillation counter (Model LS6500). The relative potency of the analogue was calculated from the concentration needed to displace 50% of [26,27-*methyl*-^{3}H]-1α,25-dihydroxyvitamin D_3 from the receptor compared with the activity of 1α,25-dihydroxyvitamin D_3 (assigned a 100% value).

Assay for HL-60 Cell Differentiation

Nitro blue tetrazolium (NBT)-reducing activity was used as a cell differentiation marker. HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS. Exponentially proliferating cells were collected, suspended in fresh medium and seeded in culture plates (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ). The cell concentration at seeding was adjusted to 2×10^4 cells/mL and the seeding volume was 1 mL/well. An EtOH solution of 1α,25-dihydroxyvitamin D_3 (final concentration: 10^{-8} M) and an analogue (final concentration: 10^{-11} to 10^{-6} M) was added to the culture medium at 0.1% volume and culture was continued for 96 h at 37 °C in a humidified atmosphere of 5% CO_2–air without a change of medium. The same amount of vehicle was added to the control culture. The NBT-reducing assay was performed according to the method of Collins.[23] Briefly, cells were collected, washed with PBS, and suspended in serum-free medium. NBT–TPA solution (dissolved in PBS) was added. Final concentrations of NBT and TPA were 0.1% and 100 ng/mL, respectively. Then, the cell suspensions were incubated at 37 °C for 25 min. After incubation, cells were collected by centrifugation and resuspended in FCS. Cytospin smears were prepared, and the counter-staining of nuclei was done with Kemechrot solution. At least 500 cells per preparation were observed.

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