New Metabolic Pathway of (24R)-24,25-Dihydroxyvitamin D₃: Epimerization of the 3-Hydroxy Group

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A new metabolic pathway of (24R)-24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] was clarified in the in vivo experiments. After the administration of 24,25(OH)₂D₃ to rats, a new monoglucuronide of a vitamin D metabolite was obtained from the bile together with 24,25(OH)₂D₃ 3- and 24-glucuronides. The genin of the metabolite was identified as 3-epi-24,25(OH)₂D₃ in comparison with the synthetic sample based on the data from ¹H, NMR, GC/MS, and LC/atmospheric pressure chemical ionization-MS. The conjugation position was determined to be the 24-hydroxy group by the LC/electrospray ionization-MS and -MS/MS experiments with derivatization. To our knowledge, this is the first reported instance of the epimerization of the 3-hydroxy group of vitamin D compound with no hydroxy group at the 1α-position.

Key words 3-epi-24,25-dihydroxyvitamin D₃; in vivo metabolite; synthesis; LC/MS; glucuronide

(24R)-24,25-Dihydroxyvitamin D₃ [24,25(OH)₂D₃] is one of the major metabolites of vitamin D₃, which has been reported to increase bone volume³ and strength⁴ at pharmacological doses. The metabolite is expected to be a new antosteoporosis medicine, and much interest is focused on the metabolism of 24,25(OH)₂D₃. It is reported that 24,25(OH)₂D₃ is oxidized on its side chain to give 23,25-dihydroxy-24-oxo-vitamin D₃ via 25-hydroxy-24-oxo-vitamin D₃ and then conjugated with glucuronic acid at the 23-hydroxy group in dogs administered 24,25(OH)₂D₃.⁵ Recently, we reported that 24,25(OH)₂D₃-3-glucuronide (G) and -24G were obtained as biliary metabolites of rats administered 24,25(OH)₂D₃ per os.⁶ In the present paper, we report on the identification of a new metabolite using LC/multistage tandem MS (LC/MS²; Finnigan MAT LCQ) and GC/electron ionization (EI)-MS (Finnigan GCQ).

Bile was collected from Wistar strain rats (males: ca. 170 g; females: ca. 140 g, 7 w) which were administered 24,25(OH)₂D₃ (0.5 mg) and treated as described in a previous paper.² Briefly, the bile specimen was passed through an Isolute C18 cartridge, washed with H₂O, and then the steroids were eluted with EtOH. The eluate was subjected to piperidinohydroxypropyl Sephadex LH-20 column chromatography, and the conjugates were eluted with 0.1 M AcONH₄ in 90% EtOH. The eluate was applied to an Isolute C18 cartridge to remove AcONH₄. The desired compounds were eluted with EtOH, which was evaporated under a N₂ gas stream. The residue was subjected to HPLC (Jsphere ODS-

H₈O [150×4.6 mm i.d.], MeCN–0.5% AcONH₄ [pH 5.0] [1:2 v/v], 1.0 ml/min, UV 265 nm), and a new peak eluted at 12.7 min was observed together with 24,25(OH)₂D₃-3G (retention time [tₚ] 8.1 min) and -24G (tₚ 14.8 min). The peak fraction was collected (12.5–14.0 min), diluted with H₂O, applied to an Isolute C18 cartridge in the manner described above to remove any inorganic salts, and the obtained residue was further purified by HPLC (Jsphere ODS-H₈O, MeCN–2% NaClO₃ [pH 3.0] [2:3 v/v], 1.0 ml/min, UV 265 nm, tₚ 13.1–14.3 min). After neutralization with 2% NaHCO₃ and dilution with H₂O, the fraction obtained was treated as described above. The EtOH eluate was evaporated and part of the residue was subjected to photodiode array UV-HPLC using the above conditions and enzymatic hydrolysis with β-glucuronidase.³ As a result, it was confirmed that the metabolite was a glucuronide of a vitamin D metabolite: it showed the typical UV absorption spectra of the vitamin D-triene structure (λₘₐₓ 268 nm, λₘₐₓ 230 nm), and completely disappeared and a new single peak emerged (Jsphere ODS-H₈O, MeCN–H₂O [3:2 v/v], 1.0 ml/min, UV 265 nm, tₚ 10.7 min) after treatment with the enzyme.

In the LC/electrospray ionization (ESI)-MS spectra of the intact metabolite (Developol ODS HDG-5 [150×2.0 mm i.d.], MeCN–10 mM AcONH₄ [1:2 v/v], 0.3 ml/min, tₚ 11.3 min), the base ion peak was observed at m/z 610 ([M+NH₄]⁺) and 591 ([M–H]⁻) in the positive- and negative-ion modes, respectively. The product ion mass spectrum using m/z 610 as a precursor ion showed the following product ions: m/z 593 [M+H]⁺, 575 [593–H₂O]⁻, 557 [593–2H₂O]⁻, 417 [genin+H]⁺, 399 [417–H₂O]⁻, and 381 [417–2H₂O]⁻. All the ESI-MS² spectral data of the metabolite were completely agreed with those of synthetic 24,25(OH)₂D₃-24G or -25G, but its tₚ was different from those of two glucuronides. These data indicated that the metabolite was a monoglucuronide of a dihydroxylated vitamin D₃ metabolite, and furthermore the structure of the genin was very similar to 24,25(OH)₂D₃. Therefore we inferred that the genin was a stereoisomer of 24,25(OH)₂D₃, such as 24S,25(OH)₂D₃, which was obtained from incubation of 24,25(OH)₂D₃ with chick kidney homogenate.⁷ However, the GC/MS chromatographic behavior of 24S,25(OH)₂D₃ and that of the genin were different (Rtx-5MS capillary column [25 m×0.25 mm i.d., 0.25 mm df], trimethylsilylated [TMS]-genin derivative: tₚ 21.9 min, TMS-24S,25(OH)₂D₃ derivative: tₚ 19.5 min, TMS-24S,25(OH)₂D₃ derivative: tₚ 19.7 min).

It is well known that the reaction of vitamin D compounds with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) gives 2-epimers, 6S- and 6R-adducts, because the reagent attacks at the s-cis diene of vitamin D from the β- and α-sides, respectively.⁸ It is also obvious that the 6S-adduct forms in preference to 6R-one in the case of compounds with no hydroxy group at the 1α-position. The PTAD adduct of the genin of the metabolite showed two equivalent overlapped peaks (YMC-Pack Pro C18 [150×3.0 mm i.d.], MeCN–H₂O [3:2 v/v], 0.4 ml/min, tₚ 4.4 min) in LC/atmospheric pressure chemical ionization (APCI)-MS monitoring at m/z 574 ([M+H–H₂O]⁺), which was quite different from the data for 24,25(OH)₂D₃ (tₚ 3.9 [6R] and 5.0 min [6S]) with a ratio of ca. 1: 5 (Fig. 1). This result suggested that the conformation of the A-ring of the genin was different from that of the usual
vitamin D compounds. We obtained ca. 7 µg of the genin from 7 rats in the manner described above, and its \(^1\)H-NMR (600 MHz) spectral data suggested that the genin was 3-epi-24,25(OH)_2D_3. That is, except for the chemical shift of the proton at the 3-position (the genin: 3.89 ppm, 24,25(OH)_2D_3: 3.94 ppm), no clear difference between the two compounds could be observed. Although the A-ring of 24,25(OH)_2D_3 exists essentially in the \(\alpha\)-chair form, that of its 3-epimer is inferred to exist in the \(\beta\)-chair form, because the A-ring is stabilized when the 3-hydroxy group is in the equatorial position. Therefore it was inferred that the difference between 24,25(OH)_2D_3 and the genin in the reaction with PTAD was caused by the different conformation.

To identify the genin more reliably, we synthesized 3-epi-24,25(OH)_2D_3 based on the Lythgoe coupling\(^7,8\) of the A-ring fragment 5 with the C/D-ring fragment 7 (Chart 1). The A-ring fragment 5 was synthesized from \(S\)-(+)epichlorohydrin (1) according to the method we had previously developed.\(^3,10\) The conversion of 1 to epoxide 2 and epoxide-opening of 2 with allylmagnesium chloride followed by silylation gave silyl ether 3. The silyl ether 3 was subjected to oxidative deprotection, hydroalumination followed by treatment with iodine, and palladium-catalyzed cyclization to give dienol 4, which was then converted to the required A-ring fragment 5 by the known method.\(^9\) On the other hand, the C/D-ring fragment 7 was synthesized by the method of Schröttner et al.\(^11\) via the Inhoffen-Lythgoe diol (6) derived from vitamin D_2.\(^12\) According to the Hoffman La Roche procedure,\(^9\) the A-ring fragment 5 was allowed to react with the C/D-ring fragment 7 to give triene 8 almost quantitatively. Finally, desilylation of 8 with tetrabutylammonium fluoride furnished 3-epi-24,25(OH)_2D_3.\(^13\) The \(^1\)H-NMR spectrum of
the synthetic 3-epi-24,25(OH)₂D₃ gave a multiplet peak of H-3 at 3.89 ppm, which agreed with that of the genin of the biliary metabolite. Furthermore, their behavior in LC/APCI-MS after derivatization with PTAD and GC/MS was in complete agreement. From these results, the genin was identified as 3-epi-24,25(OH)₂D₃.

The conjugation position was determined by the LC/ESI-MS' after derivatization with PTAD or acetic anhydride in pyridine. Through this method, the PTAD adducts of the conjugated vitamin D metabolites are cleaved at the C6–7 bond of the vitamin D skeleton and provide characteristic product ions in MS/MS/MS: in the case where the glucuronic acid is at the A-ring, a product ion at m/z 474 is observed, while in the case where it is at the side chain, only a corresponding ion at m/z 298 is observed. The product ion mass spectrum of the PTAD adduct of the metabolite showed m/z 298 but not 474, which indicated that the possible conjugation position was the 24- or 25-hydroxy group. The ESI-MS spectrum of the acetylated metabolite showed the molecular-related ions at m/z 792 [M+NH₄]⁺ and 773 [M−H]⁻ in the positive- and the negative-ion modes, respectively, indicating the presence of four sec- and one tert-hydroxy groups as the intact form in the metabolite. These results showed that the conjugation position was the 24-hydroxy group.

Based on these data, the metabolite isolated from rat bile was identified as 3-epi-24,25(OH)₂D₃ 24G (Fig. 2). The epimerization of the 3-hydroxy group of vitamin D compounds has been reported in 1,25-dihydroxyvitamin D₃ incubated in human keratinocytes or rat osteosarcoma cells, and 3-epi-1,25(OH)₂D₃ was recently isolated from the serum of rats treated with 1,25(OH)₂D₃. To the authors' knowledge, the present paper is the first reported instance of the isolation and characterization of a 3α-hydroxyvitamin D metabolite with no hydroxy group at the 1α-position.

The yield of 3-epi-24,25(OH)₂D₃ 24G was ca. 300 ng from 1 ml of bile (mean, n=4), which was much larger than that of 24,25(OH)₂D₃-3G (30 ng) or -24G (120 ng). These data indicate that the epimerization of the 3-hydroxy group is one of the important pathways in vitamin D metabolism. The mechanism of this epimerization is now under investigation in our laboratories.

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**REFERENCES AND NOTES**

13. [30] + 29.3° (c 0.345, CHCl₃). ¹H-NMR (300 MHz, CDC₁₁): δ 0.56 (3H, s, H-18), 0.94 (3H, d, J=6.3 Hz, H-21), 1.17, 1.22 (3H each, s, H-26, 27), 3.33 (1H, dd, J=6.6, 4.8 Hz, H-24), 3.89 (1H, brt, J=4.0, 8.4 Hz, H-3), 4.84 (1H, d, J=4.1 Hz, H-19), 4.56 (1H, bs, H-19), 6.04 (1H, d, J=11.1 Hz, H-7), 6.24 (1H, d, J=11.1 Hz, H-6). El-MS m/z: 416.3270 (M⁺) (Calcld for C₂₂H₃₆O₇: 416.3290).