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Development of 14-*epi*-19-Nortachysterol and Its Unprecedented Binding Configuration for the Human Vitamin D Receptor

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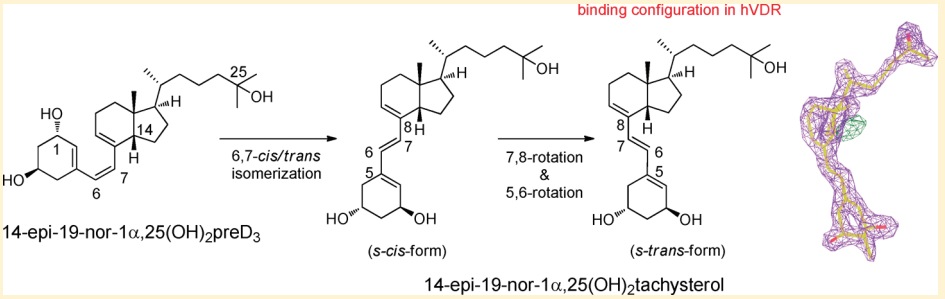
Supporting Information

ABSTRACT:



In the study of the synthesis of 14-*epi*-19-norprevitamin D_3, we found 14-*epi*-19-nortachysterol derivatives through C6,7-*cis*/*trans* isomerization. We also succeeded in their chemical synthesis and revealed their marked stability and potent VDR binding affinity. To the best of our knowledge, this is the first isolation of stable tachysterol analogues. Surprisingly, 14-*epi*-19-nortachysterol derivatives exhibited an unprecedented binding configurations for the ligand binding pocket in hVDR, C5,6-*s*-*trans* and C7,8-*s*-*trans* triene configurations, which were opposite the natural C7,8-ene-configuration of 1α,25$(OH)_2D_3$.

INTRODUCTION

There are several isomers of vitamin D in its biosynthesis pathway; provitamin D, vitamin D, lumisterol, tachysterol, and so on.[1] It is well-known that the most biologically active compound for mammals is the metabolite of vitamin D_3, 1α,25$(OH)_2D_3$ (**1**), which is a ligand of the specific nuclear receptor (vitamin D receptor, VDR), regulates gene transcription, and exhibits various biological responses as a hormone.[2,3] Among them the minor isomers and unstable isomers including their metabolites are not well-established, and details of their biological properties remain to be uncovered. For therapeutic evaluation, most scientists have administered the analogues of the major isomer of vitamin D_3, although it contains 6% of its previtamin D form, 1α,25$(OH)_2$preD_3 (**P1**),[4,5] which is generated from **1** in thermal equilibrium through a [1,7]-hydride shift (Scheme 1).[6]

Regarding this equilibrium, 14-*epi*-vitamin D_3 shows a unique characteristic: the pre-form, 14-*epi*-previtamin D_3 is major and dominant over 14-*epi*-vitamin D_3 in equilibrium, and 14-*epi*-previtamin D_3 should be isolated as the stable compound.[5] Recently, we have focused on the biological activity of the previtamin D form by synthesis of 14-*epi*-1α,25$(OH)_2$previtamin D_3 (**14-*epi*-P1**) and its analogues using this inverse equilibrium.[7] Among these compounds, the 2α-methyl-substituted analogue (2α-methyl-**14-*epi*-P1**) indicated moderate VDR binding activity (8.4% of the natural hormone, Table 1) and transactivation activity of osteocalcin promoter in HOS cells. These results provided important information; compounds in pre-form could have potent genomic activity and encouraged us in the further synthesis of new derivatives. Throughout these studies, however, we were concerned about the minor isomer in equilibrium, **14-*epi*-1**, which must be transformed from isolated **14-*epi*-P1** during biological evaluation and might show some biological activity. To clarify the precise activity of the previtamin D form, we designed new target compounds, 14-*epi*-1α,25$(OH)_2$-19-norprevitamin D_3 (**14-*epi*-19-norP1**), which led neither to the [1,7]-hydride shift nor to thermal equilibrium as natural vitamin D_3. By comparison of the activities of **14-*epi*-19-norP1** and **14-*epi*-P1**, we could understand the real activity of previtamin D form; therefore, we started the synthesis of **14-*epi*-19-norP1** and its 2-methyl substituted analogues.

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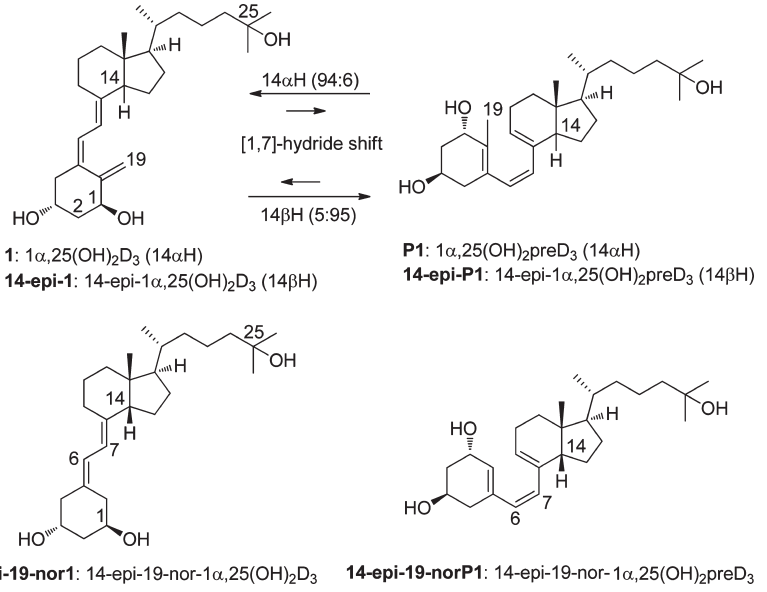
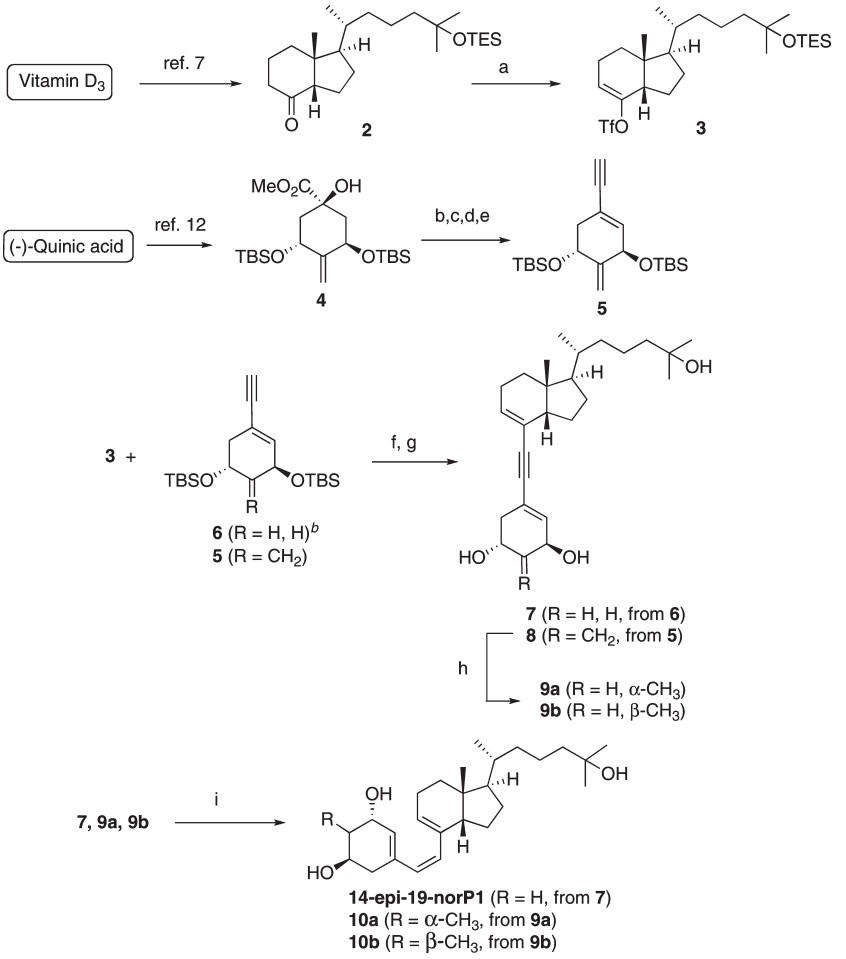
Scheme 1. Equilibrium of Vitamin D_3, 14-*epi*-Vitamin D_3, and Their 19-Nor Analogues



Scheme 2. Synthesis of 14-*epi*-19-Norprevitamin D_3 Analogues[a]



[a] Reagents and conditions: (a) LDA, $PhN(Tf)_2$, THF, yield 82%; (b) $POCl_3$, pyridine, yield 85%; (c) DIBAL-H, toluene, yield 87%; (d) TPAP, NMO, MS4A, CH_2Cl_2; (e) $TMSCHN_2$, nBuLi, THF, yield 61% (2 steps); (f) $Pd(PPh_3)_2(OAc)_2$, CuI, Et_2NH, DMF; (g) TBAF, THF, yield 80% (**7**, 2 steps), yield 99% (**8**, 2 steps); (h) $(Ph_3P)_3RhCl$, H_2, benzene, CH_2Cl_2, yield 43% (**9a**), yield 47% (**9b**); (i) Lindlar cat. quinoline, MeOH, H_2, yield 39%, conversion yield 73% (**14-*epi*-19-norP1**), yield 89% (**10a**), 57% (**10b**). [b] Reference 9e.

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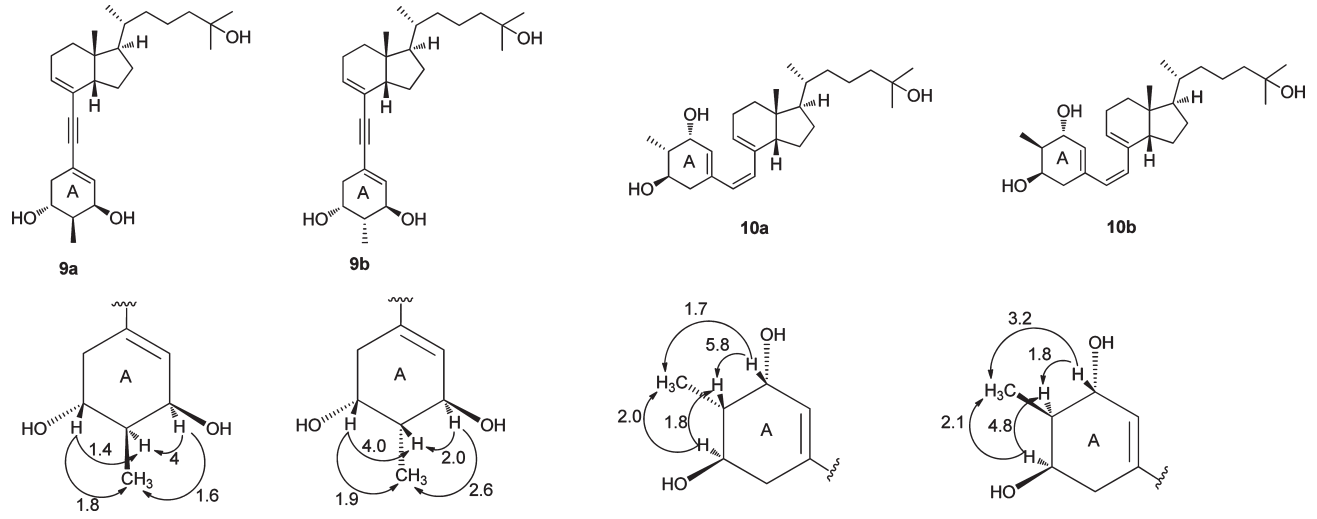
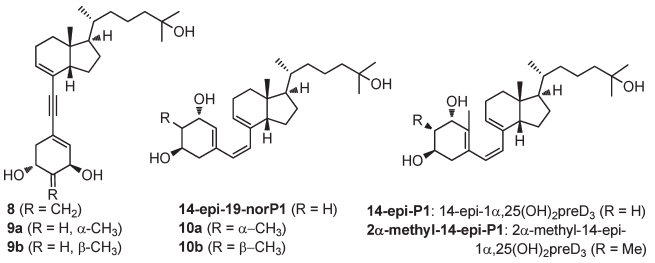


Figure 1. Representative NOE values of compounds **9a**, **9b**, **10a**, and **10b**.

Table 1. Relative Binding Affinity for VDR



compound	VDR[a]
1α,25(OH)$_2$D$_3$ (**1**)	100
8	53
9a	21
9b	9.3
14-*epi*-19-norP1	1.2
10a	1.0
10b	2.9
14-*epi*-P1[b]	0.5
2α-methyl-14-*epi*-P1[b]	8.4

[a] The potency of **1** is normalized to 100. [b] Reference 7.

RESULTS AND DISCUSSION

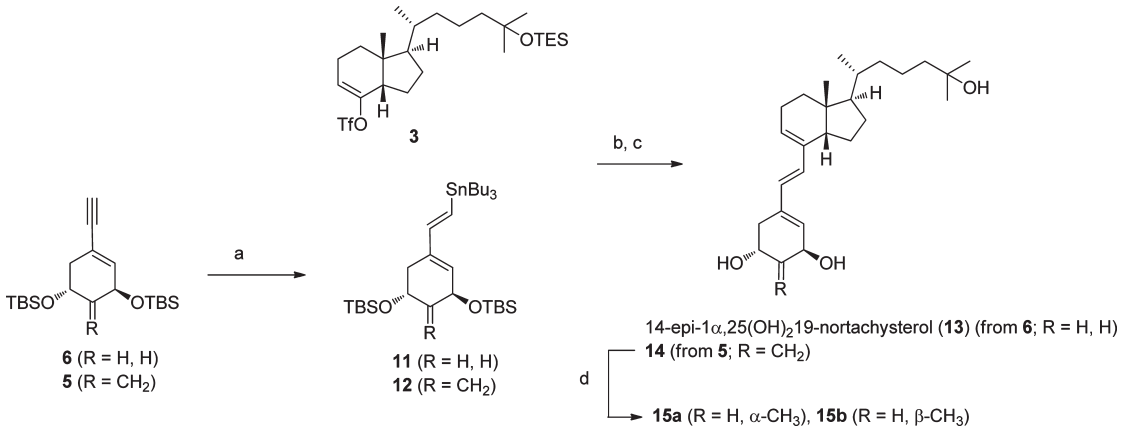
19-Norprevitamin D$_3$ analogues were previously developed by Okamura,[4,8] Mouriño,[9] Gotor,[10] and De Clercq's groups,[11] and we followed their methodology using the coupling reaction between the A-ring fragment and the CD-ring fragment (Scheme 2). The CD-ring fragment **3** was obtained through the known compound **2**[7] from vitamin D$_3$ using LDA and then PhNTf$_2$ in one step.[9a] The known compound **6** was used for the A-ring precursor,[9e] and compound **5**, used for the 2-methyl substituted A-ring precursor, was derived from (−)-quinic acid through the known compound **4** by dehydration with POCl$_3$, reduction of methyl ester, oxidation to aldehyde, and then homologation with TMSCHN$_2$.[8c,12] Thus, we had both A- and CD-ring fragments, and we attempted the Sonogashira coupling reaction.[8a] These reactions proceeded smoothly and gave the coupled compounds which were successively reacted with TBAF to afford the fully deprotected compounds **7** and **8** in excellent yield. By selective reduction of the 2-methylene moiety of **8** with Wilkinson's catalyst,[12] 2-methyl substitution was effectively performed without any influence on the other conjugated unsaturated bonds, and the resultant diastereomers **9a** and **9b** were separated using HPLC. Finally, the partial reduction of C6,7-alkyne moiety of **7**, **9a**, and **9b** with Lindlar catalyst afforded the three target compounds **14-*epi*-19-norP1**, **10a**, and **10b**.[8b]

The stereochemistry of the C2-position in **9a**, **9b** and **10a**, **10b** was determined by NOE experiments. The representative NOE values are described in Figure 1, which reasonably explained the stereochemistry, and we determined that **9a** and **10a** have 2α-methyl substitution and **9b** and **10b** have the 2β-methyl substitution.

Using the new compounds obtained above, we tested their human VDR (hVDR) binding affinity,[13,14] and the results are summarized in Table 1. Ene-yne-enes **8**, **9a**, and **9b** showed moderate affinity, and compound **8** with the 2-methylene substitution was better than the 2-methyl-substituted analogues of diastereomers **9a** and **9b**. Compounds in the pre-form, **14-*epi*-19-norP1**, **10a**, and **10b** showed low affinity, lower than 2α-methyl-14-*epi*-1α,25(OH)$_2$previtamin D$_3$ (2α-methyl-**14-*epi*-P1**), but slightly better than **14-*epi*-P1**.[7] Judging from these results, for the hVDR binding affinity of 2α-methyl-**14-*epi*-P1**, there should be some contribution of its isomer (2α-methyl-**14-*epi*-1**) in the equilibrium (Table 1).

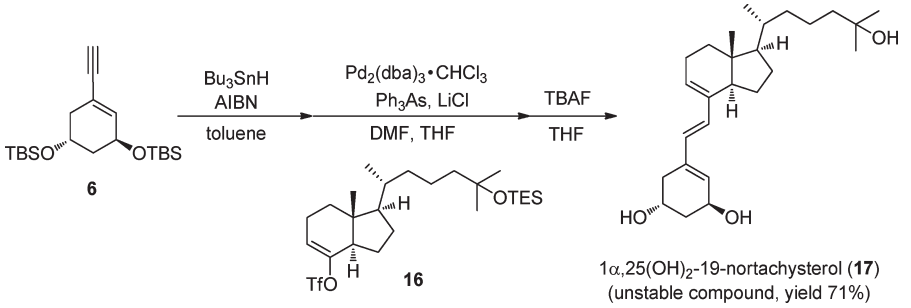
Next, we examined the stability of the new compounds in pre-form with the conjugated triene system (**14-*epi*-19-norP1**, **10a**, and **10b**), which were thought to be labile. We found that transformation occurred in the presence of acidic protons, at least in 1 mM HCl solution. Using HPLC analysis, we were able to isolate the transformed compounds, and their spectral data strongly suggested the *cis/trans* isomerization of C6,7-double bonds to give 19-nortachysterol skeleton. To confirm

Scheme 3. Synthesis of 14-*epi*-19-Nortachysterol Analogues[a]



[a] Reagents and conditions: (a) Bu_3SnH, AIBN, toluene; (b) $Pd_2(dba)_3 \cdot CHCl_3$, Ph_3As, LiCl, DMF, THF; (c) TBAF, THF, yield 46% (**13**, 3 steps), yield 62% (**14**, 3 steps); (d) $(Ph_3P)_3RhCl$, H_2, benzene, CH_2Cl_2, yield 45% (**15a**), yield 47% (**15b**).

Scheme 4. Synthesis of 1α,25(OH)$_2$-19-Nortachysterol



the tachysterol structures, we embarked upon the chemical synthesis of the most probable compounds, 14-*epi*-19-nortachysterol analogues (**13**, **15a**, and **15b**, Scheme 3). As in the above synthesis, we utilized the intermediates **5** and **6** as A-ring precursors, and the reaction with tributyltin hydride in the presence of AIBN transformed them into vinylstannanes **11** and **12** after basic column chromatography, which were immediately coupled with CD-ring fragment **3** under Stille coupling conditions. The coupled compounds were deprotected using TBAF, and we were able to synthesize 14-*epi*-1α,25(OH)$_2$-19-nortachysterol (**13**) and its 2-methylene-substituted compound (**14**). Regioselective reduction of compound **14** was successfully accomplished with Wilkinson's catalyst to give 2-methyl-substituted diastereomers **15a** and **15b**.[12] The spectral data of the compounds **13**, **15a**, and **15b** were identical to the corresponding data of isomerized products derived from **14-*epi*-19-norP1**, **10a**, and **10b** under acidic conditions.

The new compounds of the 14-*epi*-19-nortachysterol skeleton indicated particular stability in comparison with natural tachysterol, which is easily converted to vitamin D_3 by UV irradiation[15] and oxidized by O_2.[16] To the best of our knowledge, these compounds are the first example of stable tachysterol analogues.

Additionally, 1α,25(OH)$_2$-19-nortachysterol (**17**), synthesized with **6** and **16**[11] in the same manner, was unstable and slowly decomposed even under neutral conditions at room temperature, and it was impossible to determine its biological properties (Scheme 4). Above all, since the 14-*epi*-CD-ring system, *cis*-hydrindane, prefers the C8,9-endo double bond rather than C7,8-exo double bond,[17] it is thought that 14-epimerization is essential for stabilizing the tachysterol skeleton.

Table 2. Relative Binding Affinity for VDR[13,14]

compound	VDR[a]
1α,25(OH)$_2$D$_3$ (**1**)	100
13	15
14	83
15a	71
15b	48

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

Here again, we investigated the hVDR binding affinity of tachysterol analogues and, as we expected, the affinity was greatly improved and C2-substitution had a marked effect (Table 2).[18] As in the case of ene-yne-ene compounds in Table 1, 2-methylene substitution (compound **14**) had the highest binding affinity for the hVDR.[12]

At this point, we were interested in the interaction between our new synthetic molecules and hVDR, and X-ray

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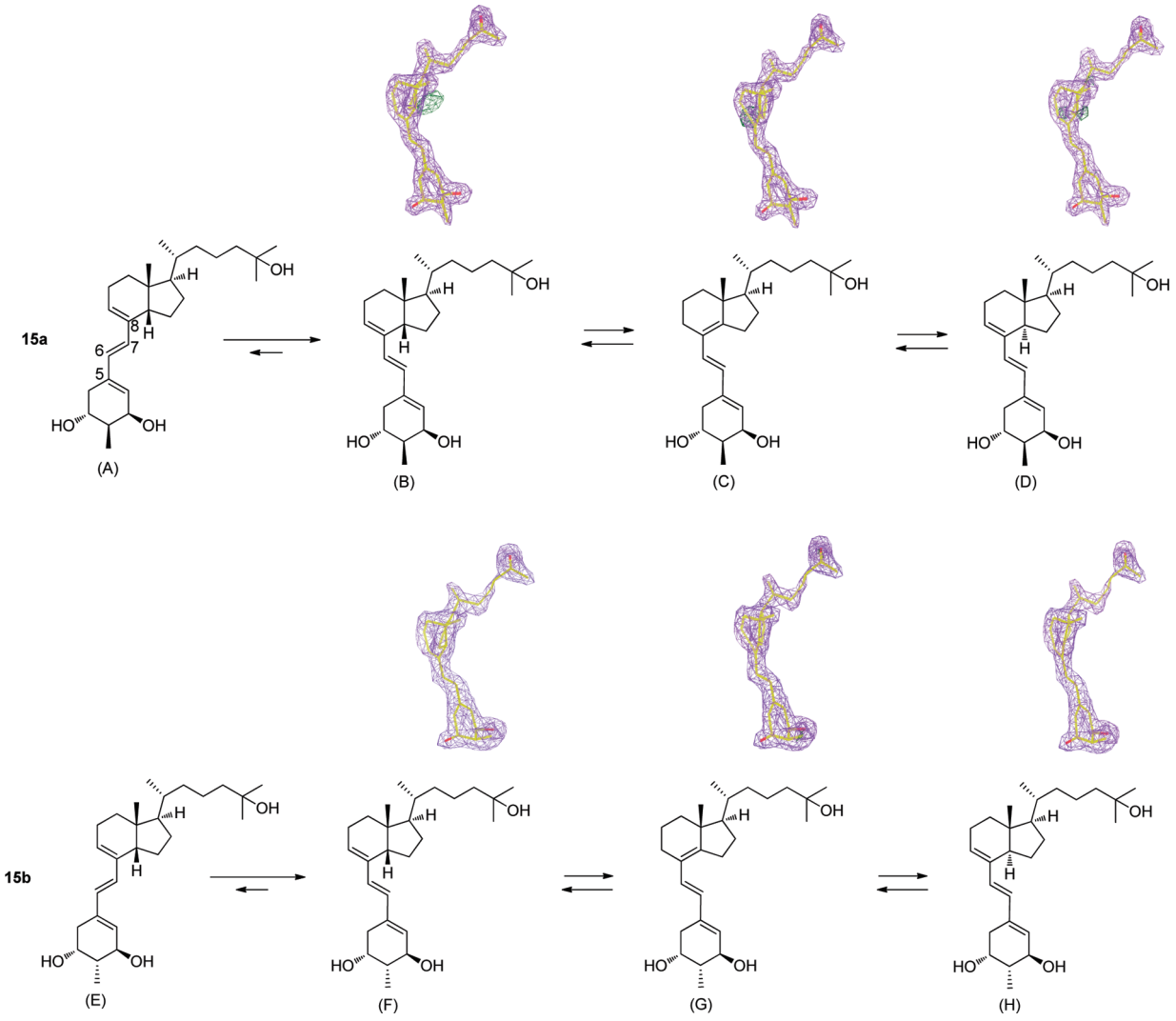

Figure 2. Binding configurations and crystallographic data of **15a** and **15b** in hVDR. The electron density omit maps contoured at 1.0σ are shown above the molecules, respectively (B−D, F−H). These pictures show $F_o - F_c$ maps in purple and $2F_o - F_c$ maps in green.

co-crystallographic analyses were performed using the complex of compound **15a** and **15b** with the ligand binding pocket (LBP) of the hVDR. Surprisingly, as shown in Figure 2, they revealed that the compounds fit the LBP with the C7,8-*s*-*trans* diene configuration as not (A) but (B), and not (E) but (F), which were opposite the natural C7,8-ene-configuration of 1α,25-$(OH)_2D_3$ (**1**). Also, there were three possible configurations of the CD-ring, and each electron density map corresponding to each configuration is shown in Figure 2. These show the mixture of the three configurations of the CD-ring (B, C, and D for **15a** and F, G, and H for **15b**) in Figure 2. The occupancy of each configuration of the CD-ring cannot be refined by the limit of the resolution.[19]

Concerning the orientation of the 2-methyl substitution, which was thought to make a hydrophobic interaction with Phe150, Leu233, and Ser237 on the α-side,[18b] compound **15b** made it on the β-side (F, G, H in Figure 2). Therefore, C5,6-*s*-*trans* configuration was preferred regardless of the stereochemistry of 2-methyl substitution. As far as we know, this binding configuration is unprecedented among the ligand molecules for VDR, and it is worth noting that this unique binding mode exhibited comparable VDR binding affinity to the natural hormone. Furthermore, we focused on the linker configuration between the A-ring and the CD-ring (from C5 to C8 positions) of the above molecules. By X-ray co-crystallographic analyses of **9a** and **9b**, their ene-yne-ene linkers also fit the LBP of the hVDR, and the 2-methyl substitutions were located on the α-side regardless of their C2-stereochemistries (Figure 3).

The superposed binding configuration among 1α,25$(OH)_2D_3$ (**1**), compounds **9a**, and **15a**(B) in the LBP of the hVDR was described, and the C5−C8 linkers successfully placed themselves in the specific space sandwiched between Ser275 and Trp288 on one side and Leu233 on the other side (Figure 4).[2] Also, the three hydroxy groups (C1, C3, and C25 positions) of the compounds well overlapped, respectively, and the CD-ring moiety indicated various conformational changes including the orientation of C20-methyl groups. These results showed the suitability of the linker and flexibility of the CD-ring structure; the appropriate distribution of both the A-ring and the side chain in the LBP was critical for high affinity.[20]

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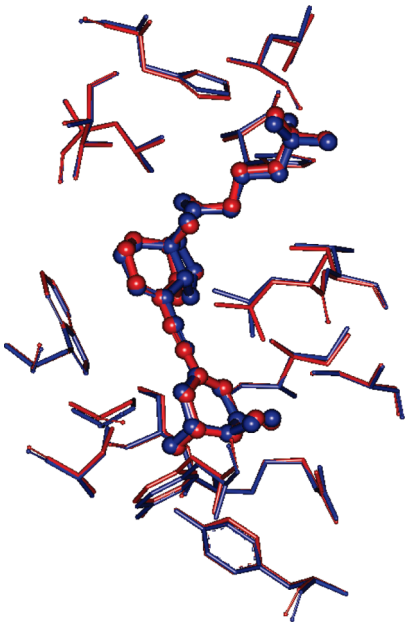

Figure 3. Superimposed three-dimensional structures of **9a** and **9b** based on X-ray crystallographic analysis in the hVDR ligand binding pocket. The **9a** complex is shown in blue and the **9b** complex in red. Protein Data Bank accession numbers are 3AUQ for **9a** and 3AUR for **9b**.

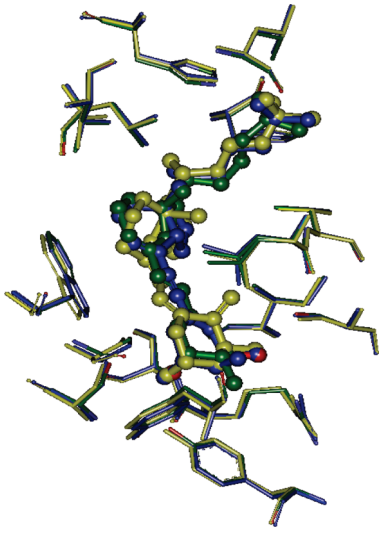

Figure 4. Superimposed three-dimensional structures of **1** and **9a** (X-ray analyses) as well as **15a** (molecular modeling)[14] in the hVDR ligand binding pocket. The **1**, **9a**, and **15a** complexes are shown in yellow, blue, and green, respectively.

CONCLUSIONS

In conclusion, we disclosed 14-*epi*-19-nortachysterol derivatives from 14-*epi*-19-norprevitamin D_3 by proton-mediated C6,7-*cis*/*trans* isomerization and succeeded in their chemical synthesis. They showed marked stability and potent hVDR binding affinity with the unprecedented binding configuration for hVDR. To the best of our knowledge, this is the first example of the isolation of stable tachysterol analogues, and revealed their unique binding configuration for hVDR. We believe that this new skeleton could have potential as a new drug candidate. Further studies of other new tachysterol analogues and their thermodynamic and biological properties are now in progress.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures, spectral data for all new compounds (^{1}H NMR, ^{13}C NMR, IR, HRMS), and crystal data (Protein Data Bank accession numbers 3AUQ for **9a** and 3AUR for **9b**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Binding affinity to VDR was evaluated using a $1\alpha,25(OH)_2D_3$ assay kit (Polarscreen Vitamin D Receptor Competitor Assay, Red, Cat. No. PV4569) purchased from Invitrogen.

(14) Supporting Information.

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(19) In Figure 2, all structures were determined on the basis of the electron density map. The X-ray crystallographic data in Figure 2 are not registered in the PDB due to the low resolution for the occupancy refinement of each isomer.

(20) EC_{50} values of osteocalcin promoter transactivation activity in human osteosarcoma (HOS) cells under serum free conditions for compounds **9a**, **13**, and **15a** were 0.04, 1.06, and 0.27 nM, respectively, when $1\alpha,25(OH)_2D_3$ (**1**) showed 0.03 nM as the positive control.