Synthesis and Biological Activities of 14-*epi*-MART-10 and 14-*epi*-MART-11: Implications for Cancer and Osteoporosis Treatment

ATSUSHI KITTAKA[1], HIDEKI HARA[1], MASASHI TAKANO[1], DAISUKE SAWADA[1], MIDORI A. ARAI[1], KENICHIRO TAKAGI[2], TAKAYUKI CHIDA[2], YOSHIFUMI HARADA[2], HIROSHI SAITO[2], KAZUYA TAKENOUCHI[2], SEIICHI ISHIZUKA[2], KEIKO HAYASHI[3], SHINICHI IKUSHIRO[3], TOSHIYUKI SAKAKI[3], TAKAYUKI SUGIURA[1] and TAI C. CHEN[4]

[1]*Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Sagamihara, Kanagawa 229-0195;*
[2]*Teijin Institute for Bio-Medical Research, Teijin Pharma Ltd., Tokyo 191-8512;*
[3]*Faculty of Engineering, Toyama Prefectural University, Toyama 939-0398, Japan;*
[4]*Boston University School of Medicine, Boston, MA 02118, U.S.A.*

Abstract. *The 14-epimer of MART-10, namely 14-epi-MART-10 (14-epi-2α-(3-hydroxypropyl)-1α,25-dihydroxy-19-norvitamin D_3) and its 2-epimeric analog (14-epi-MART-11) were efficiently synthesized using the Julia coupling reaction to connect between the C5 and C6 positions (steroid numbering). An A-ring precursor was prepared from (–)−quinic acid as shown in the previous MART-10 synthesis. The novel 14-epi-CD−ring coupling partner with an elongated two carbon unit as a sulfone was synthesized from 14-epi-25-hydroxy Grundmann's ketone in good yield. The subsequent coupling reaction followed by a deprotection step afforded a mixture of 14-epi-MART-10 and 14-epi-MART-11 in 40% yield. To separate 14-epi-MART-10 and 14-epi-MART-11, each primary hydroxyl group was esterified with a pivaloyl group and the resulting pivalates 2α and 2β were separated by high performance liquid chromatography. After the separation, the C2-stereochemistry of each (2α or 2β) was determined by ^{1}H NMR (nuclear magnetic resonance) studies including NOE (nuclear Overhauser effect) experiments. The pivaloyl group was removed under basic conditions to obtain the target molecules of 14-epi-MART-10 and 14-epi-MART-11, respectively. The VDR (vitamin D receptor)-binding affinity, HL-60 (human promyelocytic leukemia) cell differentiation activity, antiproliferative activity in PZ-HPV-7 (immortalized normal prostate) cells and transactivation activity of the osteocalcin promoter in HOS (human osteoblast cell line) cells (serum-free conditions) were investigated. In addition, the effects on bone mineral density (BMD) and the blood and urine calcium concentrations of ovariectomized (OVX) rats were examined. 14-epi-MART-10 has much greater antiproliferative and cell differentiation activities compared to 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$$D_3$).*

Correspondence to: Atsushi Kittaka, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Sagamihara, Kanagawa 229-0195, Japan. Tel: +81 426853713, Fax: +81 426853713, e-mail: akittaka@pharm.teikyo-u.ac.jp

Key Words: Synthesis, 14-*epi*-MART-10, 19-norvitamin D_3, Julia coupling, PZ-HPV-7 cells, HL-60 cells.

The potent calcemic effect of 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$$D_3$, **1**) prevents the application of pharmacological doses for cancer chemotherapy, even though 1α,25(OH)$_2$$D_3$ is able to inhibit cancer cell growth. Therefore, several thousand elaborate vitamin D_3 analogs have been synthesized to develop safer drugs than 1α,25(OH)$_2$$D_3$ (1-7). In our group, the systematic synthesis of vitamin D_3 analogs with C2-modifications has been initiated and a number of C2-modified analogs with greater vitamin D receptor (VDR)-agonist activity than 1α,25(OH)$_2$$D_3$ have been successfully synthesized (8-12). We have also synthesized several highly potent VDR-antagonists, which belong to a series of TEI-9647 analogs with C2α-functionalization as well as 24-alkyl modification on the lactone ring (13). (23*S*,24*S*)-2α-(3-Hydroxypropoxy)-24-propylvitamin D_3-26,23-lactone has been found to exhibit approximately 850-fold greater antagonistic activity (IC_{50}=7.4 pM) than the parent antagonist, TEI-9647 (IC_{50}=6.3 nM) (14, 15). The mechanism of the enhanced C2α-effects on VDR binding has been explained by X-ray crystallographic analyses of the VDR-ligand complexes (16).

The active 19-norvitamin D_3, 1α,25-dihydroxy-19-norvitamin D_3 (**2**, Figure 1) is known to have a selective activity profile, combining high potency in inducing differentiation of malignant cells with very low or no bone calcification activity (17, 18).

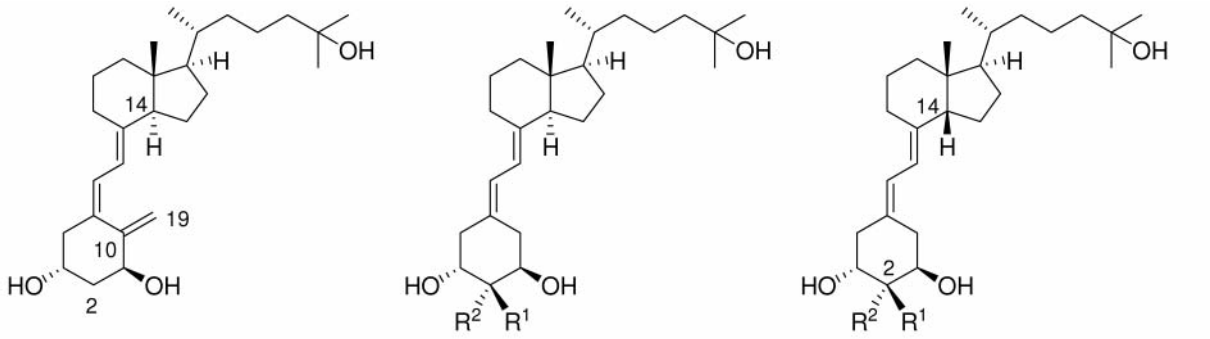

Figure 1. *Structures of active vitamin D_3 (1α,25(OH)$_2$D$_3$) (**1**); 19-nor-1α,25(OH)$_2$D$_3$ (**2**); MART-10, MART-11, 14-epi-19-nor-1α,25(OH)$_2$D$_3$ (**3**); 14-epi-MART-10, and 14-epi-MART-11.*

The C2-modified active 19-norvitamin D_3 analog, MART-10 (2α-(3-hydroxypropyl)-1α,25-dihydroxyvitamin D_3), is 36 times more potent than 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$D$_3$) (**1**) in inducing HL-60 (human promyelocytic leukemia) cell differentiation (19, 20). MART-10 is also *ca*. 500- to 1000-fold and 10-fold more active than **1** in inhibiting the proliferation of immortalized PZ-HPV-7 normal prostate cells and the invasion of PC-3 prostate cancer cells, respectively (21). Removal of the 10(19)-methylene group from the natural hormone **1** reduces the VDR-binding affinity due to its loss of hydrophobic interaction with the ligand binding domain (LBD) of the VDR. The binding affinity of **2** has been reported to be only 30% and 17% of **1** for the porcine VDR (22) and for the calf thymus VDR (19), respectively. MART-10, however, showed almost the same level of VDR-binding affinity as **1** due to the addition of the 2α-(3-hydroxypropyl) group. X-ray crystallographic analysis of the VDR-(2α-(3-hydroxypropyl)-1α,25-dihydroxyvitamin D_3) complex has clearly demonstrated that the terminal hydroxyl group of 2α-(3-hydroxypropyl)-1α,25-dihydroxyvitamin D_3 forms hydrogen bonding with Arg-274 and replaces one of the water molecules in the ligand binding domain of the VDR to stabilize the complex (16).

Bouillon's group found that 14-*epi*-19-norvitamin D analogs, TX522 and TX527, possess a much enhanced antiproliferative action on breast cancer cells *in vitro* and *in vivo*, with much lower calcemic effects compared to **1** (23). The X-ray crystallographic analysis of the VDR-TX522 complex revealed that TX522 binds to the hVDR with modified contacts of C12–Val300 and C22–Ile268 compared with **1**, which may increase interactions between the VDR and coactivators (24). Therefore, two C2-substituted 19-norvitamin D_3 epimers, 14-*epi*-MART-10 and 14-*epi*-MART-11 were synthesized and their biological activities were evaluated *in vitro* and *in vivo*.

Materials and Methods

Chemistry. The Julia coupling reaction was used to connect the A-ring structure **10** to the 14-*epi*-CD-ring structure **9** as described previously for the synthesis of MART-10 (19). First, the A-ring part **10** was prepared from (−)-quinic acid using radical allylation for a carbon-carbon bond formation at the C2 position (19, 25). Epimerization at the C14 position of 25-hydroxy Grundmann's ketone **4** (26) under basic conditions generated a mixture of 14*S*-ketone **5** and 14R-ketone **4** (the starting material) with a ratio of 6 to 1 at equilibrium at room temperature (27). The major isomer **5** with the 14S configuration was isolated by silica-gel column chromatography in 75% yield. This isomer was treated with MOM-Cl (methoxymethyl chloride) to protect the 25-hydroxyl group to form **6** in 89% yield. The subsequent two-carbon unit elongation at the C8 position of **6** was accomplished through Horner-Wadsworth-Emmons reaction, yielding α,β-unsaturated ester (7) (Figure 2). The E/Z (entgegen/zusammen) ratio of the olefinination was 50/1 and the stereochemistry of the major isomer **7** was determined by NOE (nuclear Overhauser effect) experiments, in which NOEs were observed between vinylic H7 and H14 and also between H14 and H18. The ester **7** was reduced by DIBAL-H (diisobutylalminium hydride) to yield allylic alcohol **8**. The subsequent sulfonylation with 2-mercaptobenzothiazole under Mitsunobu conditions followed by molybdenum(VI)-catalyzed oxidation afforded sulfone **9** in 82% yield (Figure 2), which was the substrate for the Julia coupling reaction with the A-ring ketone **10**.

The ketone **10** and sulfone **9** were coupled using LiHMDS (lithium hexamethyldisilazide) in THF (tetrahydrofuran) at –78˚C, and then the protecting groups of the coupling products were removed in acidic conditions to obtain 14-*epi*-2-(3-hydroxypropyl)-19-norvitamin D_3 (**11**) as a diastereoisomeric mixture due to the C2 stereochemistry. The products were converted to pivalates 2α-**12** and

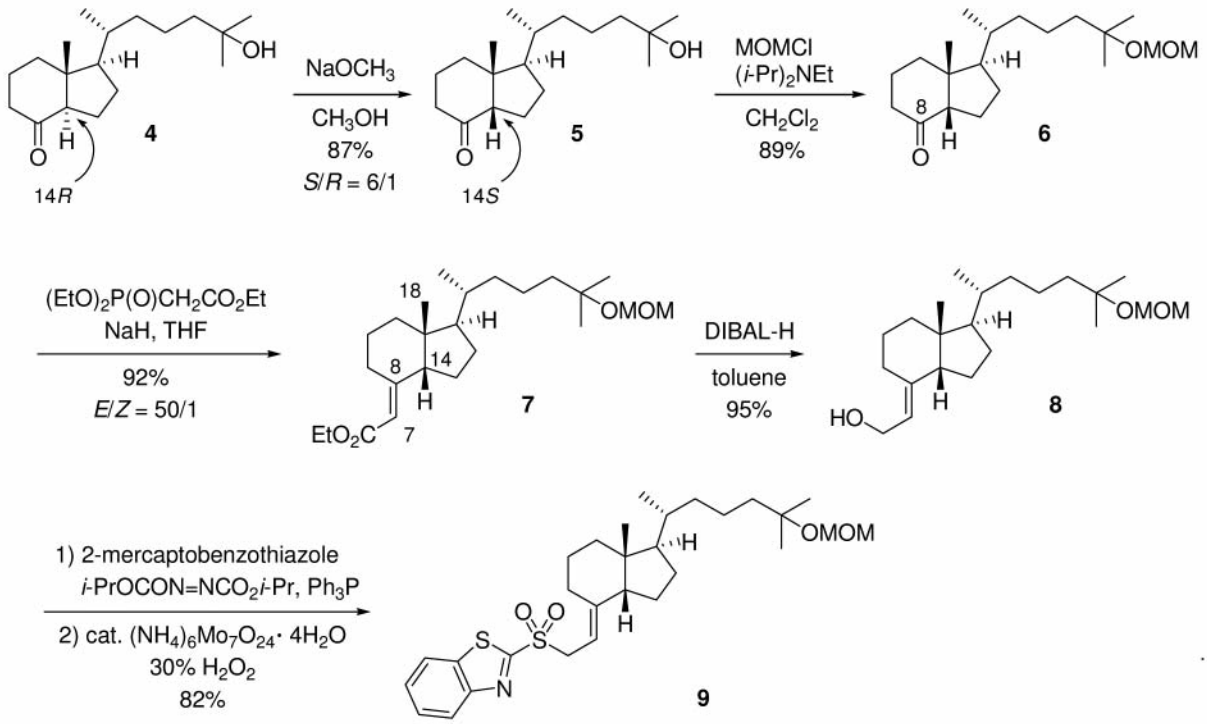

Figure 2. *Synthesis of the 14-epi-CD-ring sulfone* ***9*** *from 25-hydroxy Grundmann's ketone* ***4*** *(substrate for the Julia coupling). MOMCl: methoxymethyl chloride; THF: tetrahydrofuran; DIBAL-H: diisobutylalminium hydride; cat: catalyst.*

2β-**12**, to be separated from each other by HPLC (high performance liquid chromatography). The stereochemistries of the C2 position of 2α-**12** and 2β-**12** were determined by ^{1}H NMR (nuclear magnetic resonance) experiments, including NOE observation, as shown in previous publications of MART-10 and MART-11 synthesis (19, 20). Finally, the pivaloyl group was removed from each isomer under basic conditions to obtain the target molecules, 14-*epi*-MART-10 and 14-*epi*-MART-11 (Figure 3).

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 analog to be tested were dissolved in 50 μL of absolute ethanol in 12×75-mm polypropylene tubes. Chick intestinal VDR (0.2 mg) and 1 mg of gelatin in 1 mL of phosphate buffer solution (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. One milliliter of 40% polypropylene glycol 6000 in distilled water was added to each tube, which was mixed vigorously and centrifuged at 2,260×*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 analog 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) (28).

HL-60 cell differentiation and transactivation of human osteocalcin promoter. The ability of the three 14-*epi*-19-norvitamin D analogs to induce HL-60 differentiation (23, 29) and VDR transactivation of the osteocalcin promoter in HOS (human osteoblast cell line) cells (30) was also compared to the natural hormone.

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 fetal calf serum (FCS). Exponentially proliferating cells were collected, suspended in fresh medium and seeded in culture plates (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA). The cell density at seeding was adjusted to 2×10^4 cells/mL and the seeding volume was 1 mL/well. An ethanol solution of 1α,25-dihydroxyvitamin D_3 (final concentration: 10^{-8} M) and an analog (final concentration: 3×10^{-12} 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 (29). Briefly, cells were collected, washed with phosphate-buffered saline (PBS), and suspended in serum-free medium. NBT/TPA (12-*O*-tetradecanoylphorbol 13-acetate) 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.

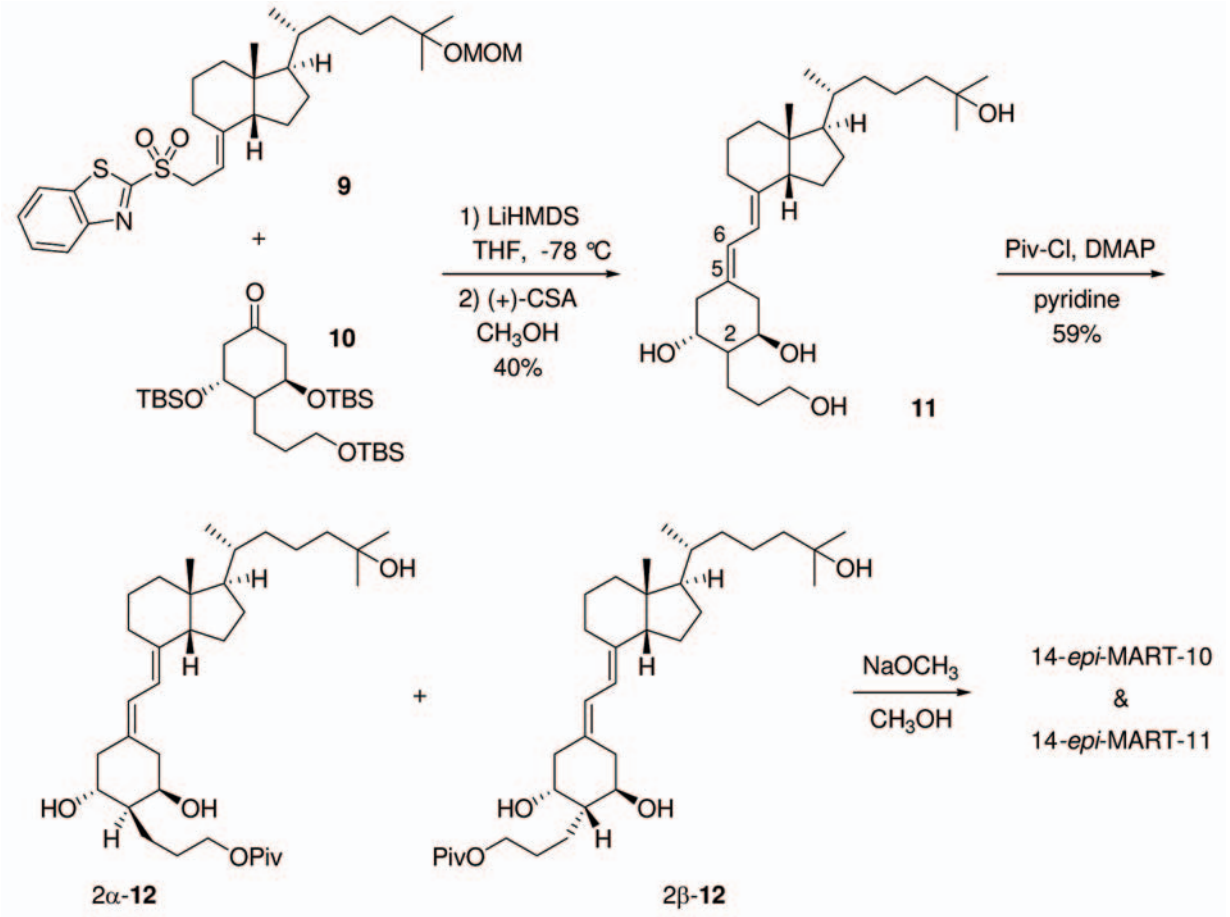

Figure 3. *Julia coupling reaction between A-ring ketone **10** and CD-ring sulfone **9** to connect C5-C6 positions and subsequent separation of C2-isomers as pivalates 2α-**12** and 2β-**12**. Deprotection of each pivaloyl group gave 14-epi-MART-10 and 14-epi-MART-11, respectively. OMOM in **9**: methoxymethoxy group; LiHMDS: lithium hexamethyldisilazide; THF: tetrahydrofuran; CSA: camphorsulfonic acid; Piv-Cl: pivaloyl chloride; DMAP: N,N-dimethyl-4-aminopyridine.*

The human osteocalcin gene promoter fragment –838/+10 was cloned into the reporter plasmid pGL3 (Promega) as described previously (30). Human *VDR* and *RXR* gene were cloned into expression vector pCDNA3 (Invitrogen). HOS cells maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% FCS (fetal calf serum) (Invitrogen). Prior to transfections, the cells were plated in a 96-well plate at the density of 400,000 cells per well in the Opti-MEM (Opti-MEM I Reduced-Serum Medium) (Invitrogen). The cells were transfected with human osteocalcin reporter vector (pGL3-hOc: 100 ng/well), human *VDR* and *RXR* expression vector (pCDNA-*hVDR*, pCDNA-*hRXR*: 10 ng/well) and phRL-TK (Promega: 25 ng/well) using 50 μL of Lipofectamine 2000 reagent (Invitrogen). After incubation at 37˚C for 3 h, the cells were treated with ethanol vehicle and various concentrations of compounds (from 0.1 pM to 100 nM). After incubation at 37˚C for 24 h, the cells were collected, washed with PBS(–), and the luciferase activity of the cells was quantitated by luminomator (Berthold) using Dual-Glo luciferase assay reagent (Promega).

Antiproliferative effects. The effects of 1α,25(OH)$_2$D$_3$ (**1**), 14-*epi*-19-nor-1α,25(OH)$_2$D$_3$ (**3**), 14-*epi*-MART-10 and 14-*epi*-MART-11 on cell proliferation were studied in cultured PZ-HPV-7 prostate cells obtained from ATCC (Manassas, VA, USA) and grown in serum-free keratinocyte growth medium supplemented with pituitary extract and epidermal growth factor as described previously (31).

[^{3}H]-Thymidine incorporation into DNA was used as an index of cell proliferation. Briefly, when PZ-HPV-7 cells reached about 50% confluence, media with growth factors were replaced with fresh basal media without growth factors (31), and the cells were grown for an additional 24 h. Cells were then treated with **1**, **3**, 14-*epi*-MART-10 and 14-*epi*-MART-11. The final concentrations of these compounds incubated with cells were from 10^{-6} M to 10^{-10} M. Eighteen hours later, the media were replaced with 0.5 mL of fresh basal medium containing methyl-[^{3}H]-thymidine and incubated for 3 hours at 37˚C. [^{3}H]-Thymidine incorporation into DNA was stopped by placing the plates on ice. Unincorporated [^{3}H]- thymidine was then removed and the cells were washed three times with ice-cold PBS. DNA labeled with [^{3}H]-thymidine and other macromolecules were precipitated with ice-cold 5% perchloric acid for 20 min and then extracted with 0.5 mL of 5% perchloric acid at 70˚C for 20 min. The radioactivity in the extracts was determined by a liquid scintillation counter as described previously (31).

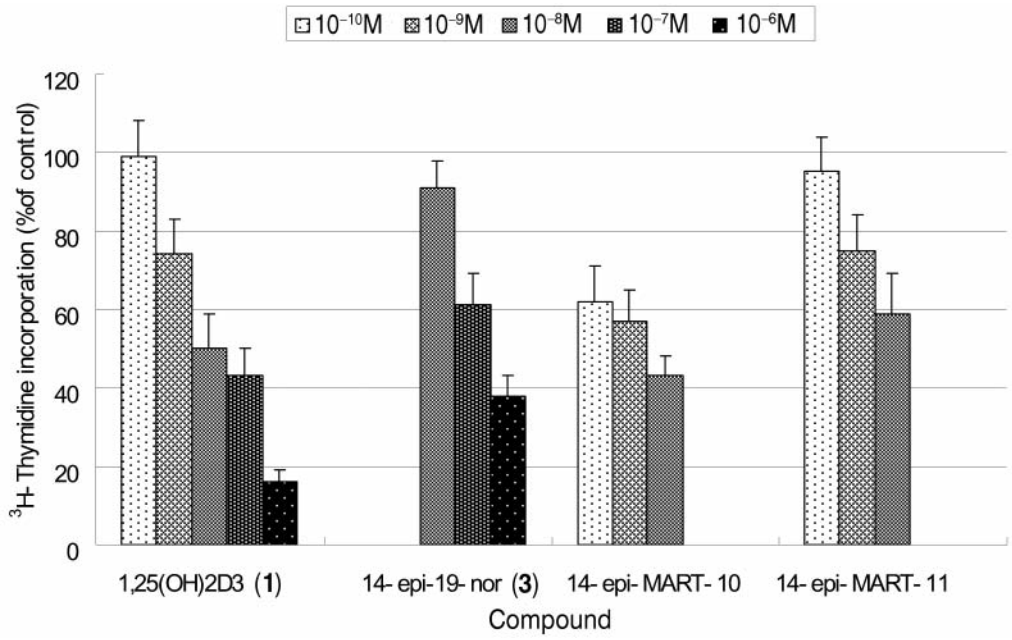

Figure 4. *Antiproliferative activity of 1α,25(OH)$_2$D$_3$, 14-epi-19-nor-1α,25(OH)$_2$D$_3$ (**3**), 14-epi-MART-10 and 14-epi-MART-11 in PZ-HPV-7 prostate cells.*

Effects on bone mineral density (BMD) and calcemic activity. The effect on calcemic activity in the serum and urine as well as on bone mineral density (BMD) was studied using ovariectomized (OVX) adult female Sprague-Dawley (SD) rats (Charles River Laboratories Japan Inc.). SD female rats at 12 weeks of age were ovariectomized (OVX) or sham-operated. Four weeks after the operation, the animals were orally administered the tested compound 5 times/week for 4 weeks. Under halothane anesthesia, the BMD of the fourth through fifth lumbar vertebrae (L4-L5) was measured by dual-energy X-ray absorptiometry (QDR 2000, Hologic, Inc., Waltham, MA, USA). The calcium (Ca) concentration was measured with an autoanalyzer (7180 Clinical Analyzer, Hitachi, Tokyo, Japan). The animal blood was drawn from the abdominal aorta under ether anesthesia, and the blood samples were centrifuged to obtain the supernatants which were stored at –20˚C until Ca measurement. To collect the animal urine, the animals were housed in metabolic cages for 24 h after the last administration of drugs.

Results and Discussion

VDR-binding affinity, induction of HL-60 cell differentiation and osteocalcin promoter transactivation activity. As previously reported, the binding affinity of MART-10 for the calf thymus VDR was almost at the same level as **1**, although 1α,25-dihydroxy-19-norvitamin D$_3$ (**2**) had only 17% of the binding affinity for the VDR compared to 1α,25(OH)$_2$D$_3$ (19). As shown in Table I, the relative binding affinity of **3** for the chick intestinal VDR was only 4% of the binding for **1**. The introduction of the 2α-(3-hydroxypropyl) group to **3**, as in 14-*epi*-MART-10, increased the binding affinity up to 27%. On the other hand, the 2β-(3-hydroxypropyl) group did not improve the binding affinity of 14-*epi*-MART-11 for the VDR.

Table I. *In vitro effects of 14-epi-19-norvitamin D$_3$ analogs on VDR binding affinity, induction of HL-60 cell differentiation activity and transactivation activity of osteocalcin promoter in HOS cells.*

Compound	VDR-binding affinity[a,b]	HL-60 cell differentiation[a,c]	Transactivation activity of osteocalcin promoter[a,d]
Natural hormone (**1**)	100	100	100
14-*epi*-19-norVD$_3$ (**3**)	4	53[e]	13
14-*epi*-MART-10	27	794	388
14-*epi*-MART-11	3	87	54

[a]The potency of **1** was normalized to 100. [b]chick intestinal VDR (ref. 28). [c]Determined by a NBT (nitro blue tetrazolium) assay (ref. 29); relative activity was calculated at ED$_{50}$ (effective dose 50). The data are the means of three separate experiments. d VDR-mediated gene regulation in the human osteoblast cell line (HOS / serum free conditions). [e](ref. 23).

Table I shows that **3** was only half as active as the natural hormone **1** in the induction of HL-60 cell differentiation, whereas, 14-*epi*-MART-10 was almost 8-fold more active and 14-*epi*-MART-11 was slightly less active than **1**. Similarly, **3** had only 13% of the transactivation activity of the natural hormone. Again, the introduction of the 2β-(3-hydroxypropyl)

group was not as effective as introducing the 2α-(3-hydroxypropyl) group to **3** at increasing the transactivation activity. For example, 14-*epi*-MART-10 was 3.9 times and 30 times more potent than the natural hormone and **3**, respectively, whereas, 14-*epi*-MART-11 was about half as active as the natural hormone and only four times more active than **3**.

Antiproliferative activity on PZ-HPV-7 prostate cells. The results are shown in Figure 4. The activity of **3** was lower compared to **1**, whereas the introduction of the 3-hydroxypropyl group to the C2-position of **3** recovered the potency and 14-*epi*-MART-11 showed almost the same activity as **1**. Interestingly, 14-*epi*-MART-10 was nearly 10-50 times more active than **1**.

Although 14-*epi*-MART-10 had lower VDR-binding affinity than **1**, it had higher transactivation activity (Table I), which was in agreement with its higher potency than **1** in the ^{3}H-thymidine incorporation and HL-60 cell differentiation assays. CYP(cytochrome P450)24A1-dependent 24-hydroxylation is the first step in the degradation and the termination of various biological activities of **1** and its analogs. Since both MART-10 and 14-*epi*-MART-10 are much more resistant than **1** to CYP24A1-dependent metabolism (unpublished observation), they would have a longer half-life in the cells and in the circulation. This could explain its greater biological activity than **1** *in vitro* and *in vivo* in spite of its lower VDR-binding affinity.

In vivo effects of 14-epi-MART-10 on bone mineral density and calcemic activity. Because 14-*epi*-MART-10 was a potent osteocalcin transactivator in the HOS cells (Table I), the effect on BMD and calcemic activity were investigated and the results are summarized in Table II.

Although **3** at a 1.0 μg/kg/day dose had no effect on BMD, 14-*epi*-MART-10 at 0.1 μg/kg/day induced a marked increase in BMD in the OVX rats under the same treatment regimen without significant calcemic and calciurinic side-effects. At the higher dose (1.0 μg/kg/day) of 14-*epi*-MART-10, however, a calcemic effect became evident.

Table II. *In vivo activity of 14-epi-MART-10*.

Animal group	Compound	Dose (μg/kg)	BMD (g/cm^2)	Ca concentration (mg/dL)	
				Blood	Urine
Sham	Vehicle	–	0.2447±0.0068	10.4±0.24	1.8±1.4
OVX	Vehicle	–	0.2154±0.0158	9.7±0.13	0.8±0.2
OVX	14-*epi*-19-nor VitD$_3$ **3**	1.0	0.2125±0.0197	9.6±0.27	1.5±1.2
OVX	14-*epi*-MART-10	0.1	0.2328±0.0217	10.0±0.17	3.9±1.9
		1.0	0.2462±0.0164	12.4±0.33	20.0±5.5

Ovariectomized (OVX) or sham-operated Sprague-Dawley rats orally administered test compounds 5 times/week for 4 weeks. BMD: Bone mineral density; vehicle: 5% ethanol in saline with 0.2% Triton® X 100.

Conclusion

The novel 14-*epi*-19-norvitamin D$_3$ analogs with the 2-(3-hydroxypropyl) group, 14-*epi*-MART-10 and 14-*epi*-MART-11 can be synthesized efficiently utilizing the Julia coupling reaction to connect between the C5 and C6 positions. Compared to **3**, 14-*epi*-MART-10 shows improved biological activities, including VDR binding affinity, HL-60 cell differentiation activity, osteocalcin transactivation activity in HOS cells, antiproliferative activity in PZ-HPV-7 prostate cells and enhancement of the BMD of OVX rats without significant calcemic effects at 0.1 μg/kg/day dose *in vivo*. 14-*epi*-MART-10 would be a good candidate model compound for designing new and more potent drugs for cancer chemotherapy and/or osteoporosis treatment.

Acknowledgements

We thank Ms. Junko Shimode, Ms. Akiko Tonoki, and Ms. Ayako Kawaji (Teikyo University, Japan) for the spectroscopic measurements. This study was supported in part by Grants-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (No. 17590012 and 19590016 to AK), a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, (No. 20790019 to DS).

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Received January 29, 2009
Revised April 24, 2009
Accepted May 14, 2009