The Difference between 14-Epi-previtamin D₃ and 14-Epi-19-norprevitamin D₃: Their Synthesis and Binding Affinity for Human VDR

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Abstract. The synthesis of 14-epi-1α,25(OH)₂previtamin D₃, 14-epi-19-nor-1α,25(OH)₂previtamin D₃, and their 2-substituted analogs is described. The vitamin D receptor (VDR) binding affinity was further evaluated and 2α-methyl substituted 14-epi-1α,25(OH)₂previtamin D₃ had 17-fold more potent affinity than 14-epi-1α,25(OH)₂previtamin D₃. In the comparison of these compounds, the effects of thermal equilibrium, with or without 19-carbon at the A-ring, and their CD-ring structures are discussed.

Vitamin D has a unique characteristic, in that it is present in thermal equilibrium with previtamin D via a [1,7]-sigmatropic hydrogen shift. In this equilibrium, the stereochemistry of C14 is the key: vitamin D is a more stable and major compound than previtamin D, while 14-epi-previtamin D is the key: vitamin D is a more stable and major compound than hydrogen shift. In this equilibrium, the stereochemistry of C14 is still in thermal equilibrium; even though it is isolated as a pure compound, it could possibly be isolated as a stable compound. Consequently biological information on the previtamin D form could be obtained. However, as 14-epi-1α,25(OH)₂previtamin D₃ (14-epi-P1) is a major isomer and is dominant over 14-epi-1α,25(OH)₂vitamin D₃ (14-epi-1), and it could possibly be isolated as a stable compound. Consequently biological information on the previtamin D form could be obtained. In terms of chemistry, thermal equilibrium works via a [1,7]-sigmatropic hydrogen shift, which needs both a double bond and a hydrogen at the appropriate 1,7-positions. A compound without a 19-methyl group, 19-norvitamin D (27,28), cannot be in thermal equilibrium or transformed into its previtamin D form, because there is not a double bond or a hydrogen at the appropriate 1,7-positions needed for equilibrium; therefore, 19-norvitamin D should be isolated at a stable pure form, with no pre-form compound. Equally, 14-epi-19-norprevitamin D, in which the 19-methyl group is deleted from 14-epi-previtamin D, should be isolated as a stable pure form without any compounds in the vitamin D form, because thermal equilibrium is impossible, and it should be possible to obtain biological information on the pure pre-form compound. The comparison of 14-epi-1α,25(OH)₂previtamin D₃ (14-epi-P1) and et al. reported that 1α,25(OH)₂preD₃ (P1) would be an agonist for rapid responses (11-16), such as stimulation of intestinal Ca²⁺ transport (transcalcitriachia) (17-20), activation of protein kinase C (PKC) (21-23) and mitogen-activated protein kinases (MAP kinases) (24-26), and other non-genomic actions; therefore, previtamin D is of interest for vitamin D chemotherapy, however, there are only a few reports on previtamin D₃, and details remain to be uncovered. As shown in Figure 1, P1 is an unstable minor compound in thermal equilibrium with 1, and is easily transformed back into the more stable vitamin D₃. Inevitably, it is difficult to isolate P1 in its pure form and evaluate its activity rendering it difficult to study. On the other hand, 14-epi-1α,25(OH)₂previtamin D₃ (14-epi-P1) is a major isomer and is dominant over 14-epi-1α,25(OH)₂vitamin D₃ (14-epi-1), and it could possibly be isolated as a stable compound. Consequently biological information on the previtamin D form could be obtained. However, as 14-epi-P1 is still in thermal equilibrium, even though it is isolated as a pure compound, it would soon contain a small quantity of the minor isomer, 14-epi-1, generated by thermal equilibrium. Accordingly, the biological results could be partially contributed to the small impurity of the vitamin D form, which should have even more potent genomic activity than the previtamin D form. In terms of chemistry, thermal equilibrium works via a [1,7]-sigmatropic hydrogen shift, which needs both a double bond and a hydrogen at the appropriate 1,7-positions. A compound without a 19-methyl group, 19-norvitamin D (27,28), cannot be in thermal equilibrium or transformed into its previtamin D form, because there is not a double bond or a hydrogen at the appropriate 1,7-positions needed for equilibrium; therefore, 19-norvitamin D should be isolated at a stable pure form, with no pre-form compound. Equally, 14-epi-19-norprevitamin D, in which the 19-methyl group is deleted from 14-epi-previtamin D, should be isolated as a stable pure form without any compounds in the vitamin D form, because thermal equilibrium is impossible, and it should be possible to obtain biological information on the pure pre-form compound. The comparison of 14-epi-1α,25(OH)₂previtamin D₃ (14-epi-P1) and...
14-epi-19-nor-1α,25(OH)₂previtamin D₃ (14-epi-19-norP₁) should provide with more precise chemical and biological information on the previtamin D skeleton. Here, the synthesis and comparison of VDR binding affinity of 14-epi-P₁ and 14-epi-19-norP₁, and their analogs with the C2-functional group are reported and the contribution of the 19-methyl group and 14-epimerization is discussed.

Materials and Methods

Chemistry (retrosynthetic analysis). 14-epi-P₁ should be prepared from 14-epi-1, a known compound, by thermal isomerization in equilibrium, so the synthesis of 14-epi-1 prepared according to reference 2 was the temporary first target (29-31). This strategy would also help to provide information on the equilibrium between vitamin D₃ and previtamin D₃. The 14-epi-1 was divided into two fragments, the CD-ring fragment 3 and A-ring fragment 4, which could be coupled by the Roche coupling method (Figure 2) (7, 32, 33).

19-Norprevitamin D₃ analogs were previously developed by Okamura (1, 34-36), Mouriño (37-41), Gotor (42-44), and De Clercq’s groups (9), and their methodology was followed using the coupling reaction between the CD-ring fragment 5 and A-ring fragment 6 under Sonogashira coupling conditions, and subsequent partial reduction of the C6,7-alkyne moiety of 2.

Synthesis of CD-ring fragment. For 14-epi-1, the CD-ring fragment 3 was synthesized from the known ketone, triethylsilyl (TES)-protected 25-hydroxy Grundmann’s ketone (7) (Figure 3) (45-47). According to the literature, epimerization of H14 was successfully conducted by NaOMe with recovery of the starting material, which was easily separated by column chromatography (2). For 14-epi-19-norP₁, the CD-ring fragment 5 was obtained through the above compound 3 (29-31) using lithium diisopropyl amide (LDA) and then N-phenylbis(trifluoromethanesulfonimide) (PhNTf₂) in one step (37). Thus, the CD-ring fragment could be obtained in short steps.

Synthesis of A-ring fragment. Previously, we found that 2α-alkyl and 2α-(ω-hydroxyalkyl) substitution afforded great improvements for VDR binding affinity and the subsequent genomic actions (48-51). We therefore decided to synthesize analogs with 2α-substitutions (methyl, 3-hydroxylpropyl and 3-hydroxypropoxy groups) for more detailed investigation in this study (Figure 4) (29, 30).

For the 14-epi-1 analogs, the 2α-substituted A-ring fragments (4a-c) were prepared from the known epoxide 8 derived from methyl α-D-glucoside (29, 49, 52-54). According to the literature method, 8 was transformed to the enyne 9a-c, which had various alkyl groups at the 2α-position by nucleophilic epoxide ring-opening reaction. Next, the enyne 9a-c reacted with nBuLi and then (CH₂O)n to give alcohol 10a-c, then, by hydroalumination and iodination 11a-c was formed, and cyclization proceeded smoothly.
to produce a six-membered A-ring 12a-c (55-58). The resultant hydroxy group was converted into phosphine oxide 4a-c in three steps. As above, it was possible to prepare 2α-substituted A-ring fragments in good overall yield.

For 14-epi-19-norP1, compound 16, for the 2-methyl substituted A-ring precursor, was derived from (−)-quinic acid through the known compound 13 by dehydration with POCl₃ (to 14), reduction of methyl ester (to 15), followed by oxidation to aldehyde, and then homologation with trimethylsilyldiazomethane (TMSCHN₂) (to 16) (36,47).

**Coupling reaction and isomerization.** Using the CD- and A-ring fragments prepared above, the coupling reaction (Figure 5) was attempted (2, 7, 32, 33). For the 14-epi-1 analogs, under basic conditions using nBuLi, the A-ring fragment 4a-c with small excess quantities worked well, and the coupled product 17a-c was obtained in moderate yield. Then, all the silyl groups in 17a-c were removed in one step with hydrogen fluoride (HF)/MeCN, and considerable quantities of deprotected compounds remained in the vitamin D form (14-epi-1a-c) at this stage; however, once they were heated at 80°C in benzene, isomerization was found to proceed smoothly by ¹H NMR (nuclear magnetic resonance) observation. After two hours, most of the vitamin D form had been converted to the previtamin D form, and isomerization seemed to reach thermal equilibrium, in which the ratio of the compounds was about 5/95 (vitamin D/previtamin D) based on ¹H NMR studies. Using high performance liquid chromatography (HPLC), the mixture of both forms was separated, and it was possible to obtain 14-epi-P1a-c, which was used for further biological studies.

For the 14-epi-19-norP1 analogs, the known compound 6 and the above prepared 16 were used for the A-ring precursor (41), and the Sonogashira coupling reaction was attempted (34). These reactions proceeded smoothly and gave coupled compounds, which
successively reacted with tetrabutylammonium fluoride (TBAF) to afford fully deprotected compounds 2 and 17 in excellent yield. By selective reduction of 17 with Wilkinson’s catalyst (47), 2-methyl substitution was performed effectively, and the resultant diastereomers 18α and 18β were separated using HPLC. Finally, the partial reduction of the C6,7-alkyne moiety of 2, 18α and 18β produced the three target compounds 14-epi-19-norP1, 14-epi-19-norP1αβ and 14-epi-19-norP1αβ (35).

VDR binding assay. The VDR binding affinity of the vitamin D3 analogs was tested by two methods. [26,27-Methyl-3H]-1α,25-dihydroxyvitamin D3 (specific activity 6.623 TBq/mmol, 15,000 dpm, 15.7 pg, Amersham Biosciences, Uppsala, Sweden) and various solution of 1α,25-dihydroxyvitamin D3 and compounds (1 mM in purchased from Invitrogen (Carlsbad, California, USA). To test the VDR binding assay.

Table I. Relative binding affinity for VDR of 2-substituted 14-epi-pre-form and 14-epi-19-nor-pre-form compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>VDR binding affinity</th>
<th>Compound</th>
<th>VDR binding affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α</td>
<td>100</td>
<td>14-epi-P1</td>
<td>0.5</td>
</tr>
<tr>
<td>14-epi-P1α</td>
<td>8.4</td>
<td>14-epi-19-norP1αβ</td>
<td>1.0</td>
</tr>
<tr>
<td>14-epi-P1β</td>
<td>1.4</td>
<td>14-epi-19-norP1αβ</td>
<td>2.9</td>
</tr>
<tr>
<td>14-epi-P1c</td>
<td>0.2</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

*The potency of 1 was normalized to 100; bchick intestinal VDR; chuman VDR.

measured (384 nm, emission: 595 nm, excitation: 535 nm, time: 250 ms/well, TriStar LB941, Berthold Japan; Tokyo, Japan). All the compounds were evaluated with N=2 within the range from 10–6 M to 10–10 M. IC50 values were calculated by using the average of the measured values. The activities of each compound were shown as relative values in which the activity of the natural hormone 1 was normalized to 100%.

Results and Discussion

The VDR binding affinity is summarized in Table I, and all of the pre-form compounds showed lower activity than the natural hormone 1. In the comparison of 14-epi-P1 and its 2-substituted analogs, two compounds indicated higher affinity than 14-epi-P1, and especially, the 2α-methyl substituted 14-epi-P1α exhibited 8.4%, 17-fold, greater activity than 14-epi-P1. Also, from the comparison of the 19-nor compounds, 2β-methyl substitution (14-epi-19-norP1αβ) gave positive effects on VDR binding affinity; however, 2α-methyl substituted analog (14-epi-19-norP1αβ) showed almost the same affinity as 14-epi-19-norP1.

From the comparison between 14-epi-P1 and 14-epi-19-norP1, the latter presented slightly better affinity. Norman et al. reported the biological properties of 1α,25(OH)2-cis-isotachysterol (19) (Figure 6), whose binding affinity for chick VDR was 0.4% (14), and judging from the above results, 19-carbon in pre-form compounds should contribute less to VDR binding affinity. Compared to 14-epi-19-
norP1α, 14-epi-P1α should be sensitive to thermal equilibrium and therefore must include a small quantity of 14-epi-P1α. In the previous studies (60, 61), 2α-methyl-1α,25(OH)2D3 (20) exhibited greater potent (4-fold) VDR binding affinity than the natural hormone 1, and its 14-epi analog (14-epi-1α) should have positive effects on their binding affinity, which is why 14-epi-P1α exhibited higher affinity than 14-epi-19-norP1α.

Regarding the CD-ring moiety, its structural change would have positive effects on genomic activity. Verlinden et al. reported the particular activity of 19-nor-1α,25(OH)2previtamin D3 (21) and its trans-decalin analog 22 (10). In that report, 21 and 22 showed 1.0% and 3.4% binding affinity for human VDR, respectively. According to the results with the present compounds, 14-epi-P1 and 14-epi-19-norP1, the CD-ring structure might have less effect on binding affinity; however, surprisingly, 22 exhibited equipotent activity in inhibiting cell proliferation and inducing cell differentiation to 1 (10). A possible explanation is that compound 22 could dissociate less quickly from the VDR than 1 and the lower affinity for vitamin D binding protein (DBP) might cause higher free concentration of 22 in the medium. Also, the docking study of 22 in the VDR

Figure 4. Synthesis of the 2α-substituted A-ring fragments. THF: tetrahydrofuran, NCS: N-chlorosuccinimide, TPAP: tetrapropylammonium perruthenate, NMO: N-methylmorpholine N-oxide. Conditions: (a) nBuLi, (CH2O)n, THF, 91% for 10a, 92% for 10b, 89% for 10c; (b) Red-Al, Et2O, then I2, THF, 73% for 11a, 70% for 11b, 75% for 11c, 75%; (c) Pd(PPh3)4, Et2N, MeCN, 93% for 12a, 89% for 12b, 82% for 12c; (d) i) NCS, Me2S, CH2Cl2, ii) PHP2, nBuLi, THF, iii) H2O2aq, 77% for 4a, 74% for 4b, 76% for 4c.
showed its unique binding style, which suggested that the pre-form compound could fit in the binding pocket and exhibit potent genomic actions (10). Therefore, the present 14-epimerized pre-form compounds would be expected to have some important biological activities, yet more biological assays are required (62).

Conclusion

2α-Substituted analogs of 14-epi-1 were synthesized and these new analogs (14-epi-P1a-c) could be isolated after thermal isomerization at 80°C. Next, 14-epi-19-nor-P1 and its 2-methyl substituted analogs were synthesized using the Sonogashira coupling method. The VDR binding affinity of the newly synthesized analogs was evaluated to compare the 14-epi-P1 analogs with the corresponding 14-epi-19-nor-P1 analogs and the 19-carbon of 14-epi-P1 was demonstrated not to be so effective on VDR binding. It is possible that the thermal equilibrium to generate the 14-epi-vitamin D form from 14-epi-P1 analogs may contribute during VDR binding assays. The structural changes of the CD-ring moiety were thought to have little effect on VDR binding affinity; however, they might have great effects on genomic activity.

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References