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Investigation of C-3 Epimerization Mechanism of 24,25-Dihydroxyvitamin D_3 in Rat Using Liquid Chromatography/Mass Spectrometry

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Studies on C-3 epimerization of 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] were performed. 3-Epi-24,25$(OH)_2D_3$ was found to circulate in the blood of rats administered 24,25$(OH)_2D_3$, which was confirmed in comparison with a standard sample based on its chromatographic behavior during inclusion high-performance liquid chromatography using γ-cyclodextrin as a mobile phase additive and data obtained from liquid chromatography (LC)/mass spectrometry (MS) and gas chromatography/MS. The C-3 epimerization mechanism was investigated in *in vitro* experiments using [3α-^{2}H]-24,25$(OH)_2D_3$. The disappearance of deuterium was monitored by LC/MS. As a result, it was clarified that the 3-epimer was formed *via* the 3-oxo-form as an intermediate. This epimerization was also found to be controlled by the enzyme existing in the liver cytosol fraction and to require NAD and NADPH as coenzymes.

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(24*R*)-24,25-Dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] is one of the major metabolites of vitamin D_3, which causes a marked increase in bone volume[1] and mechanical strength[2] in animals without hypercalcemia at pharmacological doses (Fig. 1). Based on these criteria, the metabolite is expected to be a new anti-osteoporosis medicine and much interest is focused on the metabolism of 24,25$(OH)_2D_3$. 24,25$(OH)_2D_3$ is reported to be oxidized on its side chain at the C-23 or 24 position by vitamin D 24-hydroxylase to give metabolites such as (23*S*)-23,25-dihydroxy-24-oxovitamin D_3.[3]

In previous studies, we found that in rats dosed with 24,25(OH)D_3 *per os*, a large amount of 3-epi-24,25$(OH)_2D_3$ 24-glucuronide (24G) was excreted into the bile together with 24,25$(OH)_2D_3$-3G, -24G and -3-sulfate.[4,5] The epimerization of the 3-hydroxy group was also observed in the *in vitro*[6,7] and *in vivo*[8] metabolism of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. These findings indicate that the C-3 epimerization pathway plays an important role as does the side-chain oxidation in the vitamin D metabolism. However, the existence of the free form of the 3-epimer of 24,25$(OH)_2D_3$ in biological fluids and the epimerization mechanism have not been clarified. Studies on the 3-epi-metabolites are important to develop new vitamin D medicines. For example, if 3-epi-24,25$(OH)_2D_3$ is not only excreted into the bile as a glucuronide but also circulates in the blood, some contribution of the 3-epi-form to the bone formation caused by the administration of 24,25$(OH)_2D_3$ must be considered.

In the present paper, we initially identified 3-epi-24,25$(OH)_2D_3$ in the blood of rats dosed with 24,25$(OH)_2D_3$ using inclusion high-performance liquid chromatography (HPLC), liquid chromatography (LC)/mass spectrometry (MS) and gas chromatography (GC)/MS. Second, *in vitro* experiments were performed to investigate the C-3 epimerization mechanism of 24,25$(OH)_2D_3$ using the deuterium-labeled material.

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Experimental

Materials and reagents

24,25$(OH)_2D_3$ was obtained from Duphar B.V. Co. (Amsterdam, The Netherlands) and 3-epi-24,25$(OH)_2D_3$ was synthesized by us before.[4,5] (24*R*)-1α,24,25-Trihydroxyvitamin D_3 [1,24,25$(OH)_3D_3$] was kindly provided by Chugai Pharmaceutical Co. (Tokyo, Japan). 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) was synthesized from 4-phenylurazole (Nacalai Tesque, Kyoto, Japan) and purified by sublimation.[9] Isolute C18 (EC) cartridges (500 mg; International Sorbent Tech., Ltd., Hengoed, UK) were obtained from Uniflex (Tokyo). Heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (Me-β-CD) and γ-CD were donated by Kao Co. (Tokyo) and Nihon Shokuhin Kako Co. (Tokyo), respectively. All other reagents and materials were of analytical grade.

HPLC, LC/MS and GC/MS

HPLC was performed using a Hitachi L-7110 chromatograph (Tokyo) equipped with a Shimadzu SPD-10A UV (265 nm) detector (Kyoto). LC/MS was performed using a Finnigan MAT LCQ (San Jose, CA, USA) connected to a JASCO PU-980 (Tokyo) chromatograph, and atmospheric pressure chemical ionization (APCI) was used in the positive-ion mode. The heated capillary temperature was set at 150˚C and 225˚C for analyses of the intact vitamin D compounds and the PTAD-adducts, respectively. The sheath gas flow rate was set at 80 units with a vaporizer temperature of 350˚C. The source current, the capillary voltage and the tube lens offset were 5 μA, 1 V and 10 V, respectively. For MS/MS analysis, helium was used as the collision gas and the relative collision energy was set at 20%. A J'sphere ODS H-80 column (4 μm, 15 × 0.46 cm i.d.)(YMC, Kyoto) was used at a flow rate of 1 ml/min at 30˚C for both HPLC and LC/MS.

GC/MS was performed using a Finnigan MAT GCQ, and the

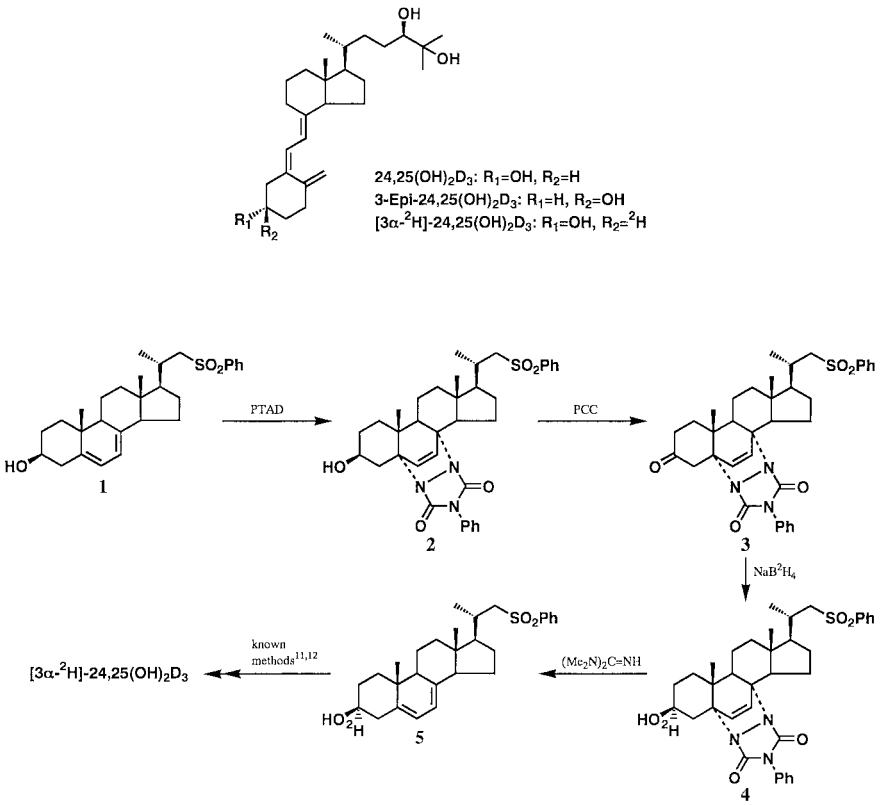

Fig. 1 Structures of $24,25(OH)_2D_3$, related compounds and intermediates in the synthesis of [3α-^{2}H]-$24,25(OH)_2D_3$.

conditions were the same as those in a previous paper.[5]

Plasma samples from rats

Male Wistar strain rats (*ca*. 170 g, 7 w, Japan S. L. C., Hamamatsu, Japan) were starved for 15 h prior to the administration of $24,25(OH)_2D_3$. A suspension of $24,25(OH)_2D_3$ (0.5 mg) in dimethylsulfoxide (0.1 ml) with saline (0.7 mg) and Tween 80 (0.2 ml) was orally given to rats. Eight hours after dosing, blood was collected in tubes from the aorta descendens under anesthesia with diethyl ether. Sodium heparin (2% v/v of blood volume) was added to the blood immediately and the sample was centrifuged at 1500*g* (15 min, 4˚C). The separated plasma was stored at –20˚C prior to use.

Pretreatment of plasma samples

The plasma sample (1 ml) was mixed with MeCN (1 ml) and subjected to centrifugation at 1500*g* for 10 min. To the supernatant was added H_2O (3 ml) and the sample was then passed though an Isolute C18 (EC) cartridge. After washing with H_2O (4 ml) and 70% MeOH (4 ml), the steroids were eluted with MeOH (3 ml), which was evaporated under a N_2 gas stream. The residue was redissolved in EtOH and stored at –20˚C.

Preparation of enzyme source

Male Wistar strain rats (*ca*. 170 g, 7 w) were used. Fresh liver and kidney tissues were homogenized in cold 0.15 M KCl to provide a final concentration of 20% (w/v). The homogenates were centrifuged at 1500*g* for 15 min and then 9000*g* for 10 min. The thus obtained supernatant (9000*g* supernatant; S-9) of the liver was further centrifuged (105000*g* for 60 min) in an ultracentrifuge (Hitachi CP 56G), and the cytosol (105000*g* supernatant) and microsomal (105000*g* precipitate) fractions were prepared. The microsomal pellets were resuspended in 10 mM potassium phosphate buffer (pH 7.5) and used. The protein concentration was determined by the method of Lowry *et al.*[10] using bovine serum albumin as a standard.

Incubation procedure

The assay medium contained $24,25(OH)_2D_3$, 3-epi-$24,25(OH)_2D_3$ or [3α-^{2}H]-$24,25(OH)_2D_3$ [10 μg in EtOH (10 μl)], $MgCl_2$ [10 μmol in 50 mM Tris–HCl buffer (pH 7.6; Tris buffer) (50 μl)], ethylenediamine tetraacetic acid·2Na [20 nmol in Tris buffer (50 μl)], coenzyme [500 nmol each in Tris buffer (50 μl)], enzyme source (2.0 mg protein) and Tris buffer in a total volume of 1.0 ml. The mixture was incubated in air at 37˚C for 2 h. The reaction was stopped by the addition of MeOH (1 ml).

Pretreatment of incubation specimens

An incubation specimen mixed with MeOH (1 ml) was subjected to centrifugation at 1500*g* for 10 min. H_2O (1 ml) was added to the supernatant, and the sample was then treated in the same way as the plasma samples.

Identification of 3-epi-24,25(OH)$_2$D$_3$

The pretreated plasma and incubation specimens were subjected to inclusion HPLC, and the eluate containing 3-epi-$24,25(OH)_2D_3$ [MeCN/H_2O (11:9, v/v) containing 4 mM γ-CD; retention time (t_R), 9.6 – 10.3 min] was collected. After concentration of the fraction to about half volume under a N_2 gas stream, the entire sample was extracted with ether (2 ml and then 1 ml, twice). The combined ethereal layer was washed with H_2O (1 ml, three times), and the solvent was then evaporated under a N_2 gas stream. A part of the residue was

subjected to LC/MS and GC/MS after derivatization.[5] Incidentally, when the substrate was recovered, the fraction between 10.6 – 11.3 min was collected.

Synthesis of [3α-^{2}H]-24,25(OH)$_2$D$_3$

General: ^{1}H-NMR spectra were obtained with a JEOL JNM-EX-270 (270 MHz) spectrometer (Tokyo). $CDCl_3$ was used as the solvent with tetramethylsilane as internal standard. The abbreviations used are s = singlet, d = doublet, m = multiplet and br = broad. Flash column chromatography was carried out with Wakogel FC-40 (20 – 40 mesh, Wako, Osaka, Japan). The synthesis of (20*S*)-22-phenylsulfonyl-23,24-dinorchola-5,7-dien-3β-ol **(1)** and the construction of the 24,25-dihydroxylated side chain were done according to the procedures by Schrötter *et al*.[11] The transformation of provitamin D into vitamin D was carried out by the usual method, irradiation by a high pressure mercury lamp through a Vycor filter followed by thermal isomerization.[12] All air-sensitive reactions were carried out under Ar or N_2.

The PTAD adduct of (20*S*)-22-phenylsulfonyl-23,24-dinorchola-5,7-dien-3β-ol **(2)**: The mixture of **1** (291 mg, 0.642 mmol) and PTAD (161 mg, 0.921 mmol) in CH_2Cl_2 (6 ml) was stirred at room temperature for 40 min. The resulting solution was subjected to flash column chromatography (30 × 1.0 cm i.d.). Elution with $CHCl_3$/MeOH (50:1, v/v) gave **2** (364 mg, 90.3%) as a colorless solid: ^{1}H-NMR δ: 0.81 (3H, s, H-18), 0.96 (3H, s, H-19), 1.24 (3H, d, J = 6.9 Hz, H-21), 4.44 (1H, m, H-3α), 6.24, 6.36 (1H each, d, J = 8.3 Hz, H-6,7), 7.28 – 7.93 (10H, m, H-Ph).

The PTAD adduct of (20*S*)-22-phenylsulfonyl-23,24-dinorchola-5,7-dien-3-one **(3)**: The mixture of **2** (402 mg, 0.640 mmol) and pyridinium chlorochromate (PCC)(414 mg, 1.92 mmol) in CH_2Cl_2 (6.5 ml) was stirred at room temperature for 2.5 h. The resulting solution was subjected to flash column chromatography (30×2.0 cm i.d.). Elution with AcOEt/CH_2Cl_2 (3:1, v/v) gave **3** (293 mg, 73.1%) as a colorless solid: ^{1}H-NMR δ: 0.88 (3H, s, H-18), 1.07 (3H, s, H-19), 1.24 (3H, d, J = 6.6 Hz, H-21), 6.25, 6.54 (1H each, d, J = 8.6 Hz, H-6,7), 7.28 – 7.93 (10H, m, H-Ph).

The PTAD adduct of [3α-^{2}H]-(20*S*)-22-phenylsulfonyl-23,24-dinorchola-5,7-dien-3β-ol **(4)**: Sodium borodeuteride (30 mg, 0.720 mmol) was added to a solution of **3** (226 mg, 0.360 mmol) in CH_2Cl_2/MeO^{2}H (2:1, v/v, 3 ml), and the mixture was stirred at room temperature for 10 min. The resulting solution was diluted with AcOEt, washed with H_2O, and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by flash column chromatography (30 × 1.0 cm i.d.)[$CHCl_3$/MeOH (50:1, v/v)] to give **4** (83 mg, 36.6%) as a colorless solid: ^{1}H-NMR δ: 0.81 (3H, s, H-18), 0.96 (3H, s, H-19), 1.24 (3H, d, J = 6.3 Hz, H-21), 6.25, 6.37 (1H each, d, J = 7.9 Hz, H-6,7), 7.28 – 7.93 (10H, m, H-Ph).

[3α-^{2}H]-(20*S*)-22-phenylsulfonyl-23,24-dinorchola-5,7-dien-3β-ol **(5)**: A solution of **4** (136 mg, 0.216 mmol) in 1,1,3,3-tetramethylguanidine (3 ml) was refluxed for 1.5 h. The resulting solution was diluted with AcOEt and washed with H_2O, chilled 5% HCl, 5% $NaHCO_3$ and H_2O, and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by flash column chromatography (30 × 1.0 cm i.d.)[$CHCl_3$/MeOH (100:1, v/v)] to give **5** (80 mg, 81.4%) as a colorless solid: ^{1}H-NMR δ: 0.60 (3H, s, H-18), 0.93 (3H, s, H-19), 1.23 (3H, d, J = 5.6 Hz, H-21), 5.36, 5.55 (1H each, br s, H-6,7), 7.57 – 7.93 (5H, m, H-Ph).

Results and Discussion

Identification of 3-epi-24,25(OH)$_2$D$_3$ in rat plasma

Our initial effort was directed toward the separation of 3-epi-24,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ using HPLC. Both compounds could hardly be separated in the usual reversed and normal phase HPLC. We have reported that the inclusion HPLC using CD as a mobile phase additive was of great advantage in the separation of isomeric steroids.[13,14] The effect of Me-β-CD and γ-CD in the separation of the two epimers were examined, and the obtained chromatograms are shown in Fig. 2. The use of Me-β-CD decreased the t_R values of both 24,25(OH)$_2$D$_3$ and its 3-epi-form, but a poor result was obtained in their separation (Fig. 2a, b). On the other hand, 3-epi-24,25(OH)$_2$D$_3$ was separated from 24,25(OH)$_2$D$_3$ (resolution value 2.4) together with the reduction of t_R by the addition of 4 mM of γ-CD in the mobile phase (Fig. 2c).

The pretreated rat plasma specimens were subjected to inclusion HPLC, and the peak corresponding to 3-epi-24,25(OH)$_2$D$_3$ was observed in the chromatogram obtained from the rats administered 24,25(OH)$_2$D$_3$ (Fig. 2d). The fraction of the putative 3-epi-24,25(OH)$_2$D$_3$ was isolated and then identified in the following two ways. First, the metabolite was subjected to LC/APCI-MS and -MS/MS after derivatization with PTAD. We have reported that this derivatization not only supplies the characteristic product ion derived from the cleavage of C-6–7 bond in the MS/MS mode but also improves the separation of 24,25(OH)$_2$D$_3$ and its 3-epi-form.[5] The derivatized metabolite gave two peaks of t_R of 5.7 and 6.2 min [MeOH/H_2O (3:1, v/v)], which were epimers at the 6-position caused by the matter that the reagent attacks at the *s-cis* diene of vitamin D from the α- and β-sides (Fig. 3a, b) and provided the product ion mentioned above at *m/z* 298, which agreed with those of the synthetic standard (Fig. 3c).

Concerning the formation ratio of the 6-epimers, it has been reported that the 6*S*-adduct forms in preference to the 6*R*-adduct in the case of compounds having only a 3β-hydroxy group in the A-ring; on the other hand, the introduction of a 1α-hydroxy group prevents the reagent from attacking the above side of vitamin D.[15] We also confirmed the following: the PTAD adduct of 24,25(OH)$_2$D$_3$ gave two peaks [MeOH/H_2O (3:1, v/v), t_R 5.0 (6*R*) and 7.4 min (6*S*)] with a ratio of *ca*. 1:5; on the contrary, in the case of its 1α-hydroxylated compound, 1,24,25(OH)$_3$D$_3$, the ratio was *ca*. 1:1 [MeOH/H_2O (7:3, v/v), t_R 5.9 and 6.3 min]. The 3-epi-24,25(OH)$_2$D$_3$-PTAD adduct gave almost equal twin peaks, which proved that this compound had a configurationally α-hydroxy group in the A-ring (Fig. 3a, b).

Second, the metabolite was subjected to GC/MS after trimethylsilylation. The data obtained from the metabolite and the synthetic standard completely agreed {t_R 24.02 min, *m/z* 632 $[M]^+$, 527 $[M–TMSOH–CH_3]^+$, 501 $[M–131]^+$, 411 $[501–TMSOH]^+$, 321 $[411–TMSOH]^+$, 131 $[(CH_3)_2COTMS]^+$}. Based on these results, the metabolite was identified as 3-epi-24,25(OH)$_2$D$_3$.

The plasma concentrations of the remaining substrate and the formed epimer were determined by comparison with a known amount of 24,25(OH)$_2$D$_3$ and the revision of recovery in the extraction of 24,25(OH)$_2$D$_3$ from rat plasma (mean ± SD: 74.9 ± 2.4%, n = 3). The concentration of 24,25(OH)$_2$D$_3$ was almost invariable (mean ± SD: 1340 ± 112 ng/ml, n = 3), while that of the 3-epi-form was variable (range: 52 – 137 ng/ml, n = 3). The present study showed that 3-epi-24,25(OH)$_2$D$_3$ is not only excreted into the bile as a glucuronide[4,5] but also circulates through the body.

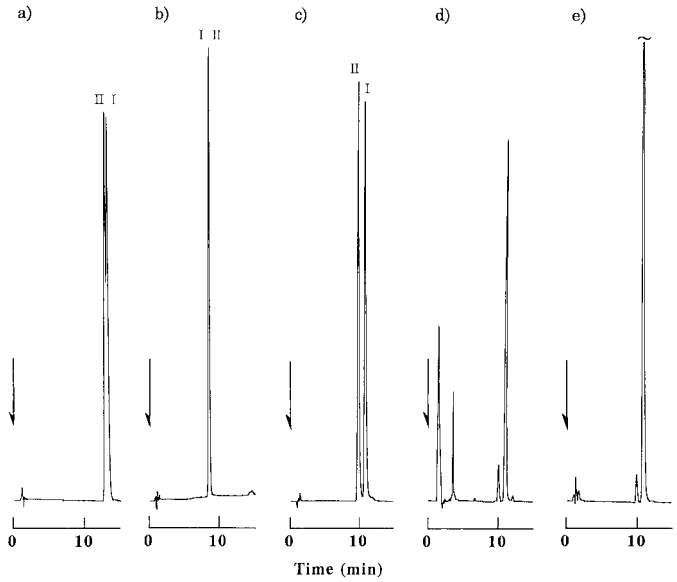

Fig. 2 Chromatograms of $24,25(OH)_2D_3$ and its 3-epimer. a, b and c, standard samples; d, from rat plasma; e, from incubation specimen (rat liver cytosol). Mobile phase: a, MeCN–H_2O (11:9, v/v); b, MeCN–H_2O (11:9, v/v) containing 4 mM Me-β-CD; c, d and e, MeCN–H_2O (11:9, v/v) containing 4 mM γ-CD. I, $24,25(OH)_2D_3$; II, 3-epi-$24,25(OH)_2D_3$.

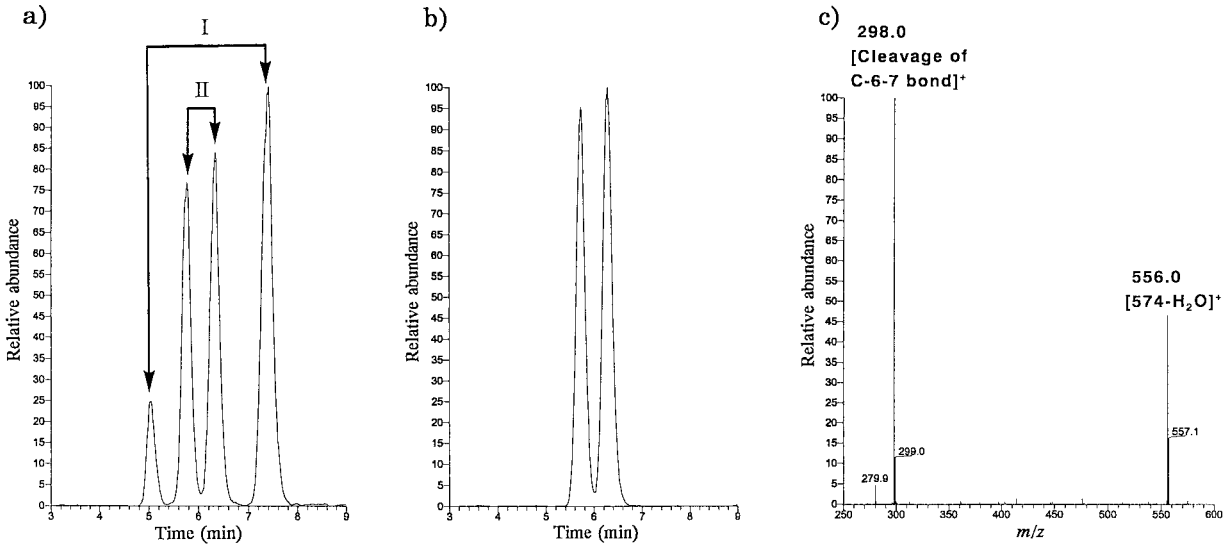

Fig. 3 LC/MS data for the PTAD adduct of 3-epi-$24,25(OH)_2D_3$. a and b: LC/MS chromatograms of standard samples (a) and from rat plasma (b){I, $24,25(OH)_2D_3$-PTAD; II, 3-epi-$24,25(OH)_2D_3$-PTAD; monitoring ion, *m/z* 574 $[M+H-H_2O]^+$}. c: product ion mass spectrum (from rat plasma) (precursor ion *m/z* 574).

Localization of epimerizing enzyme and coenzyme requirement

In order to evaluate the localization of the enzyme controlling the epimerization, rat kidney and liver S-9 fractions were incubated with $24,25(OH)_2D_3$ in the presence of NADPH and Mg^{2+}. The assay using inclusion HPLC as above showed that 3-epi-$24,25(OH)_2D_3$ was formed when the liver S-9 was used, while the kidney S-9 had no epimerizing activity. We also found that the epimer accumulated when both NADPH and NAD were added to the incubation mixture; on the contrary, the formation of the epimer was not observed with the single addition of NAD. The cytosol and microsomal fractions of the liver were then prepared and incubated in the presence of NAD and NADPH. The microsomal fraction metabolized $24,25(OH)_2D_3$ to an unknown polar metabolite, but the 3-epi-form was not detected from this incubation mixture. The cytosol fraction, on the contrary, showed the epimerizing activity, and a polar metabolite as above was not observed (Fig. 2e). These results indicate that the epimerizing enzyme exists in the cytosol fraction in the liver.

In the *in vitro* experiments with $1,25(OH)_2D_3$ using several cell lines, human keratinocytes,[6] rat osteosarcoma cells[7] and so on, had been reported to show epimerizing activity, but the subcellular localization of the enzyme has not been clarified. Although it is unclear whether the epimerizing enzymes controlling $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ are the same, the former is not a cytochrome P450 enzyme, considering its localization fraction (cytosol).

To further investigate the coenzyme requirement of the

Table 1 Coenzyme requirement of formation of 3-epi-24,25(OH)$_2$D$_3$

Coenzyme[a]	24,25(OH)$_2$D$_3$ (recovery rate[b], %)	3-Epi-24,25(OH)$_2$D$_3$ (formation rate[b], %)
None (control)	100	n.d.[c]
NAD, NADH	83.8	n.d.
NAD, NADPH	78.4 ± 3.44	2.44 ± 0.48
NAD, NADP	78.2	1.07
NADP, NADH	94.9	n.d.
NADP, NADPH	91.5	0.58
NADH, NADPH	95.5	0.75

a. Five hundred nanomoles each was added.
b. Mean of two rats, except "NAD, NADPH" (mean±SD, five rats). Percentage of 24,25(OH)$_2$D$_3$ recovered from control.
c. Not detected.

epimerization, we performed experiments in which various coenzymes were added to an incubation mixture. The results of these studies are given in Table 1, in which the amount (the peak area in the HPLC chromatogram) of 24,25(OH)$_2$D$_3$ recovered from the incubation mixture containing no coenzyme (control) was taken as 100, and the percentages of the recovered 24,25(OH)$_2$D$_3$ and the formed 3-epi-form under each condition are shown. Incidentally, the recovery rate of 24,25(OH)$_2$D$_3$ (10 μg/tube) during pretreatment was 86.3 ± 1.81% (mean ± SD, n = 6). In the presence of both NAD and NADPH, the largest amount of 3-epi-24,25(OH)$_2$D$_3$ was obtained under the examined six conditions. A relatively greater amount of the substrate was consumed in the presence of NAD, but the formation rate of the 3-epimer was varied by the coexisting reduced-form coenzymes. The 3-epi-form was detected from the incubation mixture containing NADPH, and NAD tended to be used for the epimerization of 24,25(OH)$_2$D$_3$ rather than NADP as the oxidized-form coenzyme.

Loss of deuterium at the 3α-position

The 3-epi-form is conjectured to be formed from 24,25(OH)$_2$D$_3$ *via* the 3-oxo-form as an intermediate. However, the 3-oxo-form of the vitamin D compounds cannot exist stably, because the 5,7,10(19)-conjugated-triene easily and irreversibly shifts to the 4,6,10(19)-triene in the presence of the 3-oxo-group.[16] Therefore, it is very difficult not only to synthesize the 3-oxo-form but also to isolate it from the biological matrix, even if it is formed.

24,25(OH)$_2$D$_3$ labeled with deuterium at the 3α-position is expected to be a useful tool to evaluate the existence of the 3-oxo-form. That is, if the labeled 24,25(OH)$_2$D$_3$ is converted to the 3-epi-form through the route mentioned above, the deuterium is eliminated and displaced by a proton. In this respect, we synthesized [3α-^{2}H]-24,25(OH)$_2$D$_3$ as shown in Fig. 1. The 5,7-diene system in phenyl sulfone **(1)** was protected as a Diels-Alder adduct with PTAD **(2)** prior to the oxidation of the 3-hydroxy group, because **1** was converted to the 3-oxo-4,6-diene-form by direct oxidation. The oxidation of **2** with PCC gave the 3-oxo-form **(3)** which was then subjected to treatment with sodium borodeuteride to give the desired [3α-^{2}H]-alcohol **(4)** together with an almost equal amount of by-product, which was inferred to be [3β-^{2}H]-alcohol because it was reverted to **3** on treatment with PCC. Deprotection of the 5,7-diene of **4** with 1,1,3,3-tetramethylguanidine gave [3α-^{2}H]-phenyl sulfone **(5)**, which was converted to [3α-^{2}H]-24,25(OH)$_2$D$_3$ by the method of Schrötter *et al*.[11] and the usual one.[12]

The LC/APCI-MS data for the standard 24,25(OH)$_2$D$_3$ and 3-epi-24,25(OH)$_2$D$_3$ were the same [MeOH/H$_2$O (41:9, v/v), t_R 6.6 min], in which m/z 417 $[M+H]^+$ was a base ion and an isotope peak, m/z 418, was also observed with the relative intensity of *ca*. 25% (Table 2). In the spectrum of [3α-^{2}H]-24,25(OH)$_2$D$_3$, a small peak at m/z 417 due to incomplete labeling was also detected, but the base ion peak was at m/z 418 and the purity of the labeled compound was *ca*. 96%. Incidentally, the 3-epi-form in the labeled compound was not detected in its inclusion HPLC chromatogram.

Table 2 Relative intensities of ions at m/z 417 and 418

	Relative intensity m/z 417	m/z 418
Synthetic standard		
24,25(OH)$_2$D$_3$	100	25.2
3-Epi-24,25(OH)$_2$D$_3$	100	25.8
[3α-^{2}H]-24,25(OH)$_2$D$_3$	4.0	100
From incubation specimen[a]		
Substrate (no coenzyme; control)	3.9	100
Substrate (with NAD and NADPH)	6.2	100
3-Epi-24,25(OH)$_2$D$_3$ (with NAD and NADPH)	100	36.6

a. Mean of two rats.

The *in vitro* experiments were performed using [3α-^{2}H]-24,25(OH)$_2$D$_3$ as a substrate, and the 3-epi-form and the substrate fractions were separately collected from the incubation mixture using inclusion HPLC. Their APCI-MS spectra were measured, and the relative intensities of the ions at m/z 417 and 418 were compared with those of the standards (Table 2). Although the relative intensity of the ion at m/z 418 (*ca*. 37%) was greater than that of the standard (*ca*. 26%), 3-epi-24,25(OH)$_2$D$_3$ isolated from the incubation mixture gave a base ion peak at m/z 417. The data suggested that the formation of the 3-epi-form involves a dehydrogenation process. No differences were observed between the spectrum of the substrate fraction recovered from the control (no coenzyme) and that of the standard; on the other hand, that of the substrate fraction obtained from the incubation mixture containing NAD and NADPH showed an increase in the relative intensity of the ion at m/z 417 (3.9% to 6.2%). These results suggest that 24,25(OH)$_2$D$_3$ is temporarily converted to the 3-oxo-form and then part of it reverts to 24,25(OH)$_2$D$_3$ and the remainder is converted to 3-epi-24,25(OH)$_2$D$_3$. In addition, these results indicated that a remarkable biological isotope effect was not observed in the formation of 3-epi-24,25(OH)$_2$D$_3$, because the relative intensity of the m/z 417 ion of the substrate recovered from the incubation mixture decreased in the case where the 24,25(OH)$_2$D$_3$ intermingling with the substrate was used in preference to the deuterium-labeled compound. It is unclear in what state the unstable 3-oxo-form exists; the dehydrogenation may be the rate-controlling step, and the formed product may be immediately reduced.

The reversibility of the epimerization reaction was also investigated. 3-Epi-24,25(OH)$_2$D$_3$ was incubated as above, but 24,25(OH)$_2$D$_3$ was not formed, which indicates that this epimerization proceeded from 3β to 3α unidirectionally. From the experiments using the deuterium-labeled compound, it was found that the formation of 3-epi-24,25(OH)$_2$D$_3$ consists of two process, dehydrogenation and reduction, and in the latter processes, not only the 3α- but also the 3β-hydroxy-form is produced. These data suggested that a dehydrogenase existing in the rat liver cytosol fraction is specific for the 3β-hydroxy group but not the 3α-one, which is the reason why 24,25(OH)$_2$D$_3$ is not formed from its 3-epimer.

In conclusion, we demonstrated that 3-epi-24,25$(OH)_2D_3$ was formed through the 3-oxo-form as an intermediate by an enzyme existing in the liver cytosol and then circulates in body. To our knowledge, the present paper is the first reported instance regarding the formation mechanism of 3-epi-vitamin D metabolites.

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