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Systematic studies on synthesis, structural elucidation, and biological evaluation of A-ring diastereomers of 2-methyl-1α,25-dihydroxyvitamin D_3 and 20-epi-2-methyl-1α,25-dihydroxyvitamin D_3

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Abstract

All possible A-ring diastereomers of 2-methyl-1α,25-dihydroxyvitamin D_3 (**2**) and 20-*epi*-2-methyl-1α,25-dihydroxyvitamin D_3 (**3**) were synthesized by palladium-catalyzed coupling reaction of A-ring 'enyne' synthons with CD-ring portions. The A-ring synthons were rationally synthesized via a novel and practical route, starting with methyl (*R*)-(+)- and (*S*)-(-)-3-hydroxy-2-methyl-propionate, in good yields. X-ray crystallographic analysis of 2α-methyl-1α,25-dihydroxyvitamin D_3 (**2b**) and conformational analysis of the A-ring of 2α-methyl-(**2b**) and 2β-methyl-1α,25-dihydroxyvitamin D_3 (**2f**) were carried out, and the results are described. All A-ring diastereomers (**2** and **3**), thus synthesized, were biologically evaluated both in vitro and in vivo. The biologic potency was highly dependent on the stereochemistry of the A-ring substituents. In particular, **2b** showed 4-fold higher vitamin D receptor [VDR] binding activity than the natural hormone, and its 20-epimer (**3b**) exhibited exceptionally high activity, 12-fold more potent in VDR binding, 7-fold in calcium mobilization, and 590-fold in induction of human promyelocytic leukemia (HL-60) cell differentiation as compared with the natural hormone. Further, the 20-*epi*–2β-Me-1β, 3$\alpha$$(OH)_2$ isomer (**3g**) had significant biologic potencies compared to the natural hormone despite having 1β-OH configuration. The transcriptional activities on human osteocalcin gene promoter, including VDRE in transfected mammalian cells, were also evaluated. Finally, there was a clear contrast between the effects of the 2-methyl group on the HL-60 cell differentiation- and apoptosis-inducing activities of **2** and **3**.

Keywords: 2-Methyl-1α; 25-Dihydroxyvitamin D_3; 20-*epi*-2-methyl-1α; 25-Dihydroxyvitamin D_3; Synthesis; A-ring conformation; VDR binding affinity; HL-60 cell differentiation; Apoptosis

1. Introduction

The hormonally active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (**1**), has a wide range of biologic activities including cell differentiating and antiproliferative activities, in addition to its classic role in calcium homeostasis [1], and these activities have been utilized to develop therapeutic agents for cancer, psoriasis, and osteoporosis [2]. A majority of these agents [3] are altered in the side-chain, providing many useful analogues with high potency or selective activity, whereas modification of the A-ring in recent years has afforded other useful analogues, such as ED-71 (Chugai group) [4], 19-nor analogues (DeLuca group) [5] and 1-hydroxymethyl analogues (Posner group) [6] that exhibit unique activity profiles. To investigate the relationship between the structure of A-ring and the various biologic functions of vitamin D, we synthesized A-ring modified 1α,25-$(OH)_2D_3$ analogs.

Our approach to the design and synthesis of A-ring modified analogues is based on the commonly accepted hypothesis [7] that the A-ring conformation is in dynamic equilibrium between two chair conformers, α-form and β-form in 1:1 ratio, and that the β-form, in which 1α-OH takes an equatorial position, may be responsible for the biologic activity. We

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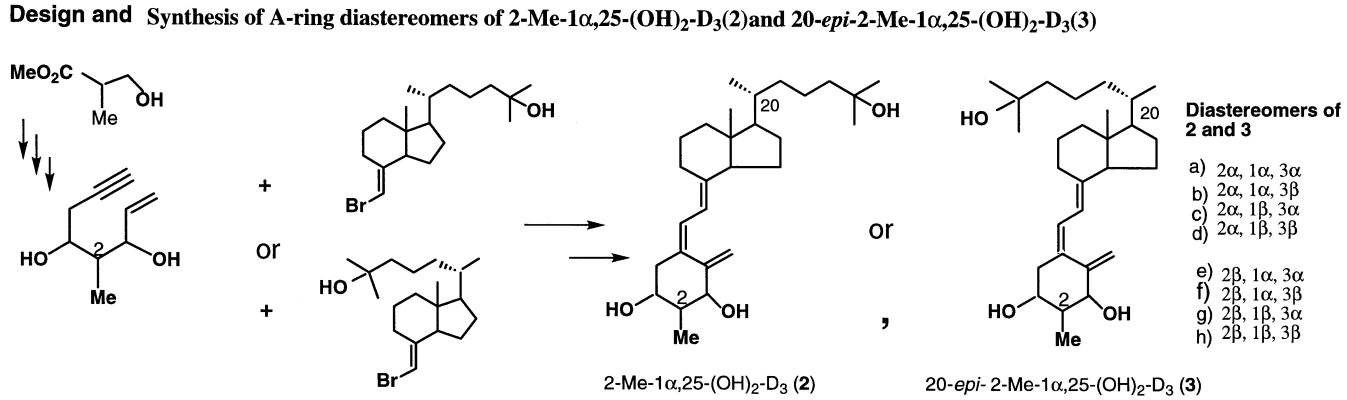
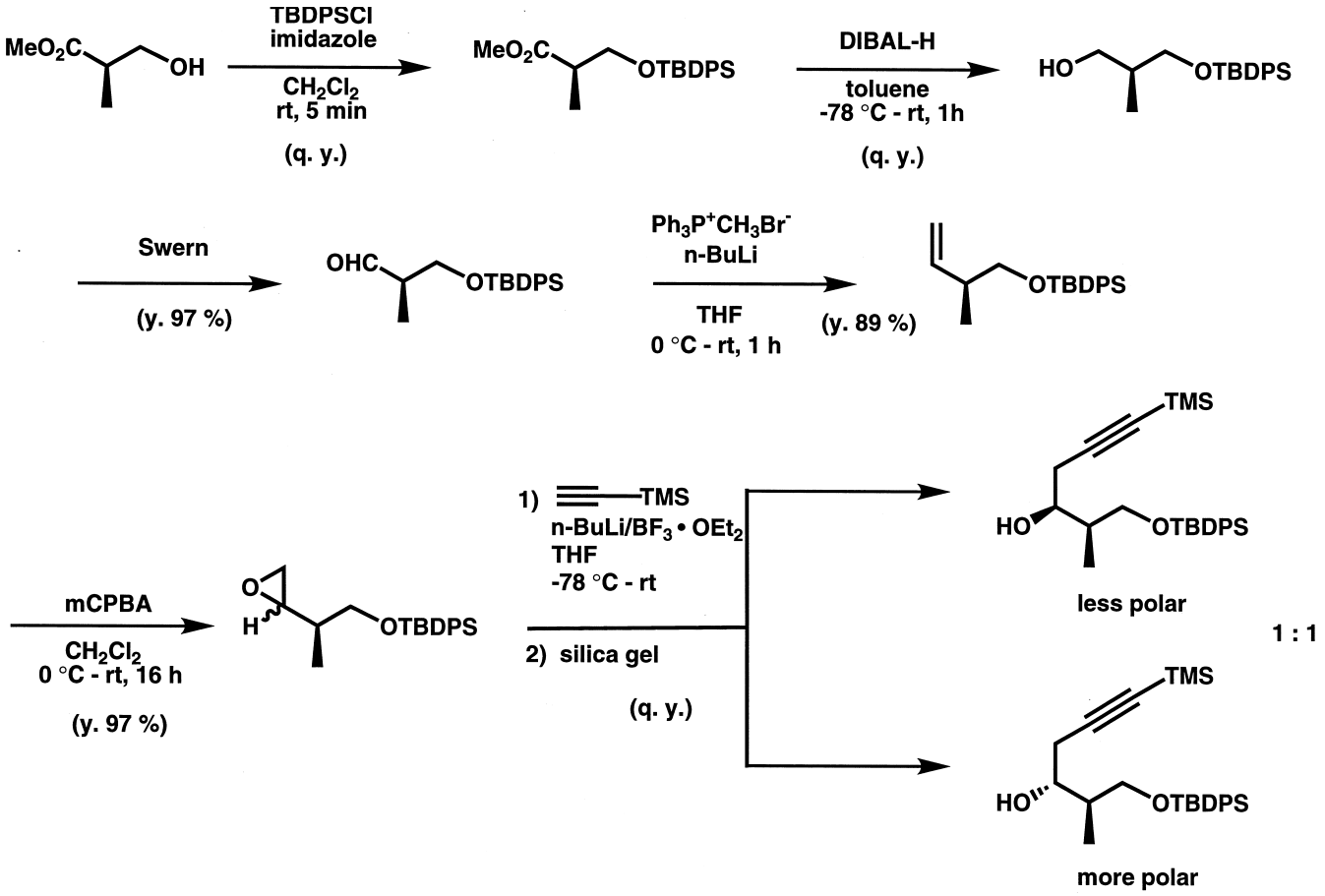


Scheme 1. Design and synthesis of A-ring diastereomers of 2-Me-1α,25(OH)$_2$-D$_3$ (**2**) and 20-epi-2Me-1α,25(OH)$_2$-D$_3$ (**3**).

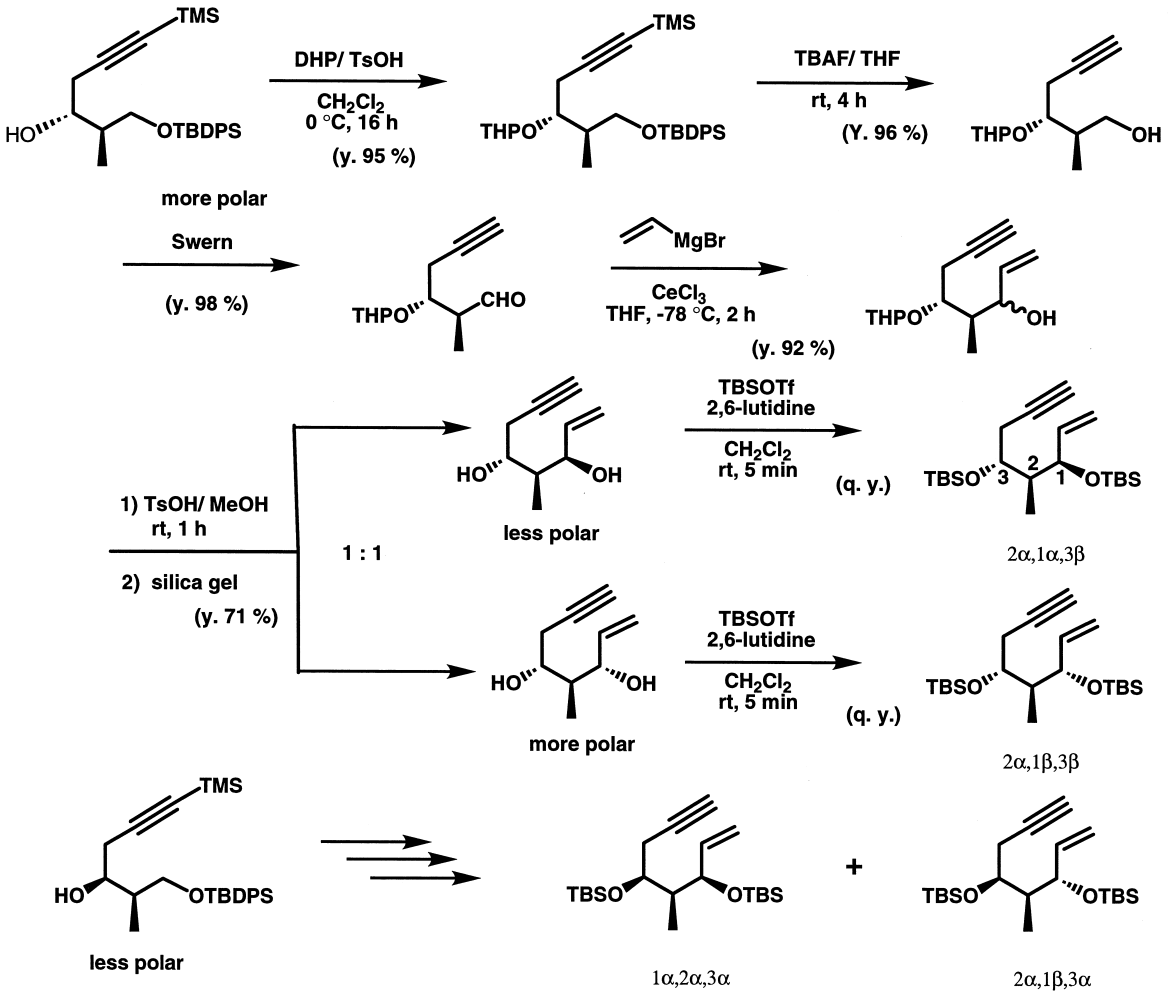
expected that the introduction of a methyl group at the 2- or 4-position would shift the equilibrium, and resultant changes, in conjunction with modification of the stereochemistry of the two hydroxy groups, would cause changes in the spectrum of biologic potency of various vitamin D functions. Therefore, we intended to synthesize all possible A-ring diastereomers of 2-methyl-1α,25-(OH)$_2$D$_3$, to evaluate their biologic activity in detail, and to establish the relationship between the A-ring structure and the potency in a wide variety of vitamin D functions.

2. Design and synthesis

For the above reasons, we first synthesized all eight possible diastereomers (**2**) of 2-methyl-1α,25-1α,25-(OH)$_2$D$_3$, and confirmed that the potency of these compounds varies depending on the configuration of the C-1 and C-3 hydroxy groups, and the stereochemistry of the C-2-methyl group. Furthermore, the remarkable effects of 2-methyl substitution on the potency prompted us to synthesize all possible diastereomers (**3**) of 20-*epi*-2-methyl-



Scheme 2. Synthesis of the 2α-methyl A-ring enynes.



Scheme 3. Synthesis of the 2α-methyl A-ring enynes.

1α,25-$(OH)_2D_3$, double modified in the A-ring and in the side chain (Scheme 1).

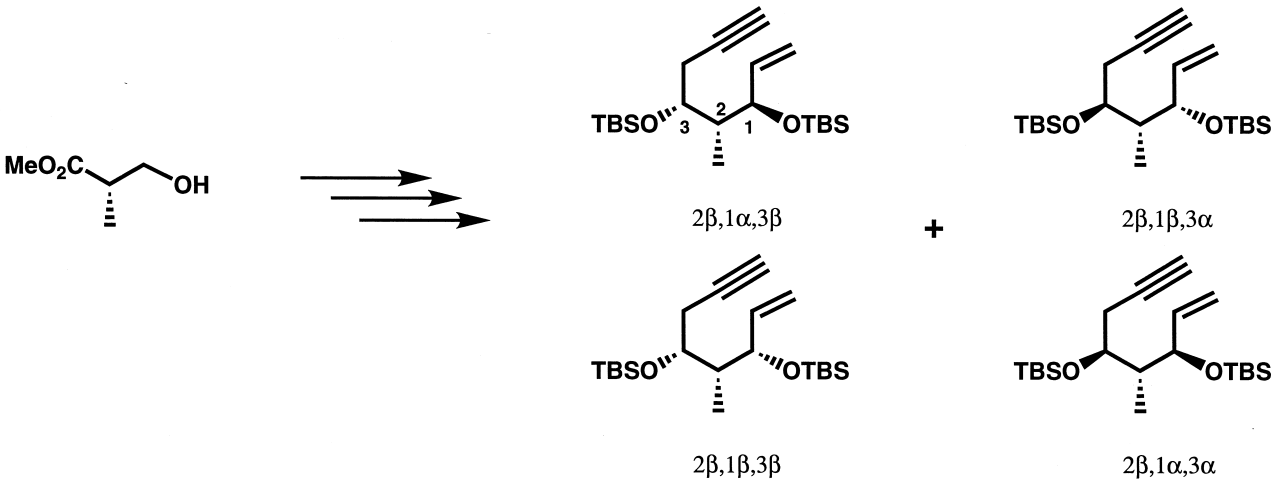
For the synthesis of analogues modified systematically in both the A-ring and the side chain, we adapted the convergent method developed by the Trost group ([8] and references cited therein) employing palladium-catalyzed coupling of the A-ring enyne synthon with the CD-ring portion. Since the synthetic route to the precursor 'enyne' of the A-ring had not been fully established, we have developed a novel and practical route to enynes for all eight possible diastereomers of 2-methyl-1α,25-$(OH)_2D_3$ [9,10].

The synthetic route as exemplified by the 2α-Me-1α-OH–3β-OH isomer is shown in Scheme 2. Commercially available methyl (R)-(-)-3-hydroxy-2-methyl-propionate was converted to the olefin in high yield in a usual manner, and the latter, on treatment with peracid, afforded the epoxide. The acetylene unit was introduced by reaction with the epoxide to give a 1:1 mixture of the alcohols. These isomers were readily separable by silica gel column chromatography, and the absolute configuration at C-3 of each isomer was determined by ^{1}H-NMR analysis of its MTPA esters [11]. The more polar (3*R*)-isomer was protected with THP, followed by treatment with TBAF to furnish the primary alcohol, which was then subjected Swern oxidation to give the aldehyde in excellent yield. The reaction of the aldehyde with Grignard reagent afforded a 1:1 mixture of the diasteromeric allylalcohols. After removal of the THP, the resulting mixture of enyne-diols, (2α,1α,3β) and (2α,1β,3β) [the Greek letters denote the configurations at C-2 (Me), C-1 (OH), and C-3 (OH), respectively] was readily separable by silica gel column chromatography, and the relative stereochemistry of the enyne-1,3-diol in each isomer was determined by ^{13}C-NMR analysis of the acetonide [12] (Scheme 3).

The less polar (3*S*)-isomer was converted to the corresponding enyne-diols (2α,1α,3α), (2α,1β,3α) via the same procedure (Scheme 4). Thus, four diastereomers of 2α-Me-enyne-diol were synthesized.

The four diastereomers of 2β-Me-enyne-diols [(2β,1α,3β), (2β,1β,3α) and (2β,1α,3α), (2β,1β,3β)] were similarly synthesized, stating with (2*S*)-propionic ester.

Finally, palladium-catalyzed coupling of the disilyl ether of these enyne-diols (for example, 2α,1α,3β enyne-

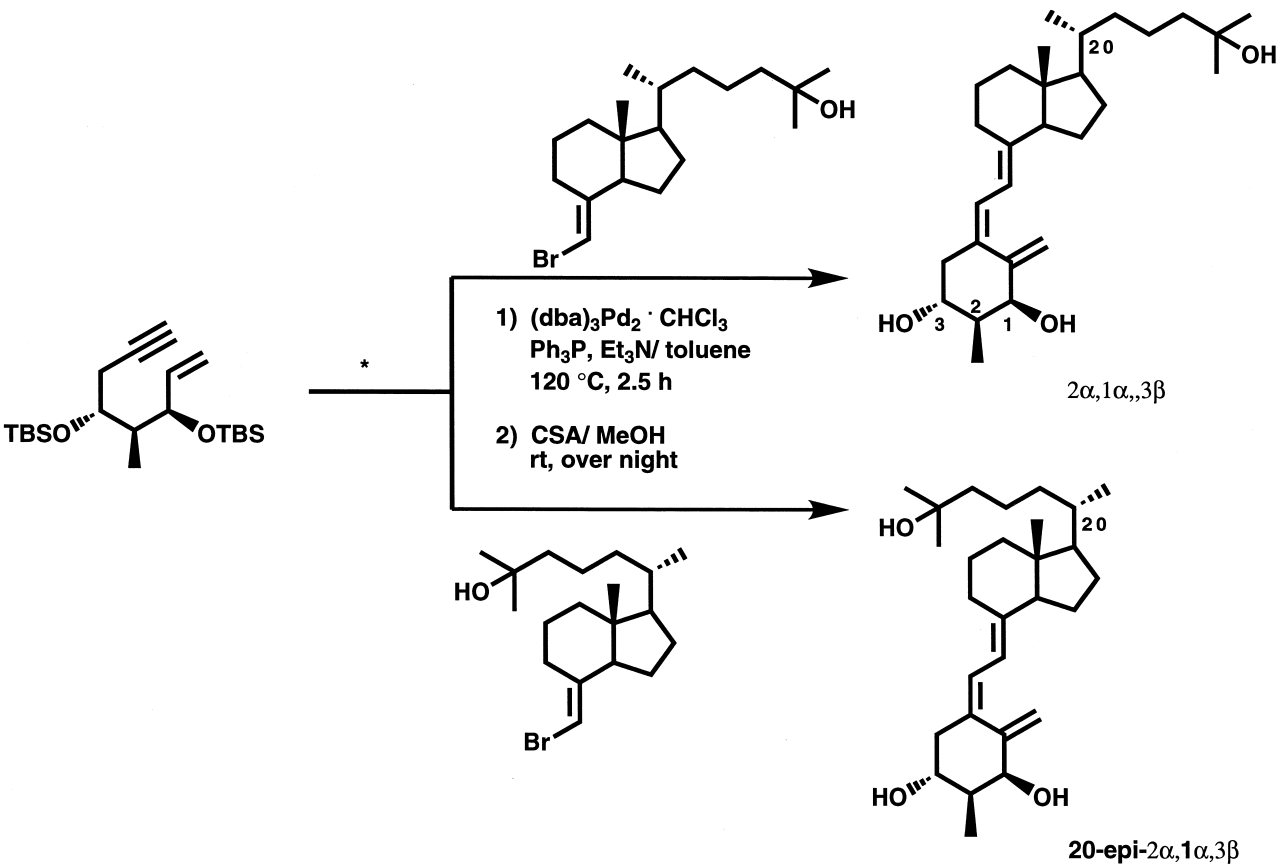


Scheme 4. The 2β-methyl A-ring enynes.

diol), with the CD-ring portion, followed by deprotection with CSA, gave all possible diastereomers of 20*R*- and 20*S*-2-Me-1,25-$(OH)_2D_3$ with the undoubted stereochemistries (2α-Me-1α,25-$(OH)_2D_3$) in good yields (Scheme 5).

3. Structural elucidation

An ORTEP drawing based on X-ray analysis of a fine crystal of 2α-methyl-1α,25-$(OH)_2D_3$ (**2b**), containing water of crystallization, is shown in Fig. 1. The results indicate



*Trost, B. M.; Dumas, J.; Villa, M. *J. Am. Chem. Soc.*, 1992, *114*, 9836.

Scheme 5. Coupling of the A-ring enyne and the CD-ring portion.

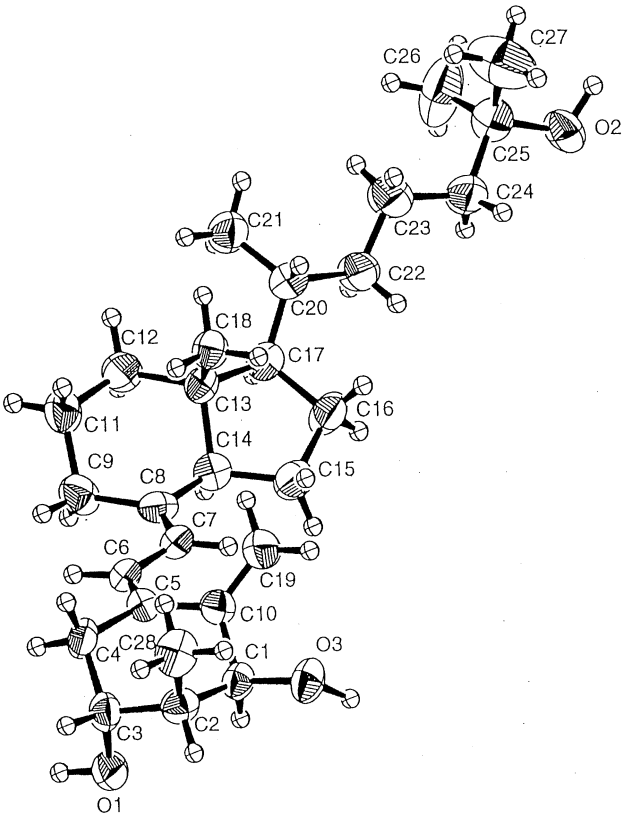


Fig. 1. ORTEP drawing based on X-ray crystallographic analysis of 2α-Me-1α,25-$(OH)_2D_3$.

that the A-ring exists in the chair β-form, in which C(1)-OH takes equatorial and both C(2)-Me and C(3)-OH take axial orientations. This conformation in the crystal, coupled with a consideration of molecular packing, suggests that water in the crystal is involved in hydrogen bonds with the C(1) and C(3) hydroxyl groups [13].

To elucidate the A-ring conformation-activity (VDR affinity) relationships, the ^{1}H-NMR spectra in $CDCl_3$ solution of the A-ring diastereomers thus synthesized were measured and analyzed in detail. In our ^{1}H-NMR analyses, the proportions of α-form to β-form were calculated [14] from two sets of coupling constants, diaxial C(1)-C(2)-H, and diaxial C(2)-C(3)-H, in addition to diaxial C(3)-C(4)-H. In every case, the A-ring is in equilibrium between chair α-form and β-form and the position of equilibrium depending on the stereochemistry. Fig. 2 shows the results of conformational analysis of 2α-methyl- and 2β-methyl-1α,25-$(OH)_2D_3$ compared with the natural hormone. In 2α-methyl-1α,25-$(OH)_2$-D_3, the α-form is predominant over the β-form (60:40), whereas the β-form is predominant over the α-form (75:25) in 2β-methyl-1α,25-$(OH)_2D_3$. Both ratios are supported by molecular mechanics calculation [15] with MM2 *(the energy differences of **2b** and **2f** are 0.95 kcal/mol and 0.93 kcal/mol, and the populations (α-form/β-form) are ca. 83/17 and 17/83 respectively). Recently, DeLuca's group [16] reported that axially oriented C(1)-OH group is required for bioactivity from a study on the A-ring conformation-activity relationship of 2-substituted-19-nor-1α,25-$(OH)_2D_3$.

4. Biologic evaluation

The basic biologic activities [9,10] of the A-ring diastereomers of 20-natural and 20-*epi*-2-Me-1α,25-$(OH)_2D_3$ were evaluated with comparison with those of the natural hormone, 1α,25-$(OH)_2D_3$. The results are summarized in Table 1.

The binding activity of 2-Me isomers to VDR of bovine thymus was highly dependent on the stereochemistry of the A-ring substituents and varied by as much as 10^4 -fold. The 1α-isomers exhibited high affinity, whereas the 1β-isomers had virtually no affinity. It is noteworthy that 2α-Me-1α,25-$(OH)_2$-D_3 (**2b**) showed 4-fold higher affinity than the natural hormone. This is the first example of an A-ring modified analog with the same side chain as natural hormone, having a significantly higher affinity than the natural hormone. This isomer also exhibited 4-fold greater potency in elevation of rat serum calcium concentration. The rank order of potency in HL-60 cell differentiation was almost parallel to that of VDR affinity (**2b** was twice as potent as the natural hormone). In binding to DBP of calf serum, the 1β-isomers showed high affinity, whereas the 1α-isomers had poor affinity, in accordance with reported data. Furthermore, in the 20-*epi* series, the potency also depended on the stereochemistry of the A-ring substituents. Among them, 20-*epi*-2α-methyl-1α,25-dihydroxyvitamin D_3 (**3b**), doubly modified by 2-methyl substitution and 20-epimerization, exhibited exceptionally high potency. It showed 12-fold higher VDR affinity, 6.5-fold higher calcium mobilization and 590 times higher potency in HL-60 cell differentiation, as compared with the natural hormone, and is one of the most potent analogues reported to date (comparable to KH-1060). In binding to DBP, the 20-*epi* isomers had approximately 300 times less affinity than the natural hormone. It is noteworthy that the VDR affinity of 20-*epi*-2β,1α,3β-isomer (**3f**) was comparable to that of the natural hormone, and that cell differentiation activity toward HL-60 cells was higher in **3f** and 20-*epi*-2α,1α,3α-isomer (**3a**) in addition to **3b**. It is also noteworthy that the 20-*epi*–2β,1β,3α isomer (**3g**) had biologic activities [VDR [7], HL-60 [190], Ca [19] compared to the natural hormone [100]] despite having 1β-OH configuration.

With regard to the in vivo activities (intestinal calcium transport and bone calcium mobilization in a D-deficient state), the 20-natural and 20-epi-2α,1α,3β isomers (**2b** and **3b**) both had significantly higher potency than the natural hormone, but no effect of 20-epimerization was observed in this case. The results are shown in part in Table 2.

Finally, to clarify further biologic activity of the analogues synthesized (**2** and **3**), their transcriptional activities on human osteocalcin gene promoter, including VDRE in transfected mammalian cells, were evaluated [17]. The rank

Figure 2 Conformations of 2α-Me- and 2β-Me-1α,25-$(OH)_2$-D_3(2b and 2f)

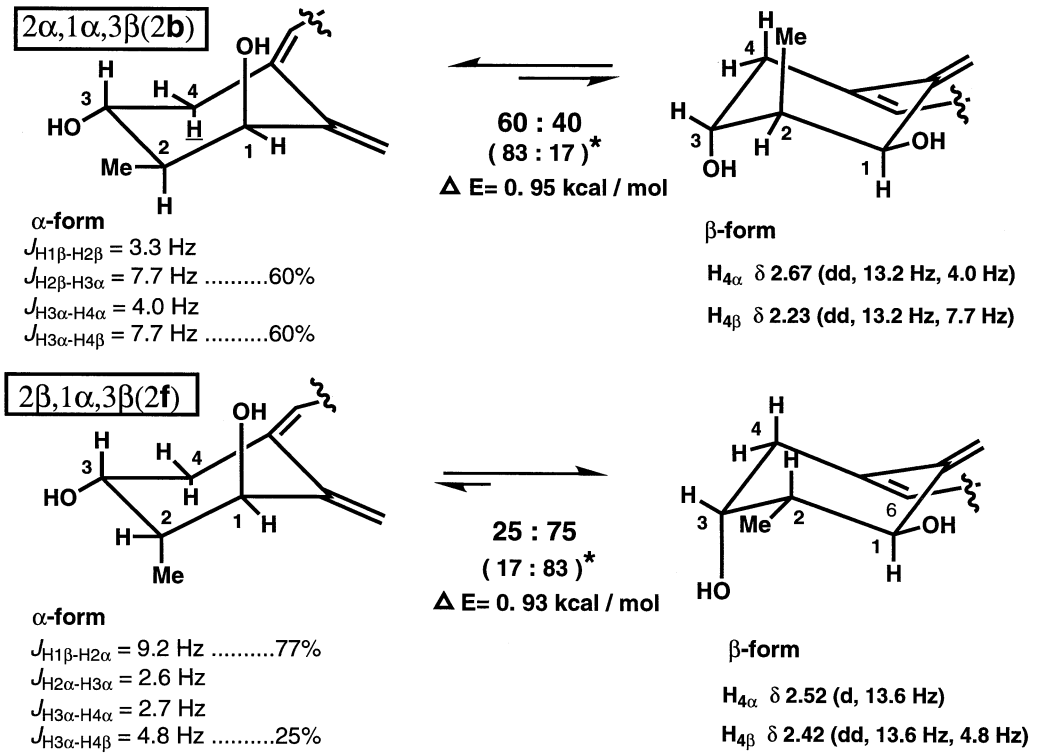


Fig. 2. Conformations of 2α-Me- and 2β-Me-1α,25-$(OH)_2D_3$ (**2b** and **2f**).

Table 1
Basic biological activity of A-ring disastereomers of 2-methyl-1α,25-$(OH)_2D_3$ (**2**) and their 20-epimers (**3**)

Compounds	VDR binding[c]	DBP binding[d]	HL-60 cell differentiation[e]	Ca mobilization[f]
1α,25-$(OH)_2$-D_3 **(1)**[a]	100	100	100	100
2α, 1α, 3α (**2a**)[b]	4	45	13	NT[g]
2α, 1α, 3β (**2b**)	400	68	200	400
2α, 1β, 3α (**2c**)	<0.1	1200	1.5	NT
2α, 1β, 3β (**2d**)	<0.1	200	1	NT
2β, 1α, 3α (**2e**)	<0.3	21	1.5	NT
2β, 1α, 3β (**2f**)	13	79	10	2
2β, 1β, 3α (**2g**)	0.8	1300	3.0	NT
2β, 1β, 3β (**2h**)	<0.1	1000	1.5	NT
20-epi-2α, 1α, 3α (**3a**)	17	<0.3	730	144
20-epi-2α, 1α, 3β (**3b**)	1200	<0.3	59 000	655
20-epi-2α, 1β, 3α (**3c**)	<0.1	<0.3	1	NT
20-epi-2α, 1β, 3β (**3d**)	<0.1	<0.3	3	NT
20-epi-2β, 1α, 3α (**3e**)	<0.1	<0.3	6	NT
20-epi-2β, 1α, 3β (**3f**)	160	<0.3	2600	115
20-epi-2β, 1β, 3α (**3g**)	7	<0.3	190	19
20-epi-2β, 1β, 3β (**3h**)	<0.1	<0.3	1	NT

[a] The activity of 1α,25-$(OH)_2$-D_3 (**1**) is normalized to 100.
[b] The Greek letters denote the configuration of substituents.
[c] Bovine thymus.
[d] Calf serum.
[e] Cell differentiation was assessed in terms of NBT reductivity.
[f] Rat serum calcium level.
[g] Not tested.

Table 2
In vivo activity of intestinal calcium transport and bone calcium mobilization of 2-methyl-1α,25-$(OH)_2$-D_3 and its 20-epimers

Stereoisomer	Amount (pmol/d/7 days)	Intestinal calcium transport (Serosa/Mucosa)	Bone calcium mobilization (Serum Ca in mg/100 ml)
20-natural series			
Exp. 1			
D-Deficient	0	3.8 ± 0.28	4.1 ± 0.26
1α,25-$(OH)_2D_3$	260	5.0 ± 0.20	5.5 ± 0.12
2β, 1α, 3β (**2f**)	260	4.3 ± 0.27	4.1 ± 0.2
	500	4.3 ± 0.16	5.0 ± 0.09
Exp. 2			
D-Deficient	0	2.34 ± 0.83	3.9 ± 0.2
1α,25-$(OH)_2D_3$	260	5.61 ± 0.63	6.1 ± 0.23
2α, 1α, 3β (**2b**)	65	5.9 ± 0.30	5.8 ± 0.08
	260	5.0 ± 0.37	7.6 ± 0.12
20-epi series			
Exp. 3			
D-Deficient	0	2.3 ± 0.49	4.4 ± 0.15
1α,25-$(OH)_2D_3$	130	3.6 ± 0.13	5.4 ± 0.19
	260	4.9 ± 0.33	5.5 ± 0.26
2β, 1α, 3β (**3f**)	130	3.8 ± 0.32	4.6 ± 0.10
	260	4.6 ± 0.57	4.4 ± 0.16
Exp. 4			
D-Deficient	0	2.7 ± 0.34	4.6 ± 0.16
1α,25-$(OH)_2D_3$	260	5.1 ± 0.24	6.1 ± 0.25
2α, 1α, 3β (**3b**)	50	4.3 ± 0.7	5.8 ± 0.21
	100	5.2 ± 0.6	7.8 ± 0.23

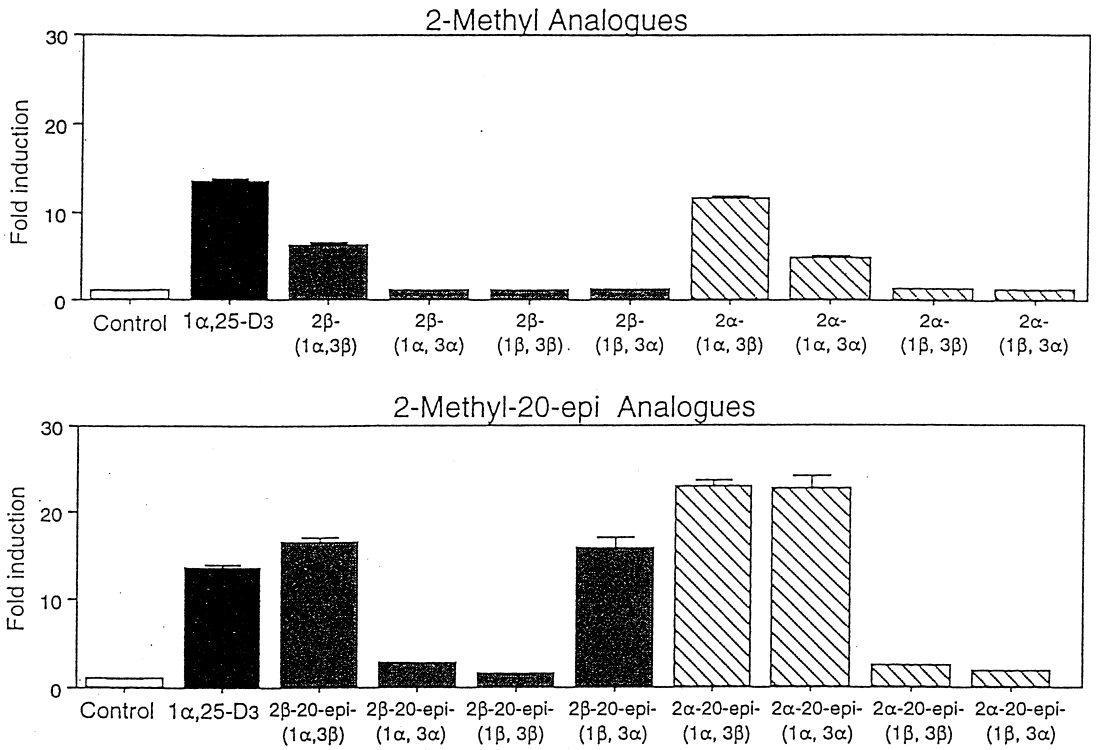


Fig. 3. Transcriptional activities of A-ring diastereomers of 2-methyl-1α,25$(OH)_2$-D_3 (**2**) and their 20-epimers (**3**) on a human osteocalcin gene in human osteosarcoma MG-63 cells.

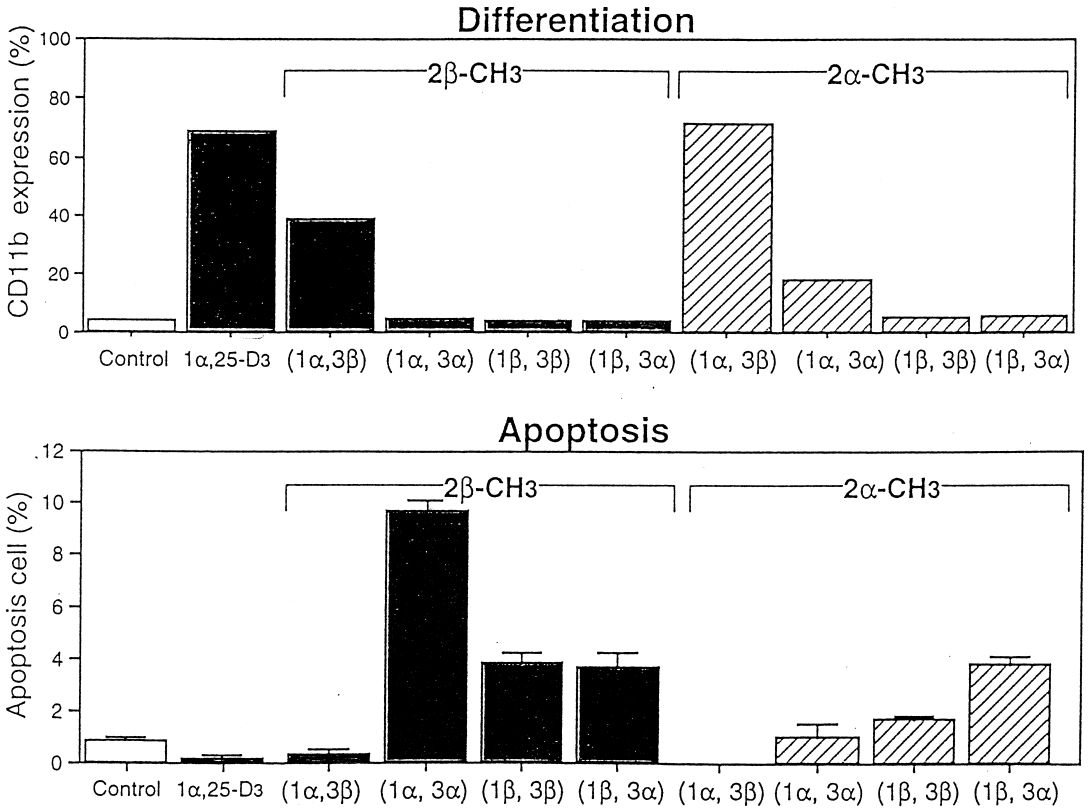


Fig. 4. Differentiation- or apoptosis-inducing effects of A-ring diastereomers of 2-methyl-1α,25(OH)$_2$D$_3$ (2) on HL-60 cells.

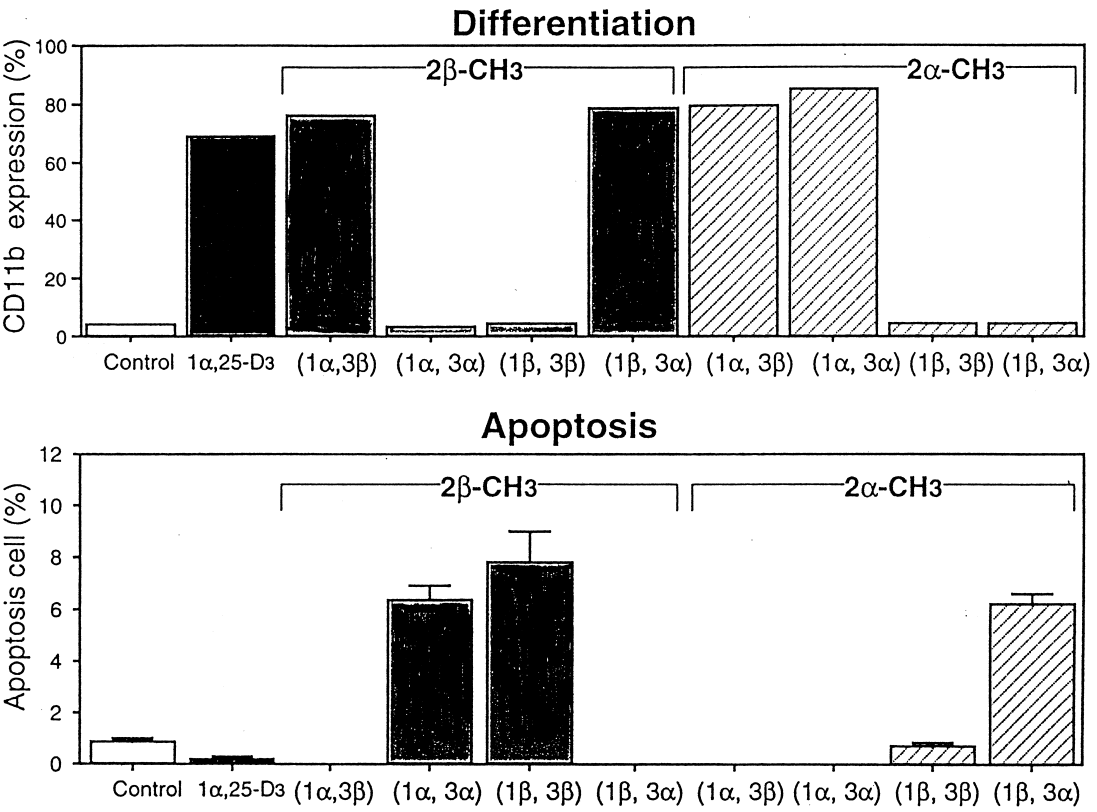


Fig. 5. Differentiation- or apoptosis-inducing effects of A-ring diastereomers of 20-epi-2-methyl-1α,25(OH)$_2$D$_3$ (**3**) on HL-60 cells.

orders of their potencies corresponded to those of VDR affinities and HL-60 differentiation activities. Fig. 3 shows a typical transcriptional activity profile toward human osteocalcin gene in human osteosarcoma (MG-63) cells. The results imply that the 2α-Me group on the A-ring may be a structure-specific motif for stimulating the VDR/VDRE-mediated genomic action of 1α,25-$(OH)_2D_3$.

Furthermore, the effects of the 2-methyl group of **2** and **3** on the activities for differentiation and apoptosis of HL-60 cells were examined [18]. Figs. 4 and 5 show a clear contrast between the cell-differentiation and apoptosis-inducing effects of 2-methyl-1α,25$(OH)_2D_3$ (**2**) and 20-*epi*-2-methyl-1α,25$(OH)_2D_3$ (**3**). The 2α-methyl isomers bearing 1α- and 3β-hydroxy groups strongly induced differentiation, but failed to stimulate apoptosis. In contrast, the 2β-methyl isomers bearing 1β- and 3α-hydroxy groups strongly stimulated apoptosis, but failed to induce differentiation of HL-60 cells. These findings provide useful information not only for studies on the structure-function relationship of 1α,25-$(OH)_2D_3$ analogues but also for the development of therapeutic agents for the treatment of leukemia and cancers.

In conclusion, two sets of A-ring diastereomers of 2-methyl-1α,25-$(OH)_2D_3$ and their 20-epimers were synthesized, and proved to be very useful tools for studies on the vitamin D biology and pharmacology.

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