Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 429–436
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Analysis of C-3 epimerization in (24*R*)-24,25-dihydroxyvitamin D_3 catalyzed by hydroxysteroid dehydrogenase

Tatsuya Higashi*, Kanako Sakajiri, Kazutake Shimada

Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

Received 1 May 2004; received in revised form 6 July 2004; accepted 8 July 2004
Available online 19 August 2004

Abstract

Studies on the C-3 epimerization in (24*R*)-24,25-dihydroxyvitamin D_3 [24*R*,25$(OH)_2D_3$] were performed using hydroxysteroid dehydrogenases (HSDs). 3-Epi-24*R*,25$(OH)_2D_3$ was formed from 24*R*,25$(OH)_2D_3$ by the catalysis of 3α- or β-HSD. These HSDs also catalyzed the C-3 epimerization in 3-epi-24*R*,25$(OH)_2D_3$ to form 24*R*,25$(OH)_2D_3$. 24*R*,25$(OH)_2D_3$ and its C-3 epimer were separated by inclusion high-performance liquid chromatography using γ-cyclodextrin (γ-CD) as the mobile phase additive or a γ-CD bonded chiral column. The product derived from the intermediate during the C-3 epimerization was isolated from the incubation specimens and identified as (7*Z*)-(24*R*)-24,25-dihydroxy-9,10-secocholesta-4,7,10(19)-trien-3-one by several instrumental analyses including ^{1}H-nuclear magnetic resonance spectrometry. The occurrence of this compound strongly proves that the formation of the C-3 epimer by HSD involves a dehydrogenation process. The present study suggests that HSDs may catalyze the C-3 epimerization of vitamin D compounds and modulate their concentrations and biological activities in animals and humans.

Keywords: (24*R*)-24,25-Dihydroxyvitamin D_3; C-3 epimerization; Hydroxysteroid dehydrogenase; 3-Oxo compound; Inclusion HPLC; LC-MS

1. Introduction

(24*R*)-24,25-Dihydroxyvitamin D_3 [24*R*,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. Based on these criteria, this metabolite is expected to be a new anti-osteoporosis medicine and much interest is focused on its metabolism in animals and humans. Although nothing but the side-chain oxidation pathway has attracted considerable attention in the metabolism of vitamin D compounds, we isolated and identified a novel metabolite modified at the C-3 position, (24*R*)-3-epi-24,25-dihydroxyvitamin D_3 [3-epi-24*R*,25$(OH)_2D_3$] in plasma and bile as the free [3] and glucuronidated forms [4,5], respectively, after the administration of 24*R*,25$(OH)_2D_3$ to rats perros. Furthermore, the C-3 epimerization has been successively observed in various vitamin D compounds over the past 5 years [6–9] and it is now accepted that the epimerization pathway plays an important role in modulating the concentrations and biological activities of vitamin D compounds in target tissue, like the well-established side-chain oxidation pathway.

To date, little is known about the enzyme responsible for the C-3 epimerization, including its localization and nature, and the identification and characterization of the enzyme is one of the most important subjects in the field of vitamin D research. Because vitamin D compounds consist of 9,10-secosteroids, the chemical properties of the C-3 hydroxy group are different from those of the common steroids (Fig. 1). We have reported that the epimerization is controlled by an enzyme existing in the liver cytosol fraction and requires both NAD and NADPH as coenzymes [3], which is analogous to the epimerization of hydroxy groups in various common steroids by hydroxysteroid dehydroge-

* Corresponding author. Tel.: +81 76 234 4460; fax: +81 76 234 4459.
E-mail address: higashi@p.kanazawa-u.ac.jp (T. Higashi).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.07.012

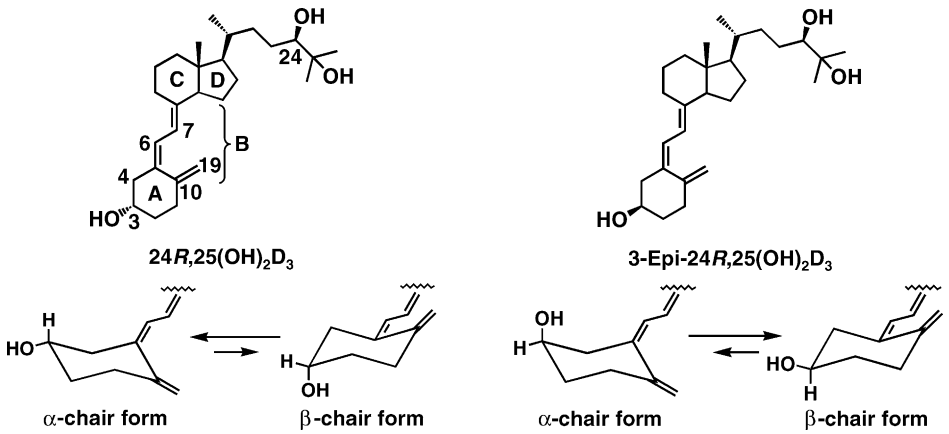

Fig. 1. Chemical structures and A-ring conformations of 24*R*,25(OH)$_2$D$_3$ and 3-epi-24*R*,25(OH)$_2$D$_3$. 24*R*,25(OH)$_2$D$_3$ are partially numbered and the rings are lettered A–D.

nases (HSDs) with respect to the subcellular localization of the enzyme and coenzyme requirement. Although the intermediate has not been isolated, it seems that common mechanisms are also observed in the formation of the C-3 epimers of 24*R*,25(OH)$_2$D$_3$ [3] as in that of the common steroids, such as digoxigenin [10] and bile acid [11]; both epimers are formed via the oxo-form as the intermediate. These data led us to hypothesize that some HSDs are the C-3 epimerizing enzymes of vitamin D compounds. The examination of the in vitro reaction of 24*R*,25(OH)$_2$D$_3$ with the HSDs is helpful for identifying the enzyme responsible for the C-3 epimerization and understanding the mechanism of the formation of the C-3 epimer. In the present study, we analyzed the reaction products of 24*R*,25(OH)$_2$D$_3$ or 3-epi-24*R*,25(OH)$_2$D$_3$ incubated with 3α- or β-HSD using high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS).

2. Experimental

2.1. Materials and reagents

24*R*,25(OH)$_2$D$_3$ was obtained from Duphar B.V. (Amsterdam, The Netherlands). 3-Epi-24*R*,25(OH)$_2$D$_3$ and (24*S*)-24,25-dihydroxyvitamin D$_3$ [24*S*,25(OH)$_2$D$_3$] were donated by Professor S. Hatakeyama (Nagasaki University, Nagasaki, Japan) and Kureha Chemical (Tokyo, Japan), respectively. (24*R*)-24,25-dihydroxyvitamin D$_2$ (internal standard, IS) was synthesized in our laboratory by the known method [12]. 3α-HSD originating from *Pseudomonas testasteroni*, β-HSD originating from *Pseudomonas testasteroni* and cholesterol oxidase (ChO) originating from *Streptomyces sp.* were purchased from Nacali Tesque (Kyoto, Japan). Alcohol dehydrogenase (AD) originating from yeast was obtained from Wako Pure Chemical Industries (Osaka, Japan). γ-Cyclodextrin (γ-CD) was donated by Nihon Shokuhin Kako (Tokyo). Oasis HLB cartridges (60 mg; Waters, Milford, MA, USA) were successively washed with AcOEt (2 ml), MeOH (2 ml) and H$_2$O (2 ml) prior to use. All other reagents and materials were of analytical grade.

2.2. Instruments

HPLC was performed using a Hitachi L-7110 chromatograph (Tokyo) equipped with a Shimadzu SPD-10A UV (265 nm) detector (Kyoto) or a Shimadzu LC-6A chromatograph equipped with a Shimadzu SPD-M6A photodiode array UV (210–360 nm) detector. Develosil 60-5 (5 μm, 250 × 4.6 mm i.d.) (Nomura Chemical, Seto, Japan) and J'sphere ODS H-80 columns (4 μm, 150 × 4.6 mm i.d.) (YMC, Kyoto) were used for the normal- and reversed-phase HPLC, respectively. Hexane-AcOEt-isopropanol (12:8:1, v/v/v) was used as the mobile phase in the normal-phase HPLC. The mobile phases in the conventional and inclusion reversed-phase HPLC were MeOH-H$_2$O (9:2, v/v) and MeCN-H$_2$O (11:9, v/v) containing 4 mM γ-CD, respectively. A chiral column, YMC CHIRAL γ-CD BR (5 μm, 250 × 4.6 mm i.d.), was also used in the reversed-phase mode with MeCN-H$_2$O (1:2, v/v) as the mobile phase. All the HPLC columns were used at the flow rate of 1 ml/min at 40 °C.

LC-MS was performed using a ThermoQuest 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 and vaporizer temperature were set at 150 and 350 °C, respectively. The sheath gas flow rate, the source current, the capillary voltage and the tube lens offset were 80 units, 5 μA, 1 and 10 V, respectively. A J'sphere ODS H-80 column was used at a flow rate of 1 ml/min and 40 °C. MeOH-H$_2$O (9:2, v/v) was used as the mobile phase.

The ^{1}H-nuclear magnetic resonance (NMR) spectra were obtained using a JEOL JNM-GSX-500 (500 MHz) spectrometer (Tokyo). CDCl$_3$ was used as the solvent and the signal of tetramethylsilane was set at 0 ppm. The abbreviations used

are s = singlet, d = doublet, dd = double doublet, m = multiplet and br = broad.

2.3. Enzymatic reaction of 24R,25(OH)$_2$D$_3$ or 3-epi-24R,25(OH)$_2$D$_3$ with HSD

The assay medium contained 24*R*,25(OH)$_2$D$_3$ or 3-epi-24*R*,25(OH)$_2$D$_3$ [5 μg in EtOH (5 μl)], NAD [500 nmol in 50 mM Tris–HCl buffer (pH 7.6; Tris buffer) (50 μl)], NADPH [500 nmol in Tris buffer (50 μl)], $MgCl_2$ [10 μmol in Tris buffer (50 μl)], ethylenediamine tetraacetic acid·2Na (EDTA) [20 nmol in Tris buffer (50 μl)], enzyme [3α-HSD (5 units), β-HSD (5 units) or a mixture of 3α-HSD and β-HSD (5 units each) in Tris buffer (100 μl)] and Tris buffer in a total volume of 1.0 ml. The mixture was incubated in air at 37 °C for 2 h.

2.4. Enzymatic reaction of 24R,25(OH)$_2$D$_3$ with AD

The assay medium contained 24*R*,25(OH)$_2$D$_3$ [10 μg in EtOH (10 μl)], NAD [500 nmol in Tris buffer (50 μl)], NADPH [500 nmol in Tris buffer (50 μl)], $MgCl_2$ [10 μmol in Tris buffer (50 μl)], EDTA [20 nmol in Tris buffer (50 μl)], AD [100 units in Tris buffer (100 μl)] and Tris buffer in a total volume of 1.0 ml. The mixture was incubated in air at 37 °C for 2 h.

2.5. Pretreatment of incubation specimen

To an incubation specimen, MeOH (1 ml) and an ethanolic solution of IS (2 μg in 100 μl) were added and the mixture was centrifuged at 1500 g for 10 min. The supernatant was passed through an Oasis HLB cartridge. After washing with H_2O (2 ml), 70% MeOH (2 ml) and hexane (1 ml), the vitamin D compounds were eluted with AcOEt (1 ml), which was evaporated under a N_2 gas stream. The residue was dissolved in EtOH (200 μl) and aliquots of it were subjected to HPLC analysis.

2.6. Identification of enzymatically formed 3-epi-24R,25(OH)$_2$D$_3$ or 24R,25(OH)$_2$D$_3$

The pretreated incubation specimens were subjected to inclusion HPLC using γ-CD as the mobile phase additive, and the eluate containing 3-epi-24*R*,25(OH)$_2$D$_3$ [retention time (t_R) 12.0–12.6 min] or 24*R*,25(OH)$_2$D$_3$ (t_R 13.0–13.8 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 (0.5 ml, three times). 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. The residue was dissolved in EtOH (100 μl) and aliquots of it were subjected to photodiode array UV-HPLC (PDA-HPLC) and LC-MS analysis.

2.7. Enzymatic synthesis of (7Z)-(24R)-24,25-dihydroxy-9,10-secocholesta-4,7,10(19)-triene-3-one (compound A)

The mixture of 24*R*,25(OH)$_2$D$_3$ [100 μg in EtOH (100 μl)] and ChO (50 units) in Tris buffer (5 ml) was incubated in air at 37 °C for 2 h. The product was extracted with AcOEt (1 ml, 4 times) and the AcOEt layers were combined. After washing the AcOEt layer with H_2O (1 ml, twice), the solvent was evaporated under a N_2 gas stream. The residue was redissolved in AcOEt (200 μl) and subjected to preparative normal-phase HPLC. The eluate containing the desired compound (t_R 8.0–9.5 min) was collected and the solvent was evaporated under a N_2 gas stream.

2.8. Epimerizing activities of 3α-HSD and β-HSD

The peak area ratio [24*R*,25(OH)$_2$D$_3$/IS or 3-epi-24*R*,25(OH)$_2$D$_3$/IS] of the standard solution [24*R*,25(OH)$_2$D$_3$ (5 μg), 3-epi-24*R*,25(OH)$_2$D$_3$ (5 μg) and IS (2 μg) in EtOH (200 μl)] in inclusion HPLC was taken as 100. The peak area ratios of the remaining substrate/IS and the formed 3-epimer/IS in an incubation specimen with 3α-HSD or β-HSD were calculated and expressed as a percent.

3. Results and discussion

3.1. C-3 epimerization in 24R,25(OH)$_2$D$_3$ by HSD

HSDs are divided into several subclasses, such as 3α-HSD and β-HSD, according to the positions and configurations of the hydroxy groups that undergo the redox reaction. Epimerization of a hydroxy group in common steroids generally involves dehydrogenation and reduction process and requires two enzymes which respond to α- and β-hydroxysteroids, respectively; for example, during the formation of an α-hydroxysteroid from its β-epimer, the β-hydroxysteroid is converted to the oxosteroid by β-HSD and then it is reduced by α-HSD to form the α-epimer [13]. However, the properties of the C-3 hydroxy groups of vitamin D compounds, 9,10-secosteroids, are much different from those of the common steroids, and the A-ring can assume two chair-conformations (Fig. 1). Therefore, 24*R*,25(OH)$_2$D$_3$ may show unexpected features during the reaction with HSDs. Based on these considerations, we examined whether the C-3 epimerization occurs, when 24*R*,25(OH)$_2$D$_3$ was incubated with 3α-HSD, β-HSD or a mixture of two enzymes.

The incubation mixtures of 24*R*,25(OH)$_2$D$_3$ and the HSDs were pretreated as described in the experimental section. The recovery rates of 24*R*,25(OH)$_2$D$_3$ (5 μg/tube), 3-epi-24*R*,25(OH)$_2$D$_3$ (5 μg/tube) and IS (2 μg/tube) during pretreatment were almost quantitative and equal; 97.4 ± 6.70%, 92.6 ± 4.74% and 93.8 ± 2.48% [mean ± standard deviation (SD), n = 6], respectively. The occurrence of 3-epi-24*R*,25(OH)$_2$D$_3$ in the incubation specimens was confirmed

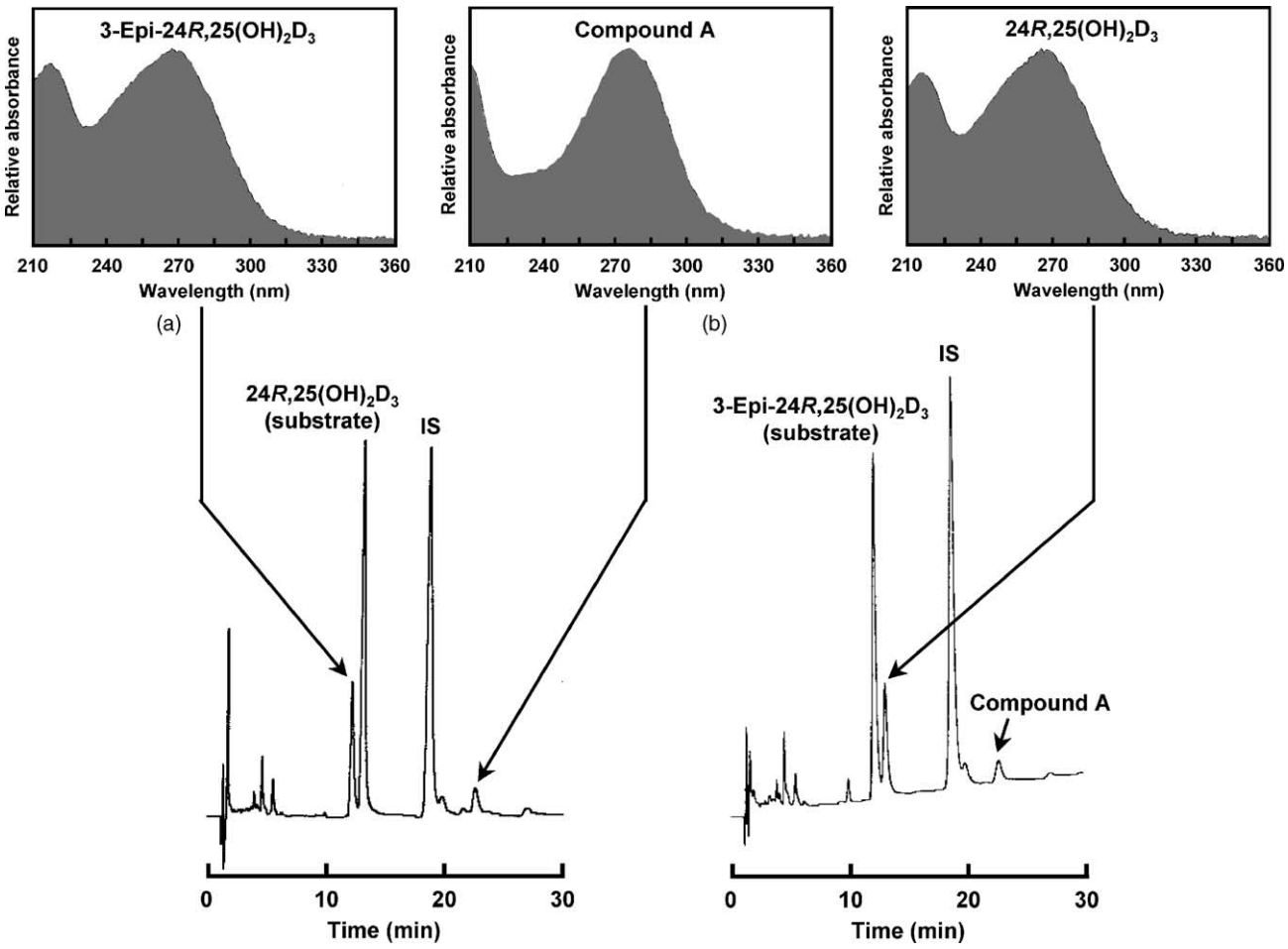

Fig. 2. Chromatograms of incubation specimen with 3α-HSD and UV spectra of enzymatic products. (a) Enzymatic products of 24*R*,25(OH)$_2$D$_3$; (b) enzymatic products of 3-epi-24*R*,25(OH)$_2$D$_3$. HPLC conditions: column J'sphere ODS H-80; mobile phase MeCN-H_2O (11:9, v/v) containing 4 mM γ-CD; detection UV 265 nm.

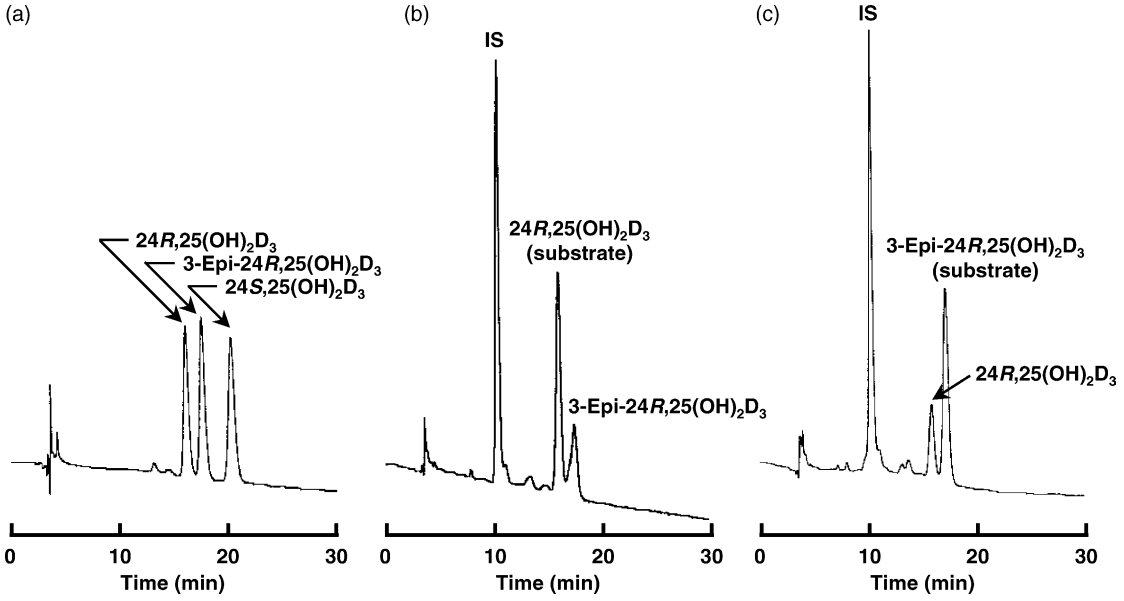

Fig. 3. Separation of 24*R*,25(OH)$_2$D$_3$, 3-epi-24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ using YMC CHIRAL γ-CD BR column. (a) Standard samples; (b) enzymatic products of 24*R*,25(OH)$_2$D$_3$ by 3α-HSD; (c) enzymatic products of 3-epi-24*R*,25(OH)$_2$D$_3$ by 3α-HSD. HPLC conditions: mobile phase MeCN-H_2O (1:2, v/v); detection UV 265 nm.

using HPLC and LC-MS. We have reported that the inclusion HPLC using γ-CD as the mobile phase additive was a significant advantage in the separation of 24*R*,25(OH)$_2$D$_3$ and 3-epi-24*R*,25(OH)$_2$D$_3$ [3], and the peak corresponding to 3-epi-24*R*,25(OH)$_2$D$_3$ (t_R 12.2 min) was observed in both chromatograms obtained from the incubation specimen with 3α-HSD (Fig. 2a) and β-HSD (the chromatogram was not shown). However, the chromatographic behavior on this inclusion HPLC alone is insufficient to prove that this product was 3-epi-24*R*,25(OH)$_2$D$_3$, because 24*R*,25(OH)$_2$D$_3$ has hydroxy groups at the 24-position as well as the 3-position; the 24*S*-isomer, 24*S*,25(OH)$_2$D$_3$ might be formed by the incubation of 24*R*,25(OH)$_2$D$_3$ with 3α-HSD or β-HSD. Although the above inclusion HPLC gave a poor result in the separation of 3-epi-24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ (t_R 12.3 min), the three compounds were satisfactorily separated when a γ-CD bonded chiral column, YMC CHIRAL γ-CD BR, was used [24*R*,25(OH)$_2$D$_3$ (t_R 16.1 min), 3-epi-24*R*,25(OH)$_2$D$_3$ (t_R 17.6 min) and 24*S*,25(OH)$_2$D$_3$ (t_R 20.3 min)] (Fig. 3a). When the HPLC using this chiral column was used for the analysis of the incubation specimens, 24*S*,25(OH)$_2$D$_3$ was not detected in the incubation mixtures with 3α-HSD (Fig. 3b) nor β-HSD (the chromatogram was not shown). These data demonstrate that the epimerization of the C-24 hydroxy group does not occur in 24*R*,25(OH)$_2$D$_3$ by the HSDs.

The fraction of the putative 3-epi-24*R*,25(OH)$_2$D$_3$ formed by the incubation of 24*R*,25(OH)$_2$D$_3$ with 3α-HSD or β-HSD was isolated and then identified by the following two methods. First, the product was subjected to PDA-HPLC; the product showed the characteristic UV absorbance (λ_{max} 267 nm and λ_{min} 230 nm) of the vitamin D structure (Fig. 2a). Second, the product was analyzed by LC-MS; the data obtained from the product and the synthetic standard completely agreed (t_R 6.4 min, *m/z* 417 $[M + H]^+$, 399 $[M + H - H_2O]^+$, 381 $[M + H - 2H_2O]^+$) (Fig. 4b). Based on these results, we concluded that the product of 24*R*,25(OH)$_2$D$_3$ incubated with 3α-HSD or β-HSD is 3-epi-24*R*,25(OH)$_2$D$_3$.

The C-3 epimerization did not proceed at all in the absence of HSD or coenzymes, which demonstrates that the epimerization is exactly the characteristic reaction by HSD, but not non-enzymatic reaction. And also the C-3 epimerization was not observed, when AD was used in place of HSD. Moreover, the C-3 epimerization in 24*R*,25(OH)$_2$D$_3$ requires only a single enzyme, 3α-HSD or β-HSD, and is much different from that in the common steroids, which generally requires two enzymes, α- and β-HSDs, as mentioned above. The use of the mixture of 3α-HSD and β-HSD did not increase the yield of the 3-epimer during the reaction of 24*R*,25(OH)$_2$D$_3$, compared with the use of 3α-HSD alone (Table 1), which also strongly proved that the single enzyme catalyzed the C-3 epimerization. However, in the presence of β-HSD which has relatively low activity for the C-3 epimerization in 24*R*,25(OH)$_2$D$_3$, the apparent epimerizing activity of 3α-HSD may lower because the substrate was consumed by β-HSD. Recently, one of the human cytosolic 3α-HSDs was reported to display significant 3β-HSD activity; 3α-HSD is able to reduce some 3-oxosteroids into both 3α-hydroxysteroids and 3β-hydroxysteroids [14]. This and our results demonstrate that the epimerization of a hydroxy group in some steroidal compounds can occur by catalysis of a single enzyme. Incidentally, to our knowledge, the epimerization of hydroxy group in steroidal compounds by single β-HSD has apparently not been reported to date.

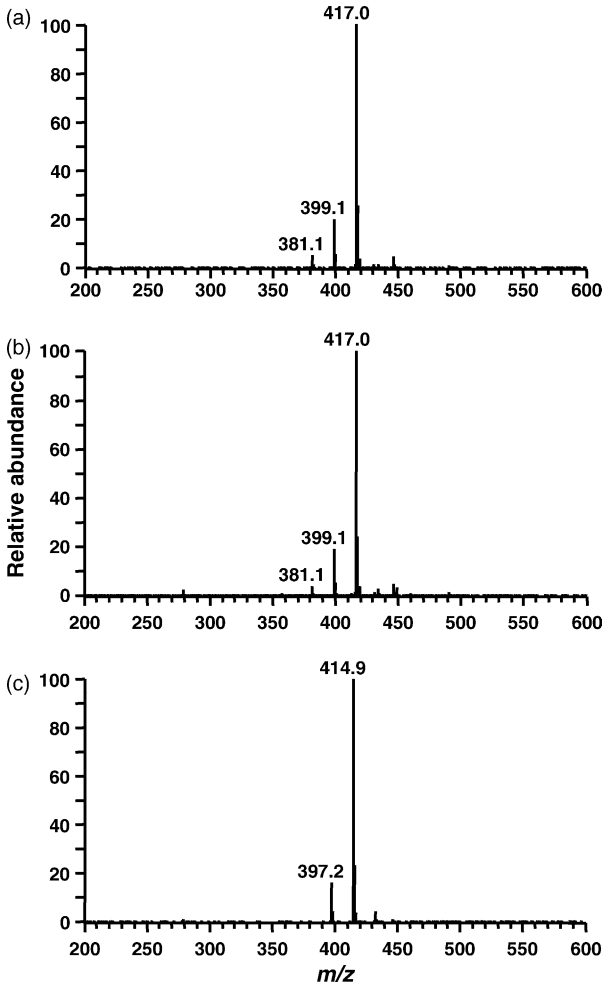

Fig. 4. LC/APCI-MS spectra of (a) 24*R*,25(OH)$_2$D$_3$, (b) 3-epi-24*R*,25(OH)$_2$D$_3$ and (c) compound A obtained from incubation specimens.

Table 1
C-3 epimerizing activities of HSDs

Enzyme/substrate	Substrate [recovery rate (%)[a]]	C-3 Epimer [formation rate (%)[a]]
3α-HSD/24*R*,25(OH)$_2$D$_3$	38.4	13.9
β-HSD/24*R*,25(OH)$_2$D$_3$	44.9	4.4
3α-HSD+β-HSD[b]/24*R*,25(OH)$_2$D$_3$	40.4	8.9
3α-HSD/3-epi-24*R*,25(OH)$_2$D$_3$	27.9	13.4
β-HSD/3-epi-24*R*,25(OH)$_2$D$_3$	44.4	6.9

[a] Mean of two incubation specimens.
[b] Mixture of 3α-HSD and β-HSD (5 units each).

3.2. Identification of 3-oxo-product derived from intermediate during C-3 epimerization

The peak derived from an unknown compound, which was named compound A, was observed at 22.5 min together with the peaks of 24*R*,25(OH)$_2$D$_3$ and 3-epi-24*R*,25(OH)$_2$D$_3$, when the sample of 24*R*,25(OH)$_2$D$_3$ incubated with 3α-HSD (Fig. 2a) or β-HSD (the chromatogram was not shown) was subjected to inclusion HPLC using γ-CD as the mobile phase additive. Compound A gave the base ion at *m/z* 415 together with its dehydrated ion at *m/z* 397 in the LC-MS analysis (Fig. 4c), which were 2 mass units smaller than those of 24*R*,25(OH)$_2$D$_3$ (Fig. 4a). This result suggested that this compound was formed by the dehydrogenation of 24*R*,25(OH)$_2$D$_3$. Compoumd A also showed the UV spectrum with λ_{max} at 275 nm and λ_{min} 227 nm (Fig. 2a), which demonstrated that the compound was not involved in the 5,7,10(19)-triene structure (λ_{max} ca. 265 nm and λ_{min} ca. 230 nm) of the vitamin D compounds.

In a previous study using [3α-^{2}H]-24*R*,25(OH)$_2$D$_3$ [3], we postulated that the 3-epimer was formed from 24*R*,25(OH)$_2$D$_3$ via the 3-oxo-form as an intermediate by the catalysis of the liver cytosol enzyme, based on the result that the deuterium at the 3α-position was displaced by a proton during the epimerization. However, the intermediate could not be isolated, because the 3-oxo-forms of the vitamin D compounds cannot stably exist; the 5,7,10(19)-triene easily and irreversibly isomerizes to the 4,7,10(19)- or 4,6,10(19)-triene in the presence of the 3-oxo-group to form the stable conjugated 4-en-3-one structure. From the result that the C-3 epimer was the main product during the reaction of 24*R*,25(OH)$_2$D$_3$ with HSD in this study, it was postulated that the reduction of the oxo-group preceded the shift of the double bond(s) to form the C-3 epimer. However, it was inferred that a part of the intermediate was converted to compound A by the shift of the double bond(s).

Yamada et al. [15] isolated the in vitro metabolite of 25-hydroxyvitamin D$_3$ having the 4,6,10(19)-trien-3-one structure, which exhibited an absorption maximum at 295 nm. This datum indicates that compound A (λ_{max} 275 nm) has an alternative structure. A ^{1}H-NMR analysis was expected to establish its structure, but the amount of compound A obtained by the reaction of 24*R*,25(OH)$_2$D$_3$ with 3α-HSD or β-HSD was not sufficient for the NMR analysis.

Because the treatment of 24*R*,25(OH)$_2$D$_3$ with oxidizing reagents, such as pyridinium chlorochromate gave poor results in the preparation of compound A; large amounts of by-products were formed, the enzymatic synthesis of compound A using ChO, which has a broad substrate specificity and can oxidize various steroids [16,17], was undertaken. The incubation of 24*R*,25(OH)$_2$D$_3$ with ChO in air at 37 °C gave a product, which co-eluted with compound A in the normal- (t_R 8.3 min) and reversed-phase HPLC (t_R 9.0 min). The UV and APCI-MS spectra of the ChO product and compound A were also identical. Based on these data, this product was confirmed to be the same as compound A. About 52 μg of compound A was produced from 100 μg of 24*R*,25(OH)$_2$D$_3$ by the treatment of 50 units of ChO.

Table 2
^{1}H-NMR data of 24*R*,25(OH)$_2$D$_3$ and compound A

	24*R*,25(OH)$_2$D$_3$	Compound A
H-3	3.94 (1H, m)[a]	–
H-4	Not assigned	5.92 (1H, s)
H-6	6.22 (1H, d, *J* = 11.2)	4.95 (2H, dd, *J* = 7.1, 7.3)
H-7	6.03 (1H, d, *J* = 11.2)	3.16 (1H, dd, *J* = 7.6, 8.0)
H-18	0.55(3H, s)	0.56 (3H, s)
H-19	4.81 (1H, d, *J* = 2.3), 5.05 (1H, brs)	5.32, 5.40 (1H each, s)
H-21	0.93 (3H, d, *J* = 6.3)	0.94 (3H, d, *J* = 6.4)
H-24	3.33 (1H, m)	3.32 (1H, m)
H-26, 27	1.16, 1.21 (3H each, s)	1.16, 1.20 (3H each, s)

[a] Chemical shifts and coupling constants are expressed in ppm and Hz, respectively.

By comparing the ^{1}H-NMR spectral data of compound A with those of 24*R*,25(OH)$_2$D$_3$ (Table 2), it was clear that compound A involved the 4-en-3-one structure; with disappearance of the signal based on H-3α of 24*R*,25(OH)$_2$D$_3$ (3.94 ppm), a new singlet peak based on H-4 emerged at 5.92 ppm in compound A. Two protons at the 6-position gave the double doublet signals based on the geminal coupling and coupling with H-7 at 4.95 ppm. The double doublet signal assigned as H-7 was also observed at 3.16 ppm. There was no significant change in the signals of the protons on the side-chain (H-21, 24, 26 and 27) between compound A and 24*R*,25(OH)$_2$D$_3$. After considering these data, compound A was identified as (7*Z*)-(24*R*)-24,25-dihydroxy-9,10-secocholesta-4,7,10(19)-trien-3-one (Fig. 5). Although the intact 3-oxo-form of 24*R*,25(OH)$_2$D$_3$ still could not be isolated, the occurrence of compound A strongly suggested that the formation of the 3-epi-form by HSD, as well as the liver cytosol enzyme [3], involves a dehydrogenation process. Judging from the chemical instability of the 3-oxo-form of vitamin D compounds, it is inferred that the dehydrogenation is the rate-controlling step, and the large part of the oxo-form is immediately reduced as soon as it is formed.

3.3. Reversibility of epimerization reaction

The reversibility of the epimerization was also investigated. 3-Epi-24*R*,25(OH)$_2$D$_3$ was incubated with 3α-HSD or β-HSD in a similar manner as with 24*R*,25(OH)$_2$D$_3$ and then the incubation specimen was analyzed by inclusion HPLC using γ-CD as the mobile phase additive. The peak corresponding to 24*R*,25(OH)$_2$D$_3$ (t_R 13.1 min) was observed in both chromatograms of the incubation specimens using 3α-HSD (Fig. 2b) or β-HSD (the chromatogram is not shown). A peak eluting at the same t_R as 24*R*,25(OH)$_2$D$_3$ was also observed in both chromatograms using the chiral column of incubation specimens with 3α-HSD (Fig. 3c) or β-HSD (the chromatogram is not shown). The PDA-HPLC (λ_{max} 267 nm, λ_{min} 230 nm, Fig. 2b) and LC-MS (t_R 6.4 min, *m/z* 417 $[M + H]^+$, 399 $[M + H - H_2O]^+$, 381 $[M + H - 2H_2O]^+$, Fig. 4a) data also demonstrated that this product was 24*R*,25(OH)$_2$D$_3$.

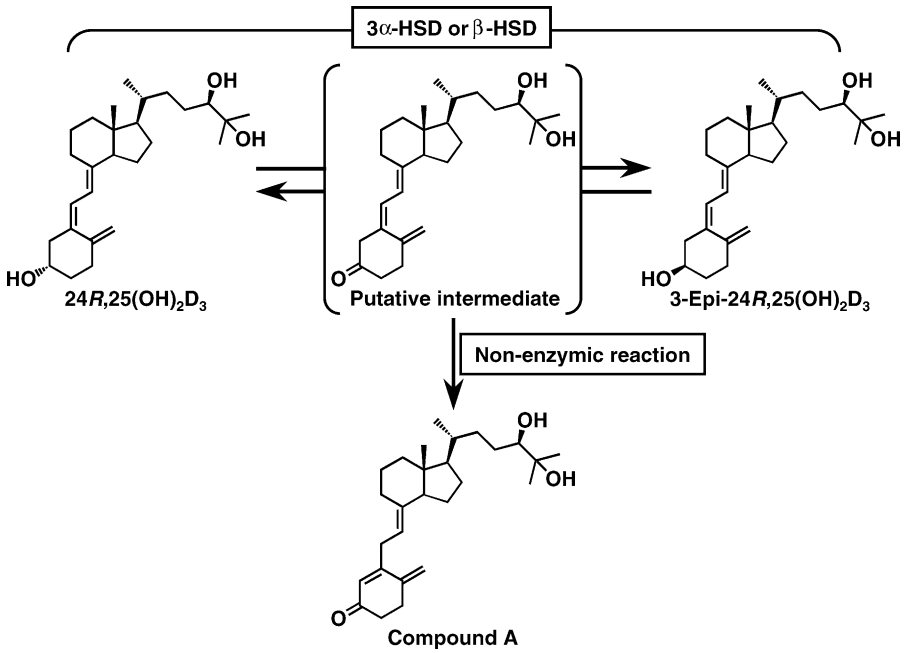

Fig. 5. Proposed C-3 epimerization mechanism in 24*R*,25(OH)$_2$D$_3$ catalyzed by HSD.

Based on these results, we concluded that 24*R*,25(OH)$_2$D$_3$ was also formed from 3-epi-24*R*,25(OH)$_2$D$_3$ by the catalysis of 3α-HSD or β-HSD. That is, the C-3 epimerization catalyzed by the HSDs is reversible, while that by the liver cytosol enzyme unidirectionally proceeded from β to α [3]. Incidentally, compound A was also formed when 3-epi-24*R*,25(OH)$_2$D$_3$ was used as the substrate (Fig. 2b).

3.4. Comparison of epimerizing activities of 3α-HSD and β-HSD

3α-HSD consumed a greater amount of 24*R*,25(OH)$_2$D$_3$ during the incubation than β-HSD (Table 1). This result demonstrates that the dehydrogenation proceeds by 3α-HSD in preference to β-HSD, though the C-3 hydroxy group of 24*R*,25(OH)$_2$D$_3$ is in the β-configuration. The amount of the 3-epimer formed by 3α-HSD was about three times greater than that by β-HSD in 24*R*,25(OH)$_2$D$_3$. 3α-HSD was also higher than β-HSD in the C-3 epimerizing activity of 3-epi-24*R*,25(OH)$_2$D$_3$. Although β-HSD alone also produced 3-epi-24*R*,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ when 24*R*,25(OH)$_2$D$_3$ and 3-epi-24*R*,25(OH)$_2$D$_3$ were used as substrates, respectively, their amounts were smaller than those expected from the relatively large consumptions of the substrates. Thus, 3α-HSD was higher than β-HSD in the C-3 epimerizing activity whenever the substrates, 24*R*,25(OH)$_2$D$_3$ or 3-epi-24*R*,25(OH)$_2$D$_3$, were used.

4. Conclusion

We demonstrated that the 3-epimer was formed from 24*R*,25(OH)$_2$D$_3$ by the catalysis of 3α-HSD or β-HSD. There is a significant similarity between the C-3 epimerization by the HSDs and that by the liver cytosol enzyme previously reported [3]; i.e., the coenzyme requirement (NAD and NADPH) and epimerization process (dehydrogenation and reduction). Although HSDs originating from *Pseudomonas testasteroni* were used in the present study, the main localization of these enzymes in animals and humans is the liver cytosol fraction [18]. These data suggest that some HSDs may catalyze the C-3 epimerization of vitamin D compounds in animals and humans.

Acknowledgements

Part of this study was supported by grants from the Ministry of Education, Science, Sport and Culture of Japan. We thank Professor S. Hatakeyama and Kureha Chemical for providing the vitamin D compounds. We also thank Nihon Shokuhin Kako for supplying the γ-CD.

References

[1] T. Nakamura, T. Kurokawa, H. Orimo, Calcif. Tissue Int. 43 (1988) 235–243.
[2] T. Nakamura, T. Kurokawa, H. Orimo, Bone 10 (1989) 117–123.
[3] T. Higashi, A. Ogasawara, K. Shimada, Anal. Sci. 16 (2000) 477–482.
[4] T. Higashi, R. Kikuchi, K. Miura, K. Shimada, H. Hiyamizu, H. Ooi, Y. Iwabuchi, S. Hatakeyama, N. Kubodera, Biol. Pharm. Bull. 22 (1999) 767–769.
[5] T. Higashi, R. Kikuchi, K. Miura, K. Shimada, H. Hiyamizu, H. Ooi, Y. Iwabuchi, S. Hatakeyama, N. Kubodera, Steroids 65 (2000) 281–294.

[6] H. Sekimoto, M.-L. Siu-Caldera, A. Weiskopf, P. Vouros, K.R. Muralidharan, W.H. Okamura, M.R. Uskoković, G.S. Reddy, FEBS Lett. 241 (1999) 278–282.
[7] M. Kamao, S. Tatematsu, G.S. Reddy, S. Hatakeyama, M. Sugiura, N. Ohashi, N. Kubodera, T. Okano, J. Nutr. Sci. Vitaminol. 47 (2001) 108–115.
[8] M. Kamao, S. Tatematsu, S. Hatakeyama, K. Ozono, N. Kubodera, G.S. Reddy, T. Okano, J. Biol. Chem. 278 (2003) 1463–1471.
[9] M. Kamao, S. Tatematsu, S. Hatakeyama, T. Sakaki, N. Sawada, K. Inouye, K. Ozono, N. Kubodera, G.S. Reddy, T. Okano, J. Biol. Chem. 279 (2004) 15897–15907.
[10] R.E. Talcott, S.J. Stohs, M.M. El-Olemy, Biochem. Pharmacol. 21 (1972) 2001–2006.
[11] H.-U. Marschall, U.C.T. Oppermann, S. Svensson, E. Nordling, E. Persson, J.-O. Höög, H. Jörnvall, Hepatology 31 (2000) 991–996.
[12] K. Katsumi, T. Okano, Y. Ono, E. Maegaki, K. Nishimura, M. Baba, T. Kobayashi, O. Miyata, T. Naito, I. Ninomiya, Chem. Pharm. Bull. 35 (1987) 970–979.
[13] E.C. Pirog, D.C. Collins, J. Clin. Endocrinol. Metab. 84 (1999) 3217–3221.
[14] S. Steckelbroeck, Y. Jin, S. Gopishetty, B. Oyesanmi, T.M. Penning, J. Biol. Chem. 279 (2004) 10784–10795.
[15] S. Yamada, K. Yamamoto, H. Takayama, T. Hayashi, C. Miyaura, H. Tanaka, E. Abe, T. Suda, J. Biol. Chem. 262 (1987) 12939–12944.
[16] D. Labaree, R.M. Hoyte, R.B. Hochberg, Steroids 62 (1997) 482–486.
[17] L.W. Guo, W.K. Wilson, J. Pang, C.H. Shackleton, Steroids 68 (2003) 31–42.
[18] H. Takikawa, M. Fujiyoshi, K. Nishikawa, M. Yamanaka, Hepatology 16 (1992) 365–371.