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Analogs of 1α,25-dihydroxyvitamin D_3 with high potency in induction of osteoclastogenesis and prevention of dendritic cell differentiation: Synthesis and biological evaluation of 2-substituted 19-norvitamin D analogs

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Abstract—In our previous papers, we found that introduction of a substituent at C(2) into 1α,25-dihydroxy-19-norvitamin D_3 (**2a**) caused dramatic changes in binding affinity for the vitamin D receptor (VDR) and in transcriptional activity compared with the parent compound. To investigate the broad biological activity of 2-substituted 19-norvitamin D analogs, we synthesized two new (20*S*)-2-hydroxyethylidene-19-norvitamin D derivatives (**3b** and **4b**) and a total of 16 A-ring-modified analogs including **3b** and **4b** were tested for the following in vitro and in vivo biological activities: (1) affinity for the VDR, (2) transcriptional activity, (3) osteoclast formation, (4) bone calcium mobilization in rats, and (5) effects on differentiation of dendritic cells (DCs). The biological effects of the analogs were compared with those of 1α,25-dihydroxyvitamin D_3 (**1a**) and 2MD, which is being developed for the treatment of osteoporosis. The efficacy of the (20*S*)-19-norvitamin D analogs with 2-hydroxyethylidene, 2-hydroxyethoxy, and 2-methyl moieties (**3b**, **5b**, **6b**, and **9b**) was more than 10-fold stronger than that of **1a** with respect to transcriptional activity, ability to induce osteoclast formation, and ability to inhibit CD86 expression, a marker of mature DCs, and was similar to that of 2MD. The (20*S*)-2β-hydroxyethoxy derivative **6b** was 2 orders of magnitude more active than **1a** and approximately twice as potent as 2MD in preventing CD86 production. The 2-epoxy derivatives **7** and **8** were relatively poor ligands for the VDR and exhibited activity lower than that of the natural hormone **1a**.

1. Introduction

1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$, **1a**] is a seco-steroid hormone having flexible structural features. 1,25-$(OH)_2D_3$ exerts its hormonal effects predominantly on intestine, bone, and kidney, where it plays a crucial role in calcium and phosphorus homeostasis and bone mineralization.[1] The active vitamin D hormone also regulates the differentiation and function of various types of cells, including cells of the immune system.[1] In addition to the roles described above, recent evidence suggests that 1,25-$(OH)_2D_3$ functions in diverse physiological processes, such as hair follicle cycling[2,3] and blood pressure regulation.[4,5]

The biological effects of 1,25-$(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), which is a member of a superfamily of nuclear hormone receptors functioning as ligand-activated transcription factors.[6] Ligand binding allows the VDR to heterodimerize with the retinoid X receptor (RXR), which is the nuclear receptor for 9-*cis*-retinoic acid, and the VDR/RXR heterodimer complex binds with high affinity to vitamin D responsive elements (VDREs) located in the promoter regions of

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target genes. Once bound to DNA, the VDR/RXR dimer recruits coactivators such as SRC-1 and DRIP, and initiates gene transcription. The newly synthesized proteins elicit the physiologic responses characteristic of vitamin D.

1,25-$(OH)_2D_3$ has been widely used as a therapeutic agent in the treatment of osteoporosis, rickets, secondary hyperparathyroidism, and psoriasis, but it has a narrow therapeutic window limited by the development of critical hypercalcemia.[1] In attempts to overcome this limitation, more than three thousand 1,25-$(OH)_2D_3$ analogs have been synthesized in a search for compounds with the required cellular effects but lacking the calcemic side effects.[7] Currently, six synthetic analogs of 1,25-$(OH)_2D_3$ are marketed for the treatment of secondary hyperparathyroidism and psoriasis. Additionally, at least three analogs are now undergoing clinical trials for the chemotherapy of osteoporosis[8,9] and various cancers.[10,11] These studies have shown that deletion of the 19-exomethylene moiety results in considerable suppression of the calcemic activity of 1,25-$(OH)_2D_3$ but preserves its cell differentiation activities.[12,13] 19-Nor-1α,25-dihydroxyvitamin D_2 (paricalcitol) has been approved for the treatment of secondary hyperparathyroidism and does not cause significant hypercalcemia. Shevde et al. found that another 19-norvitamin D analog, 2-methylene-19-nor-(20*S*)-1α,25-dihydroxyvitamin D_3 (2MD, **2b**), exhibits the same affinity for the VDR as does 1,25-$(OH)_2D_3$ and shows a preferential activity on bone relative to intestine.[9,14–16] 2MD also stimulates bone formation both in vitro and in vivo, and is now in phase II clinical trials. 2β-Hydroxypropoxy-1α,25-dihydroxyvitamin D_3 (ED-71) has a significant affinity for the VDR, one-eighth that of 1,25-$(OH)_2D_3$, and its affinity for vitamin D binding protein (DBP) is about 3-fold that of 1,25-$(OH)_2D_3$.[8,17] In an ovariectomized rat model for osteoporosis, ED-71 increases bone mass to a greater extent than does 1,25-$(OH)_2D_3$. ED-71 is a promising therapeutic candidate for treatment of osteoporosis, and phase III clinical trials are under way. Introduction of substituents at the C(2) position dramatically changes the biological profile of vitamin D analogs, as seen in 2MD, ED-71, and a series of 2-substituted 19-norvitamin D analogs synthesized in our laboratory.[18–20] Many 2-substituted vitamin D analogs exhibit increased binding affinity for the VDR and for DBP, high HL-60 cell differentiating activity, and high calcemic potency.[14,21–24]

1,25-$(OH)_2D_3$ induces osteoclast formation.[25–27] Bone-resorbing, multinucleated active osteoclasts are induced by cell-to-cell contact of osteoblasts with osteoclast precursors. Receptor activator of nuclear factor-κ B ligand (RANKL) is an essential factor for osteoclastogenesis. RANKL is expressed on the surface of osteoblasts and binds to RANK (the receptor for RANKL) present on the surface of osteoclast precursors, stimulating the differentiation, fusion, and activation of osteoclasts. 1,25-$(OH)_2D_3$ activates the expression of RANKL in the osteoblast and thus induces osteoclast formation in a dose-dependent fashion.

The VDR is found in various cells of the immune system. The immune effects of 1,25-$(OH)_2D_3$ are mainly mediated through its action on dendritic cells (DCs).[28] Recent studies have demonstrated that 1,25-$(OH)_2D_3$ inhibits the differentiation and maturation of DCs, which are critical antigen-presenting cells in the induction of T-cell-mediated immune responses.[29] In vitro, treatment of DCs with 1,25-$(OH)_2D_3$ leads to down-regulated expression of the costimulatory molecules CD40, CD80, and CD86 and to decreased IL-12 and enhanced IL-10 production, resulting in decreased T cell activation.[30] This combination of functional effects of 1,25-$(OH)_2D_3$ in DCs is, in part, linked to induction of DCs with tolerogenic properties.

In our investigation of the structure-activity relationships (SAR) of a series of 19-norvitamin D analogs, we have focused on modification of the A-ring at C(2). Modification of the C(2) position is of key importance in modulating the biological properties of vitamin D. We have synthesized a number of 2-substituted 19-norvitamin D analogs and described their biological activities.[19,20] We have found that introduction of hydroxyethylidene or hydroxyethoxy groups at C(2) causes dramatic changes in the activity profile compared with the parent vitamin D. In the VDR-binding assay, the 2β-hydroxyethoxy-19-norvitamin D analog **6b** is 5-fold more active than 1,25-$(OH)_2D_3$, and this to our knowledge is the strongest affinity for the VDR among the known 19-norvitamin D analogs; **6b** also has dramatically enhanced transcriptional activity (30-fold higher than that of **1a**).[19] In this paper, we synthesized two new 19-norvitamin D derivatives (**3b** and **4b**) and performed a detailed evaluation of the biological activities of sixteen 2-substituted 19-norvitamin D analogs with 20*R*- or 20*S*-configuration in comparison with those of the natural hormone (**1a**) and the established analog 2MD (**2b**) (Fig. 1).

2. Results and discussion

2.1. Synthesis

For the synthesis of **3b** and **4b**, we used a Wittig-Horner reaction of the A-ring phosphine oxide **11** with the 20-*epi*-25-hydroxy Grundmann's ketone **12** (Fig. 2). The A-ring phosphine oxide **11** (ca. 2:1 diastereomeric mixture), prepared from D-glucose as reported,[18] was treated with the C/D-ring ketone **12** to afford **13** (66%, ca. 3:2 diastereomeric mixture), which was selectively deprotected to yield **14** (46%). The 2-hydroxy-19-norvitamin D derivative **14** was oxidized to the 2-oxo derivatives **15** (86% as a single compound) under Swern's conditions. Cyanomethylation of **15** with diethyl (cyanomethyl)phosphonate gave **16** (99%) as an approximately 1:1 mixture of *E* and *Z* isomers at C(2), which was reduced with diisobutylaluminum hydride followed by sodium borohydride to afford the 2-hydroxyethylidene derivative **17** as a separable isomeric mixture (**17a**, 27%; **17b**, 25%). Deprotection of **17a** and **17b** with camphor sulfonic acid yielded (*E*)- and (*Z*)-hydroxyethylidene-19-norvitamin D analogs **3b** and **4b**, respectively. The

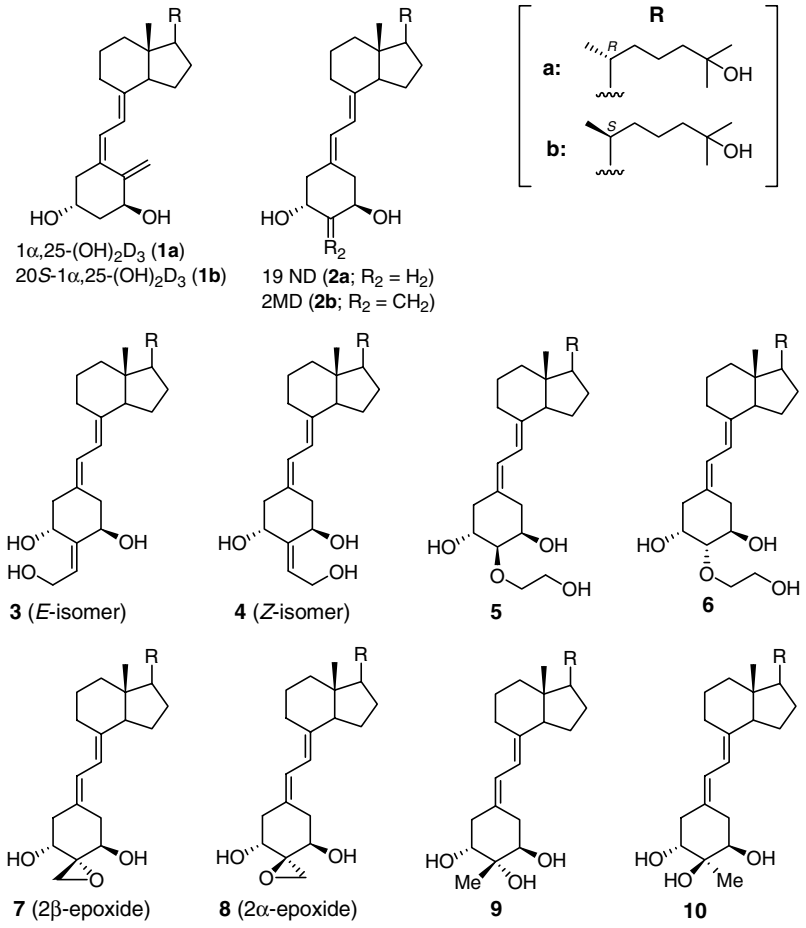


Figure 1. Structures of 1,25-$(OH)_2D_3$ and its analogs.

stereochemistries of the 2-hydroxyethylidene analogs **3b**, **4b**, **17a**, and **17b** at C(2) were determined by their phase-sensitive 2D NOESY spectra. In **3b** and **17a**, an NOE was observed between H-1 and vinyl proton (δ 5.80 for **3b**, δ 5.72 for **17a**). A correlation cross peak was observed between H-3 and vinyl proton (δ 5.83 for **4b**, δ 5.72 for **17b**) in **4b** and **17b**.

2.2. Biological activity

2.2.1. VDR affinity and transcriptional activity. The 2-substituted 19-norvitamin D analogs were examined for their affinity for the bovine thymus VDR; the results are summarized in Table 1.[19,20] The VDR-binding affinities of the analogs **3a** and **6a** relative to the natural hormone **1a** were 2-fold greater and equivalent, respectively. In the crystal structures of the human or rat VDR ligand-binding domain (LBD) in complex with 1,25-$(OH)_2D_3$ (**1a**) and its derivatives, C(19)H_2 of the ligands interacts with Leu233.[31–35] 19-Norvitamin D analogs with no 19-exomethylene group exhibited lower affinity for the VDR relative to **1a**. Introduction of 2*E*-hydroxyethylidene or 2β-hydroxyethoxyl moieties at the C(2) position compensates for the loss of the van der Waals contact at C(19)H_2 with Leu233. Docking studies of analogs **3a** and **6a** with the structural modifications described above show that the backbone carbonyl of Asp144 is hydrogen-bonded with the terminal hydroxyl group of substituents at C(2). The binding of vitamin D analogs to the VDR is markedly affected by their configuration at C(20): 20*S*-isomers have a higher affinity than do 20*R*-isomers.[7] Comparison of the receptor binding of the C(2) isomeric pairs of the 19-norvitamin D analogs **3**–**10** was consistent with the reported findings.[7] Interestingly, the (2*E*)-hydroxyethylidene-19-norvitamin D derivative **3b** with 20*S*-configuration showed an even weaker affinity for the VDR than that of the 20*R*-counterpart **3a**, whereas 20-epimerization markedly increased the binding potency for the *Z*-isomers (**4a** vs **4b**). Similar trends were also observed for (2*E*)- and (2*Z*)-ethylidene-19-norvitamin D analogs, which were first developed by DeLuca's group.[22] 19-Norvitamin D analogs **5**, **8**, and **10**, whose hydrophilic oxygen atom or hydroxyl group occupies the α-configuration at the C(2) position, bound less well to the VDR than did the corresponding counterparts **6**, **7**, and **9**. Within the VDR ligand-binding pocket (LBP), above the A-ring, a narrow space is open for hydrophobic interactions with the adjacent LBP amino acid residues Leu233, Phe150, and the phenyl ring of Tyr236. Compounds **5**, **8**, and **10** can make unfavorable interactions within the LBP, explaining their decreased binding affinity for the VDR.

The VDR-mediated transcriptional activity of the 19-norvitamin D analogs was tested by luciferase assay

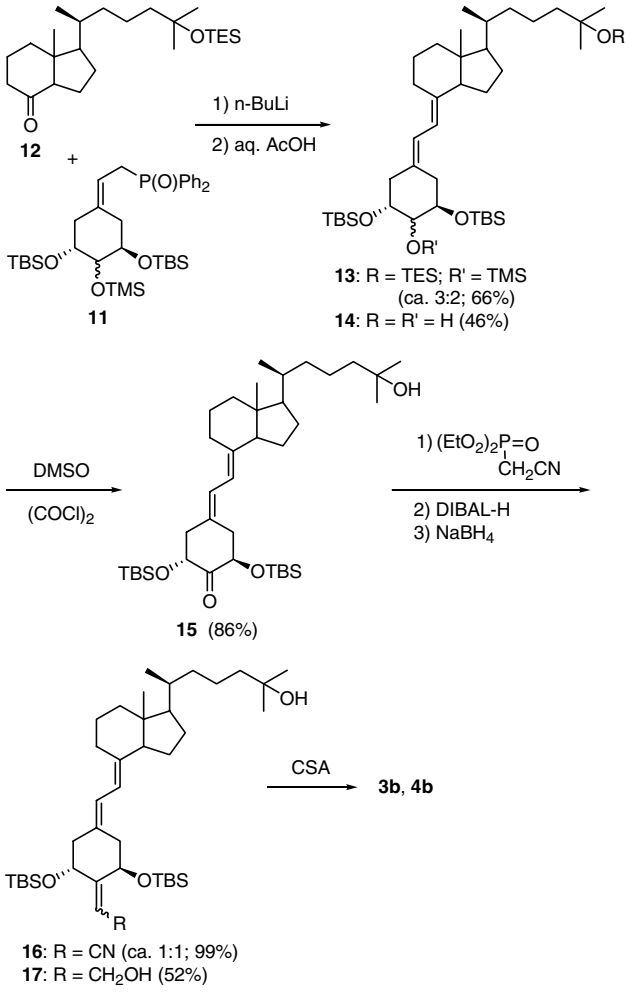
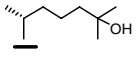
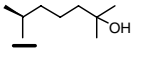


Figure 2. Synthetic scheme of the (20*S*)-2-hydroxyethylidene-19-norvitamin D analogs.

Table 1. Relative VDR affinity and transcriptional activity of 2-substituted 19-norvitamin D analogs[a]

Compound	a: 		b: 	
	VDR affinity[b]	Trans. activity[c]	VDR affinity[b]	Trans. activity[c]
1	1.0	1.0	5.0[d]	40[d]
3	2.0[d]	2.0[d]	1.6	12.5
4	0.007[d]	0.3[d]	0.02	4.0
5	0.1[d]	3.5[d]	1.0[d]	10[d]
6	1.0[d]	7.0[d]	5.0[d]	30[d]
7	0.04[d]	0.05[d]	0.5[d]	0.2[d]
8	0.003[d]	0.04[d]	0.2[d]	0.9[d]
9	0.3[d]	2.8[d]	1.0[d]	32[d]
10	0.001[d]	0.06[d]	NT	NT

NT, not tested.
[a] Potencies of 1,25-$(OH)_2D_3$ (**1a**) are normalized to 1.
[b] Bovine vitamin D receptor.
[c] Activity was assessed in terms of ED_{50}.
[d] Refs. 18–20.

with mouse osteopontin VDRE in COS-7 cells. The results are shown in Table 1 and Figure 3.[19,20] The relative activities of test compounds were assessed by the ED_{50} values calculated from their dose–response curves (Fig. 3). The two isomeric pairs **3** versus **4** or **9** versus **10** with 20*R*-configuration exhibited large differences in transcriptional activity. The four analogs **5** and **6** with the 2-hydroxyethoxy moiety proved to be much more active than **1a** in spite of their C(2)- and C(20)-configurations, although the 2α-hydroxyethoxy analogs **5** have an unfavorable hydrophilic substituent at C(2). In a previous paper,[19] to clarify the interaction between the VDR and the 2-substituted 19-norvitamin D analogs we performed two-dimensional alanine scanning mutational analysis with the 2α- and 2β-hydroxyethoxy-19-norvitamin D derivatives; the 2α-isomer **5b** showed striking differences from the native ligand **1a** and the 2β-isomer **6b**. From the alanine mutational analysis in combination with a docking model of **5b**, we proposed that **5b** docks in the VDR LBP with the A-ring α-conformation, which is different from the native hormone

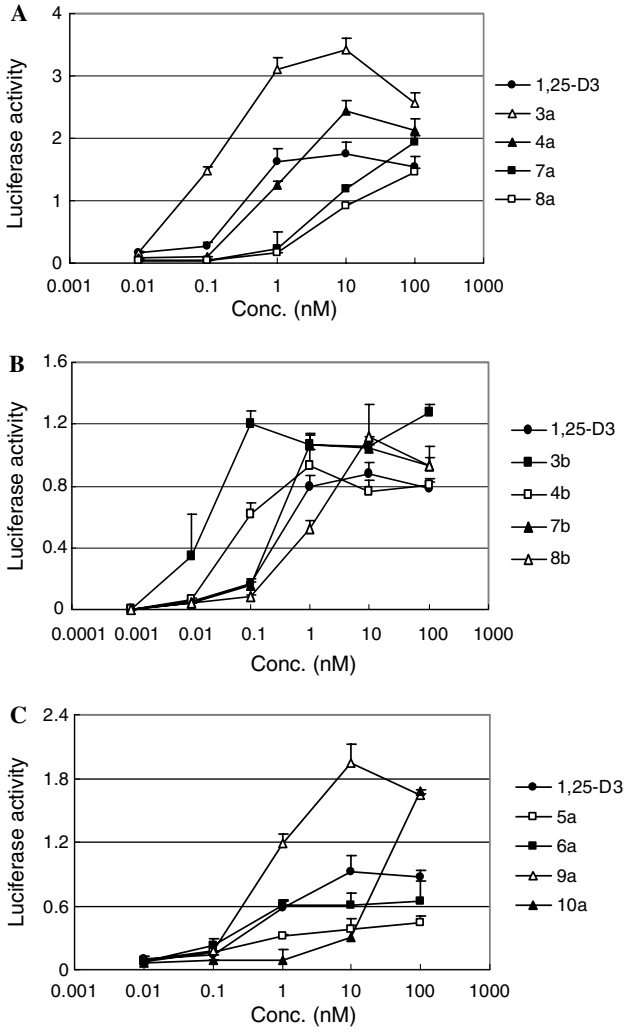


Figure 3. Dose–response effects of 1,25-$(OH)_2D_3$ and 19-norvitamin D analogs on transcriptional activity. (A) ●, 1,25-$(OH)_2D_3$ (ED_{50} = 0.3 nM); △, **3a** (ED_{50} = 0.15 nM); ▲, **4a** (ED_{50} = 1 nM); ■, **7a** (ED_{50} = 6 nM); □, **8a** (ED_{50} = 7 nM). (B) ●, 1,25-$(OH)_2D_3$ (ED_{50} = 0.25 nM); ■, **3b** (ED_{50} = 0.02 nM); □, **4b** (ED_{50} = 0.06 nM); ▲, **7b** (ED_{50} = 0.28 nM); △, **8b** (ED_{50} = 1.4 nM). (C) ●, 1,25-$(OH)_2D_3$ (ED_{50} = 1.4 nM); □, **5a** (ED_{50} = 0.4 nM); ■, **6a** (ED_{50} = 0.2 nM); △, **9a** (ED_{50} = 0.5 nM); ▲, **10a** (ED_{50} = 24 nM).

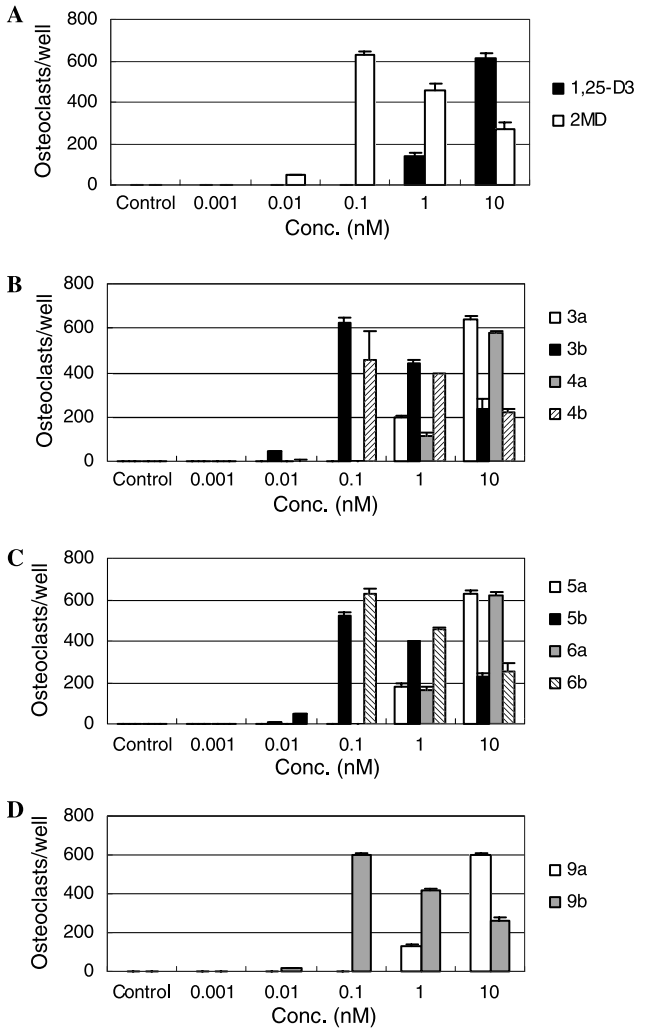


Figure 4. Effects of 1,25-$(OH)_2D_3$, 2MD, and 19-norvitamin D analogs on osteoclast formation.

1a with the A-ring β-conformation.[36] In the α-conformation, the 2α-substituent of **5b** takes an equatorial conformation and the docking model of this compound suggests that the hydroxyl group at C(2) can form a hydrogen bond with Asp144.

The analogs **7** and **8** with an oxirane at C(2) showed an extremely weak transcriptional activity and the differences between VDR binding activity and transcriptional activity were found to be small. The 2-methyl-2-hydroxy analogs **9** and **10** were more potent in transcriptional activity than were the corresponding epoxy analogs. Fujishima et al. compared 2-cyclopropyl-1α,25-$(OH)_2D_3$ and 2,2-dimethyl-1α,25-$(OH)_2D_3$, demonstrating that the VDR affinity and cell differentiation-inducing activity of analogs with cyclopropane at C(2) were similar to those of the natural hormone **1a**, whereas the 2,2-dimethyl analogs showed 3–30% of the activity of **1a**.[37,38] These results are different from those for our analogs with 2-epoxy or 2-methyl-2-hydroxy groups and suggest that an oxygen atom attached directly to the C(2) position plays a critical role in VDR binding. Among the 19-norvitamin D analogs prepared to date in our laboratory, the 2α-methyl-2β-hydroxy analog **9b** is the most potent in transcriptional activity.

Table 2. Relative potency of 1,25-$(OH)_2D_3$ and its analogs in osteoclast formation and inhibition of CD86 expression[a]

Compound	a:		b:	
	Osteoclast formation[b]	CD86 inhibition[b]	Osteoclast formation[b]	CD86 inhibition[b]
1	1.0	1.0	NT	NT
2	NT	NT	87	60
3	1.2	5.0	77	42
4	0.8	1.3	66	6
5	1.0	1.5	66	50
6	1.0	6.0	87	100
7	NT	0.2	NT	2.5
8	NT	0.13	NT	0.4
9	0.95	2.0	66	25
10	NT	NT	NT	0.43

NT, not tested.
[a] Potencies of 1,25-$(OH)_2D_3$ (**1a**) are normalized to 1.
[b] Activity was assessed in terms of ED_{50}.

2.2.2. Effect of 2-substituted 19-norvitamin D analogs on osteoclast formation. To define VDR-mediated osteoclastogenesis, the effects of 19-norvitamin D analogs on osteoclast formation were examined in a mouse co-culture system; the results are summarized in Figure 4 and Table 2. 1,25-$(OH)_2D_3$ and 2MD induced multinucleated cells that were positive for tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts), features typical of osteoclasts. 2MD was approximately 100 times more potent than 1,25-$(OH)_2D_3$, as reported by DeLuca and co-workers[15] Osteoclast formation by the 19-norvitamin D derivatives with 20*R*-configuration was dose-dependent with activity similar to that of 1,25-$(OH)_2D_3$, whereas the corresponding 20*S*-epimers showed almost the same potency as 2MD. Regardless of the type of substituent at the C(2) position, 19-norvitamin D analogs with a 20*S*-configuration were much more active than their corresponding 20*R*-counterparts in eliciting osteoclast formation. These results suggest that the 20*S*-side chain structure of 19-norvitamin D plays a crucial role for VDR-mediated osteoclast differentiation. RANKL produced by osteoblasts is an essential factor for osteoclastogenesis. Osteoclast precursors express RANK, which is the receptor for RANKL, recognize RANKL through a cell–cell contact, and differentiate into osteoclasts. 1,25-$(OH)_2D_3$ up-regulates the expression of RANKL in osteoblasts and 2MD elicits an approximately 100-fold induction of RANKL compared with 1,25-$(OH)_2D_3$.[15] In our preliminary experiments, we found that **3b**, **6b**, and **9b** strongly stimulated RANKL mRNA production in osteoblasts, showing activity similar to that of 2MD.

2.2.3. Bone calcium mobilization. 1,25-$(OH)_2D_3$ (**1a**) induces calcium resorption from bone in vivo and enhances the expression of RANKL in osteoblasts. RANKL is a key regulator of osteoclast formation.

Vitamin D-mediated formation of osteoclasts is related to bone resorption activity.

To investigate their support of calcium mobilization from bone (BCM), the calcemic activities of 19-norvitamin D analogs were tested in vitamin D-deficient rats on a low-calcium diet (Table 3). Our experimental model studied the time course of BCM at 6, 12, and 24 h after a single dose of 1 μg of 1,25-$(OH)_2D_3$ or the 2-substituted 19-norvitamin D analogs. In this model, administration of vitamin D-related compounds elevated the serum level of calcium, and different compounds showed different time courses with respect to peak calcium level. The natural hormone **1a** exhibited peak activity around 12 h after administration. 2MD also showed peak activity at 12 h and had a stronger effect than **1a** on serum calcium, concomitant with its greater potency for osteoclast formation. Comparing the times of maximal effect, the 2*E*-hydroxyethylidene derivatives **3a** and **3b** were more effective than **1a**, whereas the 2*Z*-isomers **4a** and **4b** were slightly less effective than and equal to **1a**, respectively. The four 2-hydroxyethoxy analogs, except for **5a**, were slightly more potent than **1a** on calcemic activity. In contrast with the 66-fold difference of relative potency of **9b** to **1a** in osteoclast formation, the 2α-methyl-2β-hydroxy analog **9b** exhibited only weak calcemic activity compared with **1a**. Our present data demonstrate that substitution of 19-norvitamin D at the C(2) position has variable effects on calcemic activity. These results suggest that alteration of C(20) stereochemistry is not essential for calcium-regulatory potency.

2.2.4. Effects on differentiation and maturation of dendritic cells. We studied the effect of 19-norvitamin D analogs on differentiation and maturation of dendritic cells (DCs) differentiated from mouse bone marrow macrophages obtained from bone marrow cells cultured with M-CSF for 4 days. First, the bone marrow macrophages were cultured for 3 days in the presence of GM-CSF without or with 19-norvitamin D analogs. On day 7 of culture, cells were harvested and analyzed by fluorescence-activated cell sorting (FACS) for surface expression of CD11c, which is a marker for immature DCs. Figure 5A shows a representative experiment studying surface phenotype. Expression of CD11c was not affected by the 19-norvitamin D analogs at concentrations of 10^{-10}–10^{-7} M. Similar results were obtained with 1,25-$(OH)_2D_3$-treated macrophages. This result suggests that the synthetic 19-norvitamin D analogs do not affect differentiation from bone marrow macrophages to immature DCs.

Immature DCs obtained by 2-day culture with GM-CSF can be induced to form mature DCs with potent antigen-presenting properties by incubation with lipopolysaccharide (LPS). DC maturation is accompanied by enhanced production of the maturation marker CD86. Immature DCs were cultured with LPS in the presence or absence of 19-norvitamin D analogs. The effects of the vitamin D compounds on DC maturation were compared with those of 1,25-$(OH)_2D_3$ (**1a**) and 2MD (**2b**) as positive controls. Figure 5B shows representative flow cytometric histograms of control cells and of cells cul-

Table 3. Bone calcium mobilization by 1,25-$(OH)_2D_3$, 2MD, and 19-norvitamin D_3 analogs in vitamin D-deficient rats on a low-calcium diet[a]

Compound	Serum Ca (6 h)/(mg/dL) (means ± SEM)	Serum Ca (12 h)/(mg/dL) (means ± SEM)	Serum Ca (24 h)/(mg/dL) (means ± SEM)
None (control)	4.68 ± 0.56^{b}	4.84 ± 0.45^{b}	4.90 ± 0.38^{b}
1,25-$(OH)_2D_3$ **1a**	6.73 ± 0.56^{c}	8.20 ± 0.71^{c}	7.72 ± 0.11^{c}
2MD **2b**	7.19 ± 0.74^{d}	9.57 ± 0.01^{d}	9.34 ± 0.43^{d}
None (control)	4.23 ± 0.54^{b}	4.51 ± 0.65^{b}	5.16 ± 0.44^{b}
1a	5.16 ± 0.55^{c}	6.70 ± 0.18^{c}	6.55 ± 0.69^{c}
3a	5.95 ± 0.35^{d}	6.47 ± 0.39^{d}	7.73 ± 0.89^{d}
4a	5.98 ± 0.49^{e}	6.14 ± 0.56^{e}	5.53 ± 0.28^{e}
3b	6.18 ± 0.18^{f}	7.58 ± 0.51^{f}	8.42 ± 0.39^{f}
4b	5.58 ± 0.37^{g}	6.68 ± 0.29^{g}	6.71 ± 0.31^{g}
None (control)	5.12 ± 0.57^{b}	5.17 ± 0.25^{b}	4.63 ± 0.31^{b}
1a	8.02 ± 0.62^{c}	7.19 ± 0.18^{c}	7.10 ± 0.24^{c}
5a	7.92 ± 0.16^{d}	7.20 ± 0.46^{d}	6.10 ± 0.60^{d}
6a	8.54 ± 0.24^{e}	7.04 ± 0.47^{e}	7.99 ± 0.48^{e}
5b	8.73 ± 0.61^{f}	8.32 ± 0.25^{f}	7.54 ± 0.49^{f}
6b	8.15 ± 0.67^{g}	8.79 ± 0.97^{g}	8.00 ± 0.56^{g}
None (control)	3.30 ± 0.42^{b}	4.59 ± 0.52^{b}	3.33 ± 0.37^{b}
1a	5.25 ± 0.38^{c}	7.63 ± 0.69^{c}	6.77 ± 0.43^{c}
9a	5.06 ± 0.33^{d}	6.47 ± 0.15^{d}	5.94 ± 0.09^{d}
9b	4.71 ± 0.52^{e}	7.33 ± 0.50^{e}	7.28 ± 0.74^{e}

Statistical analysis was done by Student's *t*-test. Serum Ca at 6 h, panel 1: b from c and d, $P < 0.01$; panel 2: b from c, d, f, and g, $P < 0.001$, b from e, $P < 0.01$; panel 3: b from c, $P < 0.05$, b from d and f, $P < 0.001$, b from e and g, $P < 0.01$; panel 4: b from c and e, $P < 0.001$, b from d, $P < 0.01$. Serum Ca at 12 h, panel 1: b from c and d, $P < 0.01$; panel 2: b from c, $P < 0.001$, b from d, e, and f, $P < 0.01$, b from g, $P < 0.05$; panel 3: b from c, d, f, and g, $P < 0.001$, b from e, $P < 0.01$; panel 4: b from c, d, and e, $P < 0.001$. Serum Ca at 24 h, panel 1: b from c and d, $P < 0.01$; panel 2: b from c, e, f, and g, $P < 0.001$, b from d, $P < 0.01$, b from d and f, $P < 0.01$; panel 3: b from c, d, f, and g, $P < 0.001$, b from d, $P < 0.01$; panel 4: b from c and d, $P < 0.001$, b from e, $P < 0.01$.

[a] Weanling male rats were maintained on the vitamin D-deficient diet containing 0.03% calcium and 0.6% phosphorus for 3 weeks.

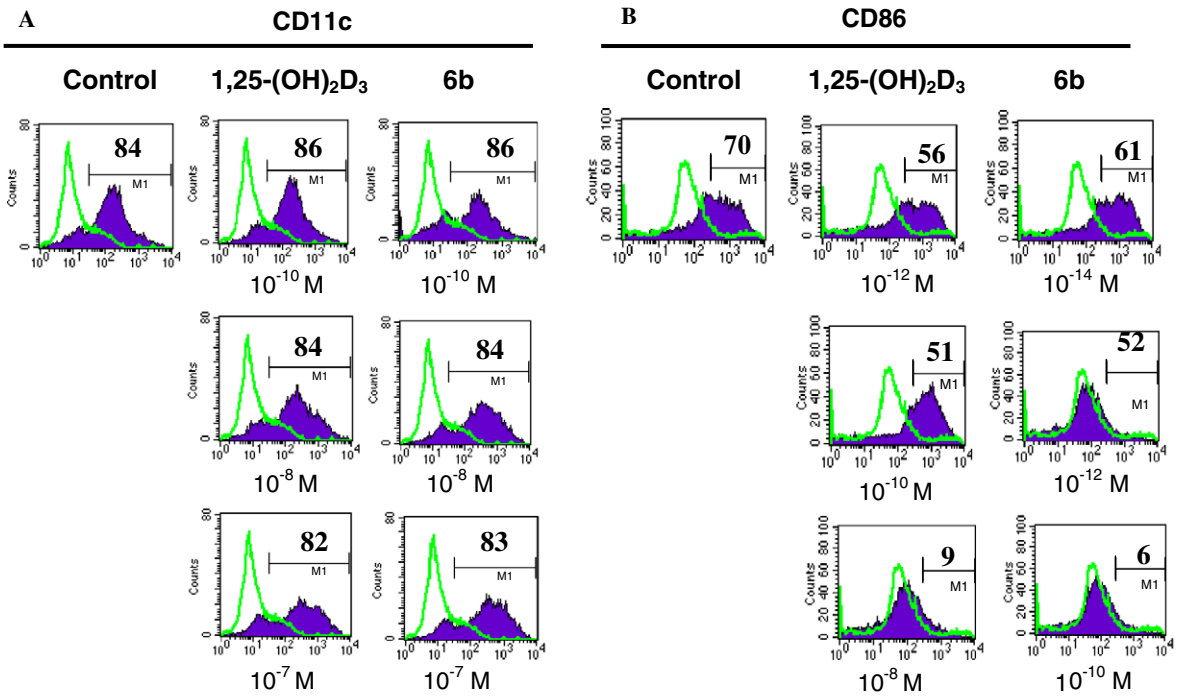


Figure 5. Flow cytometric histograms of CD11c and CD86 expressed by DCs differentiated in the absence or presence of 1,25-$(OH)_2D_3$ and **6b**. (A) Open or filled histograms: CD11c expression in the GM-CSF-untreated or treated bone marrow macrophages. (B) Open or filled histograms: CD86 expression in the LPS-untreated or treated immature DCs.

tured with 1,25-$(OH)_2D_3$ or with the 2-hydroxyethoxy derivative **6b**. Reduction of CD86 was observed in the presence of 1,25-$(OH)_2D_3$, with maximal effect occurring at 10^{-8} M. With **6b**, however, the maximal inhibitory effect was observed at 10^{-10} M. Dose-dependent inhibition of DC maturation by 1,25-$(OH)_2D_3$ and 19-norvitamin D analogs is illustrated in Figure 6, and the experiments are summarized in Table 2. 19-Norvitamin D analogs prevented the LPS-induced maturation of immature DCs, maintaining DCs at the immature stage characterized by low CD86 expression. The fifteen 19-norvitamin D analogs tested, excluding **7a**, **8a**, **8b**, and **10b**, as well as 2MD were more effective than 1,25-$(OH)_2D_3$ in inhibiting CD86 induction. The (20*S*)-2*E*-hydroxyethylidene and (20*S*)-2-hydroxyethoxy derivatives **3b**, **5b**, and **6b** exhibited 42-, 50-, and 100-fold higher potency than the natural hormone **1a**, respectively, having potency similar to that of 2MD. The rank order of potency for osteoclast formation among the eight test compounds almost paralleled that for inhibition of CD86 expression. Treatment with 19-norvitamin D analogs prevented the LPS-induced maturation of immature DCs, maintaining DCs at the immature stage.

IL-12 is a cytokine derived from antigen-presenting cells that is known to play an important role in T-cell development. To investigate the effects of 1,25-$(OH)_2D_3$ and 19-norvitamin D analogs on IL-12 mRNA expression in DCs, we performed reverse-transcription PCR on RNA extracted from DC cultures, as described in Section 4. As shown in Figure 7, 1,25-$(OH)_2D_3$ inhibited IL-12 mRNA expression in DCs in a dose-dependent manner. Addition of 10^{-9} and 10^{-10} M 2-hydroxyeth-

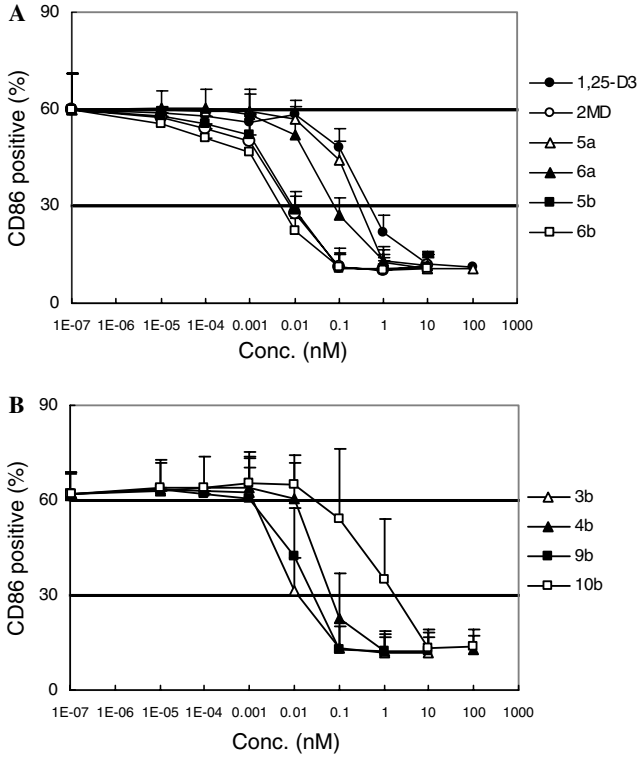


Figure 6. Dose-dependent inhibition of CD86 expression by 1,25-$(OH)_2D_3$, 2MD, and 19-norvitamin D analogs (A) ●: 1,25-$(OH)_2D_3$ (IC_{50} = 0.3 nM); ○: 2MD (IC_{50} = 0.005 nM); △: **5a** (IC_{50} = 0.2 nM); ▲: **6a** (IC_{50} = 0.05 nM); ■: **5b** (IC_{50} = 0.006 nM); □: **6b** (IC_{50} = 0.003 nM). (B) △: **3b** (IC_{50} = 0.007 nM); ▲: **4b** (IC_{50} = 0.05 nM); ■: **9b** (IC_{50} = 0.012 nM); □: **10b** (IC_{50} = 0.7 nM).

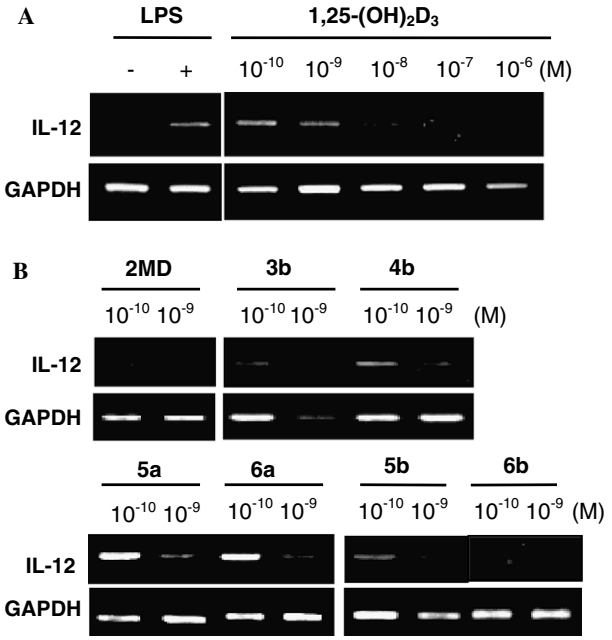


Figure 7. Effects of 1,25-$(OH)_2D_3$, 2MD, and 19-norvitamin D analogs on the expression of IL-12 mRNA in DCs.

oxy-19-norvitamin D analogs **5** and **6** to DC culture resulted in a dose-dependent reduction in IL-12 mRNA expression. Of the four 2-hydroxyethoxy derivatives, the (20*S*)-2β-hydroxyethoxy derivative **6b** markedly decreased IL-12 mRNA expression at 10^{-9} M. 2MD also exhibited activity higher than that of **1a** in suppression of IL-12 mRNA expression. The (20*S*)-2β-hydroxyethoxy analog **6b** inhibited both CD86 expression and IL-12 mRNA expression in DCs. DCs play a key role in initiation of the immune response.

Immature DCs are positive for CD11c and CD86, whereas mature DCs express up-regulated levels of CD86. Surface expression levels of CD11c were not inhibited by 1,25-$(OH)_2D_3$ or by 19-norvitamin D analogs, whereas those compounds markedly prevented up-regulation of CD86. Our data suggest that 19-norvitamin D analogs prevent differentiation from immature DCs to mature DCs. In addition, the effect of 19-norvitamin D analogs on mature DCs, leading to inhibition of IL-12 production, may be, in part, responsible for the induction of DCs with tolerogenic properties. Vitamin D analogs able to regulate DC differentiation may be useful in the treatment of autoimmune diseases and the prevention of transplant rejection.

3. Conclusion

We have synthesized a total of sixteen 2-substituted 19-norvitamin D analogs. Their profiles of biological activity were assessed in terms of affinity for the VDR, VDR-mediated transcriptional activity, and effects on osteoclast and dendritic cell differentiation in comparison with the natural hormone **1a**. The 2-hydroxyethylidene, 2-hydroxyethoxy, and 2-methyl-2-hydroxy analogs of 19-nor-1,25-$(OH)_2D_3$ are extremely interesting as they display a broad spectrum of biological activity. 20-*epi*-19-Norvitamin D analogs with these C(2) substituents are characterized by an extremely high potency in the induction of differentiation of osteoclasts and the inhibition of differentiation and maturation of dendritic cells, exhibiting an activity similar to that of 2MD. These 20*S*-19-norvitamin D analogs may be useful in the treatment of bone and autoimmune diseases.

4. Experimental

^{1}H NMR spectra were obtained on a Bruker ARX-400 spectrometer, operating at 400 MHz. Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane as an internal standard (δ 0 ppm). Abbreviations used are: singlet (s), doublet (d), triplet (t), multiplet (m), aromatic (arom), and broad signal (br). Low- and high-resolution mass spectra (LR-MS and HR-MS) were obtained with electronic ionization (EI) on a JEOL JMS-AX505HA spectrometer run at 70 eV for EI; *m*/*z* values are given with relative intensities in parentheses. UV spectra were obtained on a Beckmann DU-7500 spectrophotometer. Column chromatography was carried out on silica gel (Wako Pure Chem. Ind. Ltd. Wakogel C-200), unless otherwise indicated. All reactions, unless specifically mentioned, were conducted under an atmosphere of argon gas. Yields are not optimized. Tetrahydrofuran (THF) was distilled from Na benzophenone ketyl prior to use. Toluene was distilled from Na. Dimethylsulfoxide (DMSO), methylene chloride (CH_2Cl_2), and triethylamine were distilled from calcium hydride. *N*,*N*-Dimethylformamide (DMF) was distilled from 4 Å molecular sieves.

4.1. (20*S*)-1α-[(*tert*-Butyl-dimethylsilyl)oxy]-2-[(trimethylsilyl)oxy]-25-[(triethylsilyl)oxy]-19-norvitamin D_3 *tert*-butyl-dimethylsilyl ether (13)

To stirred solution of **11** (435.2 mg, 0.660 mmol, ca. 2:1 isomeric mixture) in dry THF (5 mL) at −78 °C was added *n*-BuLi (412 μL, 0.660 mmol, 1.6 M solution in hexane) and the resulting dark-orange solution was stirred for 15 min. To this colored solution was added a solution of **12** (173.8 mg, 0.440 mmol) in dry THF (3 mL) and the whole mixture was stirred for 2 h at −78 °C. The mixture was quenched with saturated NH_4Cl and extracted with AcOEt. The organic phase was rinsed with brine, dried over $MgSO_4$, and evaporated to dryness. The residue was purified by chromatography on silica gel (20 g) using 2% AcOEt in hexane to give **13a**, **b** (243.4 mg, 66% based on **12**) as a mixture of **13a**:**13b** = ca. 3:2 ratio, and 5% AcOEt in hexane to afford the unreacted starting material **12** (30.0 mg, 17%) and **11** (157.6 mg).

LR-MS *m*/*z* (%): 834 (no M^+), 702 (10), 645 (2), 616 (7), 570 (20), 513 (5), 484 (25), 75 (100). Compound **13a** (major): ^{1}H NMR ($CDCl_3$) δ: 0.04, 0.055, 0.058, 0.063 (each 3H, s, 4× Si–Me), 0.12 (9H, 3× Si–Me), 0.54 (3H, s, H-18), 0.56 (6H, q, J = 7.9 Hz, 3× Si–CH_2), 0.85 (3H, d, J = 6.5 Hz, H-21), 0.87, 0.88 (each 9H, s, 2× Si–*t*-Bu), 0.94 (9H, t, J = 7.9 Hz, 3× Si–CH_2CH_3), 1.19 (6H, s,

H-26, 27), 2.30 (1H, m), 2.50 (2H, m), 2.79 (1H, m, H-9), 3.54 (1H, m, H-2), 3.80 (1H, m, H-3), 3.88 (1H, m, H-1), 5.81 (1H, d, J = 11.1 Hz, H-7), 6.10 (1H, d, J = 11.1 Hz, H-6). Compound **13b** (minor): ^{1}H NMR ($CDCl_3$) δ: 0.04, 0.06 (each 3H, s, 2× Si–Me), 0.07 (6H, s, 2× Si–Me), 0.12 (9H, 3× Si–Me), 0.53 (3H, s, H-18), 0.56 (6H, q, J = 7.8 Hz, 3× Si–CH_2), 0.84 (3H, d, J = 6.6 Hz, H-21), 0.86, 0.89 (each 9H, s, 2× Si–*t*-Bu), 0.94 (9H, t, J = 7.8 Hz, 3× Si–CH_2CH_3), 1.19 (6H, s, H-26, 27), 2.10 (1H, m), 2.44 (2H, m), 2.79 (1H, m, H-9), 3.60 (1H, m, H-2), 3.80 (1H, dd, J = 8.7, 4.5 Hz, H-1), 3.94 (1H, m, H-3), 5.79 (1H, d, J = 11.2 Hz, H-7), 6.13 (1H, d, J = 11.2 Hz, H-6).

4.2. (20*S*)-1α-[(*tert*-Butyl-dimethylsilyl)oxy]-2,25-dihydroxy-19-norvitamin D_3 *tert*-butyl-dimethylsilyl ether (14)

A solution of **13** (182.5 mg, 0.218 mmol, **13a**:**13b** = ca. 3:2) in THF, AcOH, and water (v/v/v, 8:8:1, 9.5 mL) was stirred for 2 h at 0 °C and for 20 h at ambient temperature and the mixture was diluted with AcOEt. The organic layer was successively washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4. The solvent was evaporated in vacuo, and the residue was purified by chromatography on silica gel (10 g) using 2% AcOEt in hexane to give **14a** (39.1 mg, 28%) and **14b** (26.0 mg, 18%).

LR-MS *m*/*z* (%): 648 (M^+, 6), 630 (7), 498 (5), 441 (42), 75 (100). HR-MS *m*/*z*: 648.4965 (Calcd for $C_{38}H_{72}O_4Si_2$: 648.4969). Compound **14a** (major): ^{1}H NMR ($CDCl_3$) δ: 0.067, 0.077, 0.083, 0.10 (each 3H, s, 4× Si–Me), 0.54 (3H, s, H-18), 0.86 (3H, d, J = 6.6 Hz, H-21), 0.87, 0.88 (each 9H, s, 2× Si–*t*-Bu), 1.22 (6 H, s, H-26, 27), 2.27 (1H, d, J = 3.2 Hz, OH), 2.31 (1H, dd, J = 12.6, 3.7 Hz), 2.48 (2H, m), 2.79 (1H, m, H-9), 3.51(1H, m, H-2), 3.91, (1H, m, H-3), 4.00 (1H, m, H-1), 5.80 (1H, d, J = 11.1 Hz, H-7), 6.15 (1H, d, J = 11.1 Hz, H-6). Compound **14b** (minor): ^{1}H NMR ($CDCl_3$) δ : 0.06, 0.07, 0.08, 0.10 (each 3H, s, 4× Si–Me), 0.53 (3H, s, H-18), 0.86, 0.90 (each 9H, s, 2× Si–*t*-Bu, overlapped with H-21), 1.21 (6H, s, H-26, 27), 2.18 (1H, dd, J = 13.0, 4.5 Hz), 2.39 (3H, m), 2.80 (1H, m, H-9), 3.59 (1H, m, H-2), 4.00 (2H, m, H-1, 3), 5.80 (1H, d, J = 11.2 Hz, H-7), 6.18 (1H, d, J = 11.2 Hz, H-6).

4.3. (20*S*)-1α-[(*tert*-Butyl-dimethylsilyl)oxy]-2-oxo-25-hydroxy-19-norvitamin D_3 *tert*-butyl-dimethylsilyl ether (15)

To a stirred solution of oxalyl chloride (18 μL, 0.206 mmol) in dry CH_2Cl_2 (1 mL) at −78 °C was added a solution of DMSO (29 μL, 0.414 mmol) in dry CH_2Cl_2 (0.2 mL). After 5 min of stirring, a solution of **14** (61.0 mg, 0.094 mmol, **14a**:**14b** = ca. 3:2) in dry CH_2Cl_2 (1.2 mL) was added dropwise. The reaction mixture was stirred for 15 min at −78 °C, and Et_3N (131 μL, 0.940 mmol) was added. The whole mixture was stirred for 30 min at −78 °C and for 10 min at 0 °C, quenched with ice water, and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with brine, dried over $MgSO_4$, and evaporated to dryness. The residue was purified by chromatography on silica gel (5 g) using 20% AcOEt in hexane to afford **15** (52.0 mg, 86%) as a single compound.

Compound **15**: ^{1}H NMR ($CDCl_3$) δ : 0.055, 0.065, 0.069, 0.10 (each 3H, s, 4× Si–Me), 0.55 (3H, s, H-18), 0.87, 0.89 (each 9H, s, 2× Si–*t*-Bu, overlapped with H-21), 1.22 (6H, s, H-26, 27), 2.45 (1H, dd, J = 13.5, 8.7 Hz), 2.52 (1H, dd, J = 14.2, 4.1 Hz), 2.66 (1H, dd, J = 13.5, 5.5 Hz), 2.72 (1H, dd, J = 14.2, 6.3 Hz), 2.83 (1 H, m, H-9), 4.35 (1H, dd, J = 6.3, 4.1 Hz), 4.55 (1H, dd, J = 8.7, 5.5 Hz), 5.81 (1H, d, J = 11.2 Hz, H-7), 6.35 (1H, d, J = 11.2 Hz, H-6). LR-MS *m*/*z* (%): 646 (M^+, 1), 598 (11), 571 (100), 439 (29). HR-MS *m*/*z*: 571.4009 (M^+−*t*-Bu–H_2O) (Calcd for $C_{34}H_{59}O_3Si_2$: 571.4003).

4.4. (*E*)- and (*Z*)-(20*S*)-1α-[(*tert*-Butyl-dimethylsilyl)oxy]-2-cyanomethylene-25-hydroxy-19-norvitamin D_3 *tert*-butyl-dimethylsilyl ether (16)

To a stirred solution of diethyl (cyanomethyl)phosphonate (24 μL, 0.148 mmol) in dry THF (1 mL) at −40 °C was added *n*-BuLi (95 μL, 0.151 mmol, 1.58 M solution in hexane). The mixture was stirred for 15 min after which time a solution of **15** (48.7 mg, 0.075 mmol) in dry THF (1.2 mL) was added dropwise. Stirring was continued for 1.5 h at −40 °C, the mixture was quenched with saturated NH_4Cl, and extracted with AcOEt. The AcOEt layer was washed with brine dried over $MgSO_4$, and evaporated in vacuo. The residue was purified by chromatography on silica gel (5 g) using 10% AcOEt in hexane to afford **16a** (*E*-isomer) and **16b** (*Z*-isomer) (50.0 mg, 99%) as a mixture of two isomers in a ratio of ca. 1:1.

LR-MS *m*/*z*(%): 669 (no M^+), 651 (16), 594 (89), 567 (100), 519 (28), 462 (14), 408 (8). HR-MS *m*/*z*: 651.4841 (M^+−H_2O) (Calcd for $C_{40}H_{69}O_2NSi_2$: 651.4867). Compound **16a** (*E*-isomer): ^{1}H NMR ($CDCl_3$) δ : 0.054, 0.067, 0.099, 0.121 (each 3H, s, 4× Si–Me), 0.55 (3H, s, H-18), 0.83, 0.92 (each 9H, s, 2× Si–*t*-Bu), 0.86 (3H, d, J = 6.5 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.80 (1H, m, H-9), 3.12 (1H, m, H-10), 4.46 (1H, m, H-1), 4.99 (1H, t, J = 2.8 Hz, H-3), 5.47 (1H, d, J = 1.8 Hz, C=CHCN), 5.82 (1H, d, J = 11.1 Hz, H-7), 6.19 (1 H, d, J = 11.1 Hz, H-6). Compound **16b** (*Z*-isomer): ^{1}H NMR ($CDCl_3$) δ: 0.063, 0.075, 0.112, 0.132 (each 3H, s, 4× Si–Me), 0.54 (3H, s, H-18), 0.83, 0.92 (each 9H, s, 2× Si–*t*-Bu), 0.86 (3H, d, J = 6.5 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.80 (1H, m, H-9), 2.99 (1H, m, H-10), 4.57 (1H, m, H-3), 5.04 (1H, t, J = 2.8 Hz, H-1), 5.47 (1H, d, J = 1.8 Hz, C=CHCN), 5.79, (1H, d, J = 11.1 Hz, H-7), 6.32 (1H, d, J = 11.2 Hz, H-6, 7).

4.5. (*E*)- and (*Z*)-(20*S*)-1α-[(*tert*-Butyl-dimethylsilyl)oxy]-2-(2-hydroxy-ethylidene)-25-hydroxy-19-norvitamin D_3 *tert*-butyl-dimethylsilyl ether (17)

To a stirred solution of **16** (20.0 mg, 0.030 mmol, **16a**:**16b** = ca. 1:1) in dry toluene (1 mL) at −78 °C was added dropwise diisobutylaluminum hydride (60 μL, 0.060 mmol, 1.0 M solution in hexane). The mixture was stirred for 3 h at −78 °C and for 1 h at −20 °C. Additional diisobutylaluminum hydride (30 μL, 0.030 mmol) was added, and stirring was continued for

5.5 h at −20 °C. After addition of saturated potassium sodium tartrate, the mixture was poured into ice water and extracted with AcOEt. The AcOEt phase was washed with brine, dried over $MgSO_4$, and concentrated to dryness. The crude product was dissolved in ethanol (EtOH, 1 mL) and sodium borohydride ($NaBH_4$, 1.1 mg, 0.030 mmol) was added portionwise at 0 °C. After being stirred for 1 h, the mixture was poured into ice water and extracted with AcOEt. The organic layer was washed with brine and dried over $MgSO_4$. Solvent was removed in vacuo, and the residue was purified by chromatography on silica gel (4 g) using 15% AcOEt in hexane to give **17a** (5.5 mg, 27%) and **17b** (5.0 mg, 25%). The unreacted starting material **16** was recovered (7.5 mg, 38%).

Compound **17a** (*E*-isomer): ^{1}H NMR ($CDCl_3$) δ: 0.02, 0.07, 0.08 (3H, 3H, 6H, s, 4× Si–Me), 0.55 (3H, s, H-18), 0.85, 0.92 (each 9H, s, 2× Si–*t*-Bu, overlapped with H-21), 1.22 (6H, s, H-26, 27), 2.29 (2H, m, H-4), 2.79 (1H, m, H-9), 2.88 (1H, dd, J = 12.7, 4.3 Hz, H-10), 4.19 (1H, dd, J = 12.7, 6.8 Hz, CH_2OH), 4.31 (1H, dd, J = 12.7, 6.7 Hz, CH_2OH), 4.37 (1H, dd, J = 9.7, 4.3 Hz, H-1), 4.81 (1H, t, J = 3.8 Hz, H-3), 5.72 (1H, t, J = 6.8 Hz, C=CH), 5.85 (1H, d, J = 11.2 Hz, H-7), 6.15 (1H, d, J = 11.2 Hz, H-6). Compound **17b** (*Z*-isomer): ^{1}H NMR ($CDCl_3$) δ: 0.01, 0.07, 0.08, 0.09 (each 3H, s, 4× Si–Me), 0.54 (3H, s, H-18), 0.83, 0.93 (each 9H, s, 2× Si–*t*-Bu), 0.85 (3H, d, J = 6.5 Hz, H-21), 1.22 (6H, s, H-26, 27), 2.14 (1H, br t, J = ∼11.5 Hz, H-4), 2.55 (1H, dd, J = 12.3, 5.0 Hz, H-4), 2.82 (2H, m, H-9, 10), 4.22 (1H, dd, J = 12.3, 7.1 Hz, CH_2OH), 4.30 (each 1H, dd, J = 12.7, 7.0 Hz, CH_2OH), 4.47 (1H, m, H-3), 4.86 (1H, t, J = 3.1 Hz, H-1), 5.72 (1H, m, C=CH), 5.81 (1H, d, J = 11.1 Hz, H-7), 6.25 (1H, d, J = 11.1 Hz, H-6).

4.6. (*E*)- and (*Z*)-(20*R*)-1α,25-Dihydroxy-2-(2-hydroxyethylidene)-19-norvitamin D_3 (3b and 4b)

A mixture of **17a** (11.0 mg, 0.016 mmol) and (−)-10-camphor sulfonic acid (11.4 mg, 0.049 mmol) in dry MeOH (0.5 mL) was stirred for 2 h at ambient temperature. $NaHCO_3$ (5%) was added, and the solution was extracted with AcOEt. The organic phase was washed with brine, dried over $MgSO_4$, and evaporated in vacuo. The residue was chromatographed on silica gel (3 g) using 3% MeOH in AcOEt to afford **3b** (6.4 mg, 88%).

Compound **3b**: ^{1}H NMR ($CDCl_3$) δ: 0.54 (3H, s, H-18), 0.86 (3H, d, J = 6.5 Hz, H-21), 1.21 (6H, s, H-26, 27), 2.42 (2H, m, H-4), 2.81 (1H, m, H-9), 3.15 (1H, d, J = 12.8, 4.9 Hz, H-10), 4.15 (1H, dd, J = 12.4, 5.9 Hz, CH_2OH), 4.39 (2H, m, H-1, CH_2OH), 4.84 (1H, m, H-3), 5.80 (1H, m, C=CH), 5.88 (1H, d, J = 11.1 Hz, H-7), 6.29 (1H, d, J = 11.1 Hz, H-6). LR-MS *m*/*z* (%): 446 (M^+, 9), 428 (9), 410 (21), 392 (82), 374 (100), 263 (84). HR-MS *m*/*z*: 446.3407 (Calcd for $C_{28}H_{46}O_4$: 446.3396). UV λ_{max} (EtOH): 246 (ε 30,700), 254 (ε 34,800), 263 (ε 23,000) nm.

The same procedure as described above, but using **17b** (11.0 mg, 0.016 mmol) and (−)-10-camphor sulfonic acid (11.4 mg, 0.049 mmol) in dry MeOH (0.5 mL) gave **4b** (4.8 mg, 66%).

Compound **4b**: ^{1}H NMR ($CDCl_3$) δ: 0.55 (3H, s, H-18), 0.85 (3H, d, J = 6.5 Hz, H-21), 1.21 (6H, s, H-26, 27), 2.21 (1H, br t, J = ∼13 Hz, H-4), 2.33 (1H, dm, H-10), 2.70 (1H, d, J = 12.8, 4.7 Hz, H-4), 2.82 (2H, m, H-9, 10), 4.24 (1H, dd, J = 12.6, 6.4 Hz, CH_2OH), 4.38 (1H, dd, J = 12.6, 7.4 Hz, CH_2OH), 4.46 (1 H, m, H-3), 4.87 (1H, t, J = 4.2 Hz, H-1), 5.83 (2H, m, H-7, C=CH), 6.40 (1H, d, J = 11.1 Hz, H-6). LR-MS *m*/*z* (%): 446 (M^+, 9), 428 (10), 410 (31), 392 (100), 374 (79), 263 (84). HR-MS *m*/*z*: 446.3385 (Calcd for $C_{28}H_{46}O_4$: 446.3396). UV λ_{max} (EtOH): 246, 254, 263 nm.

4.6.1. Reagents and animals. 1,25-$(OH)_2D_3$ was a gift from Mercian Corp. (Tokyo, Japan). 19-Norvitamin D analogs (**3**–**10**) and 2MD were synthesized in our laboratory[18–20] and these structures are shown in Figure 1. [26,27-Methyl-^{3}H]-1,25-$(OH)_2D_3$ (specific activity 6.62 TBq/mmol) was purchased from Amersham (Buckinghamshire, UK). All vitamin D samples were dissolved in 95% EtOH. Concentrations of vitamin D samples were determined by ultraviolet (UV) spectroscopy using the molar absorptivity ε = 34,800 (λ_{max} 254 nm) for 2-ethylidene analogs (**3**–**4**), ε = 40,500 (λ_{max} 252 nm) for 2-hydroxyethoxy analogs (**5**–**6**), ε = 32,600 (λ_{max} 251 nm) for 2-epoxy analogs (**7**–**8**), and ε = 37,500 (λ_{max} 252 nm) for 2-methyl-2-hydroxy analogs (**9**–**10**). Vitamin D receptor (VDR) binding assays were carried out using a bovine thymus VDR kit (Yamasa Shoyu, Co., Ltd, Chiba, Japan). Radioactivity was determined with a TRI-CARB 1900TR Analyzer (PACKARD) liquid scintillation counter using a liquid scintillation cocktail ACS-II obtained from Amersham (Buckinghamshire, UK). GM-CSF was purchased from PeproTech EC Ltd, (London, UK). LPS was purified from Escherichia coil strain K235 as described.[39] Five- to 8-week-old male ddY mice were obtained from Japan SLC, Inc. (Shizuoka, Japan) and male weanling rats (Sprague–Dawley strain) were maintained for 3 weeks on vitamin D-deficient diet containing 0.03% Ca and 0.06% phosphorus (Teklad, Madison, USA).

4.6.2. Vitamin D receptor binding assay. Bovine thymus VDR receptors were dissolved in 0.05 M phosphate buffer (pH 7.4) containing 0.3 M KCl (45 mL) and 5 mM dithiothreitol just before use. The receptor solutions (500 μL) were pre-incubated with an increasing amount of 1,25-$(OH)_2D_3$ (10^{-11}–10^{-6} M) or 19-norvitamin D analogs (**3**–**10**) in 50 μL of EtOH for 60 min at 25 °C. [^{3}H]-1,25-$(OH)_2D_3$ (∼5000 cpm) in 50 μL of EtOH was added, and the receptor mixture was left to stand overnight at 4 °C. Two hundred microliters of dextran-coated charcoal suspension (Yamasa Shoyu Co., Ltd.) was added, and the mixture was vortexed. After 30 min at 4 °C, the bound-free (B-F) separation (separation of vitamin bound to VDR [B] from vitamin D unbound to VDR [F]) was carried out by centrifugation at 3000 rpm for 10 min. Then, 500 μL aliquot of the supernatant was mixed with ACS-II (9.5 mL) in each vial, and the radioactivity was measured with a liquid scintilla-

tion counter. Each assay was performed at least twice in duplicate. The relative VDR-binding affinity of the 19-norvitamin D analogs was calculated by comparison of the compound concentrations required for 50% displacement of [^{3}H]-1α,25-$(OH)_2D_3$ from the receptor protein.

4.6.3. Transfection and transactivation assay. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS, JRH Bioscience, Lenexa, USA). Cells were seeded in 24-well plates at a density of 2×10^4 per well 24 h before transfection. The next day, the medium was replaced with 250 μL of fresh serum-free medium (Opti-MEM). Then a DNA/Trans IT-LT1 reagent (Mirus, Madison, USA) mixture containing 0.28 μg of a receptor plasmid (SPP × 3-TK-Luc), 0.2 μg of wild-type hVDR expression plasmid (pCMX-hVDR), and 0.02 μg of the internal control plasmid containing sea pansy luciferase expression constructs (pRL-CMV) was prepared according to the manufacturer's procedures and added to each well. The SPP × 3-TK-Luc reporter plasmid contains three copies of the mouse osteopontin vitamin D response element. The cells were further incubated for 4 h and the medium was replaced with fresh DMEM containing 5% FBS pretreated with dextran-coated charcoal. The next day, transfected cells were treated with either 1,25-$(OH)_2D_3$ (10^{-7}–10^{-11} M), 19-norvitamin D analogs (10^{-6}–10^{-14} M) or EtOH vehicle and cultured for 16 h. Cells in each well were harvested with a cell lysis buffer, and the luciferase activity was measured with a luciferase assay kit (Toyo Ink, Inc., Tokyo, Japan) according to the manufacturer's instruction. Transactivation measured by the luciferase activity was normalized with the luciferase activity of the same cells determined by the sea pansy luciferase assay kit (Toyo, Ink). All experiments were done in triplicate.

4.6.4. Osteoclast differentiation assay. Bone marrow cells were obtained from tibiae of 5- to 8-week-old male mice of the ddY strain. Primary osteoblastic cells were prepared from the calvariae of newborn ddY mice as previously described.[39,40] Briefly, mouse bone marrow cells (1.5×10^5 cells/well) and primary osteoclasts (3×10^3 cells/well) were cocultured for 7 days in the presence of 1,25-$(OH)_2D_3$, 2MD or 19-norvitamin D analogs (10^{-8}–10^{-12} M) in 0.3 mL of α-MEM (Sigma, St. Louis, USA) supplemented with 10% FBS in 48-well plates. Cells were replenished on day 3 with fresh medium. Cells were then fixed with 10% formaldehyde in PBS and stained for tartrate-resistant acid phosphatase (TRAP) as described.[40,41] TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts, under microscopic examination. The results were expressed as means ± SEM of three cultures.

4.6.5. Measurement of bone calcium mobilization. Twenty-day-old weanling male rats from the low vitamin D colony were purchased from SLC, Inc. and fed the vitamin D-deficient diet containing 0.03% calcium and 0.6% phosphorus diet for 3 weeks. The rats were given 1 μg of 19-norvitamin D analogs or 1,25-$(OH)_2D_3$ in 50 μL of EtOH by jugular injection. After 6, 12, and 24 h, blood was taken to determine serum calcium and rats were euthanized. Serum calcium concentration was determined by colorimetric assay using *o*-cresolphthalein complexone (Calcium C-test Wako, Wako Pure chemical, Osaka, Japan) according to the manufacturer's instructions. Serum calcium was measured with a spectrometer model BECKMAN DU-600. Statistical analysis was done by Student's *t*-test.

4.6.6. Preparation of mouse bone marrow macrophage. Bone marrow cells obtained from 4- to 6-week-old ddY male mice were suspended in α-MEM supplemented with 10% FBS in 100-mm diameter dishes (10^7 cells/10 mL/dish) in the presence of M-CSF (100 ng/mL). After the cells were cultured for 4 days, the adherent cells were harvested by treatment with 0.05% trypsin–EDTA (Life Technology, Inc.) for 5 min. The harvested cells were resuspended in RPMI 1640 medium (Sigma) supplemented with 5% FBS. At day 0, cells were seeded in 24-well plates (2×10^5 cell /2 mL/well) with GM-CSF (5 ng/mL) in the presence of increasing concentrations of 1,25-$(OH)_2D_3$ or 19-norvitamin D analogs. On day 2, half of the cells was stained with biotin-conjugated anti-CD11c antibody followed by streptavidin–fluorescein isothiocyanate (BD PharMingen, San Diego, USA). On the other hand, the other half of the cells was replaced with fresh medium containing the same concentration of GM-CSF, 1,25-$(OH)_2D_3$, and 19-norvitamin D analogs. LPS (1 μg/mL final concentration) was added to the cultures to stimulate the maturation of dendritic cells. After further culturing for 1 day, the cells were stained with phycoerythrin-conjugated CD86 antibody (from BD PharMingen, San Diego, USA). The stained cells were assessed on a FACS can flow cytometer (Becton Dickinson, Mountain View, USA), and data were analyzed with Cell Quest software (Becton Dickinson, Mountain View, USA). All data with standard deviation (SD) are mean values for at least three independent experiments.

4.6.7. Total RNA extraction and RT-PCR analysis. For reverse-transcribed PCR analysis, bone marrow cells prepared from ddY mice were cultured as described above. At day 7, total RNA was extracted from mouse bone marrow cells by the Trizol method (Invitrogen, CA, USA) according to the manufacturer's instruction manual.

Total RNA (1 μg) was reverse-transcribed using Superscript II (Invitrogen, USA) according to the manufacturer's protocols. Aliquots of the obtained cDNA pool were subjected to PCR amplification with Go *Taq* DNA polymerase (Promega Co., USA). Primers for mouse IL-12 and GAPDH used in this study are as follows: IL-12, 5′-cagatagcccatcaccctgt-3′ (forward), 5′-acg gccagagaaaaactgaa-3′ (reverse); and GAPDH, 5′-gaaggt cggtgtgtaacggatttggc-3′ (forward), 5′-catgtaggccatgaggt ccaccac-3′ (reverse). The PCR program was as follows: an initial denaturation at 94 °C for 2 min was followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s, and then a final extension step at 72 °C for 5 min. PCR prod-

ucts were separated by 2% agarose gel electrophoresis and were stained with ethidium bromide.

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