Novel Metabolism of 1α,25-Dihydroxyvitamin D_3 with $C_{24}-C_{25}$ Bond Cleavage Catalyzed by Human CYP24A1†

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ABSTRACT: Our previous study revealed that human CYP24A1 catalyzes a remarkable metabolism consisting of both C-23 and C-24 hydroxylation pathways that used both 25(OH)D_3 and 1α,25$(OH)_2D_3$ as substrates, while rat CYP24A1 showed extreme predominance of the C-24 over C-23 hydroxylation pathway [Sakaki, T., Sawada, N., Komai, K., Shiozawa, S., Yamada, S., Yamamoto, K., Ohyama, Y. and Inouye, K. (2000) *Eur. J. Biochem. 267,* 6158−6165]. In this study, by using the *Escherichia coli* expression system for human CYP24A1, we identified 25,26,27-trinor-23-ene-D_3 and 25,26,27-trinor-23-ene-1α(OH)D_3 as novel metabolites of 25(OH)D_3 and 1α,25$(OH)_2D_3$, respectively. These metabolites appear to be closely related to the C-23 hydroxylation pathway, because human CYP24A1 produces much more of these metabolites than does rat CYP24A1. We propose that the $C_{24}-C_{25}$ bond cleavage occurs by a unique reaction mechanism including radical rearrangement. Namely, after hydrogen abstraction of the C-23 position of 1α,25$(OH)_2D_3$, part of the substrate−radical intermediate is converted into 25,26,27-trinor-23-ene-1α-(OH)D_3, while a major part of them is converted into 1α,23,25$(OH)_3D_3$. Because the $C_{24}-C_{25}$ bond cleavage abolishes the binding affinity of 1α,25(OH)D_3 for the vitamin D receptor, this reaction is quite effective for inactivation of 1α,25(OH)D_3.

The active form of vitamin D_3, 1α,25$(OH)_2D_3$,[1] plays an essential role in calcium homeostasis, immunology, and cell differentiation (*1*). The 1α,25$(OH)_2D_3$ level is precisely regulated through gene regulations of 1α-hydroxylase (CYP27B1) (*2*) and 24-hydroxylase (CYP24A1) (*3*). A huge number of in vivo and in vitro studies have revealed more than 30 vitamin D_3 metabolites from the vitamin D_3 metabolic pathways. The physiological meanings of most of the metabolites have not yet been clarified, although some of the metabolites were found to be biologically active (*4*−*7*).

As described previously (*8*−*12*), an *Escherichia coli* expression system for CYP24A1 has low backgrounds in kinetic studies of P450 relating to the metabolism of vitamin D_3 because of the absence of a P450 gene in the *E. coli* genome (*13*) and the lack of steroids in *E. coli* cells. Using the *E. coli* expression system, we revealed that rat CYP24A1 catalyzes the six-step monooxygenation of 1α,25$(OH)_2D_3$, which is designated the C-24 hydroxylation pathway (Figure 1) (*10*). In addition to this pathway, human CYP24A1 showed the alternative metabolism of 1α,25(OH)D_3 to 1α,25-(OH)D_3-26,23-lactone through the C-23 hydroxylation pathway (*14*, *11*).

Vitamin D analogues are potentially useful for clinical treatments of type I rickets, osteoporosis, renal osteodystrophy, psoriasis, leukemia, and breast cancer (*1*). With regard to the metabolism of vitamin D analogues, the metabolism in target tissues such as the kidneys, small intestine, and bones is pharmacologically essential as reported by Komuro et al. (*15*). The major metabolic enzyme of the vitamin D analogues in these tissues is considered to be CYP24A1 (*9*, *12*, *15*).

Animal experiments suggest that rat CYP24A1 and mouse CYP24A1 have extreme predominance of C-24 over C-23 (*16*). On the other hand, most of the metabolites observed in the guinea pig are included in the C23-hydroxylation pathway (*17*). These facts mean that the preclinical tests using

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[1] Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1 α,25-dihydroxyvitamin D_3; 24*R*,25$(OH)_2D_3$, 24*R*,25-dihydroxyvitamin D_3; 23*S*,25$(OH)_2D_3$, 23*S*,25-dihydroxyvitamin D_3; 1α,24,25$(OH)_3D_3$, 1α,24*R*,25-trihydroxyvitamin D_3; 23*S*,25,26$(OH)_3D_3$, 23*S*,25,26-trihydroxyvitamin D_3; 24-oxo-25(OH)D_3, 24-oxo-25-hydroxyvitamin D_3; 24-oxo-1 α,25$(OH)_2D_3$, 24-oxo-1α,25-dihydroxyvitamin D_3; 24-oxo-1 α,23,25$(OH)_3D_3$, 24-oxo-1α,23,25-trihydroxyvitamin D_3; 25(OH)D_3-26,23-lactol, 25-hydroxyvitamin D_3-26,23-lactol; 25(OH)D_3-26,23-lactone, 25-hydroxyvitamin D_3-26,23-lactone; tetranor 23(OH), 24,25,26,27-tetranor-23-hydroxyvitamin D_3; tetranor 1α,23$(OH)_2D_3$, 24,25,26,27-tetranor-1α,23-dihydroxyvitamin D_3; tetranor-23-oxo-1α (OH)D_3, 24,25,26,27-tetranor-23-oxo-1α-hydroxyvitamin D_3; trinor-23-ene-1α(OH)D_3, 25,26,27-trinor-23-ene-1α-hydroxyvitamin D_3.

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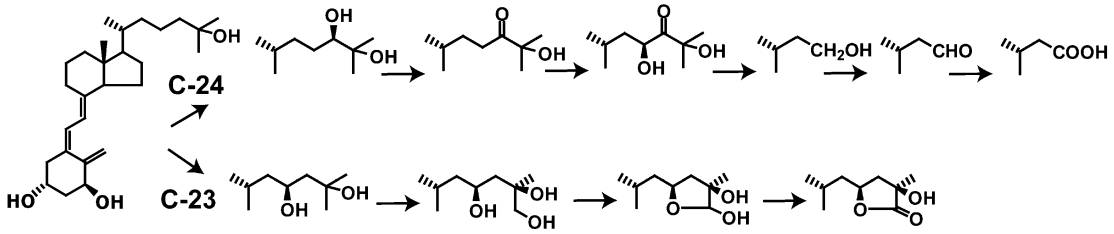

FIGURE 1: C-24 and C-23 oxidation pathways of 1α,25$(OH)_2D_3$ catalyzed by human CYP24A1.

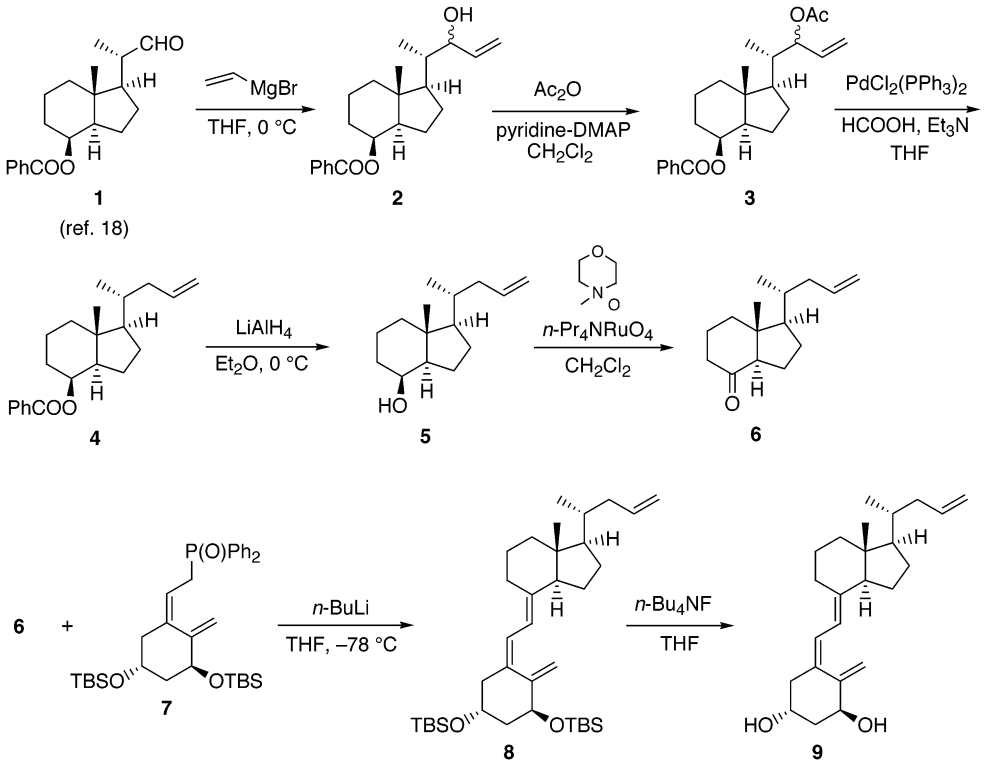

FIGURE 2: Synthesis of 25,26,27-trinor-23-ene-1α$(OH)D_3$.

animals such as the rat, mouse, and guinea pig could not correctly predict the metabolism of vitamin D analogues in the human body. Since a human kidney specimen is not easily obtained, an in vitro system containing human CYP24A1 will be required for the prediction of the drug metabolism in the human kidney.

In this paper, we present novel metabolites of 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ by human CYP24A1. Because the metabolites were less polar than the corresponding substrates, we missed the essential metabolites in the previous study (*11*). Although C−C bond cleavage via peroxide adduct including multistep oxidation is often seen in P450 reactions, a unique reaction mechanism including radical rearrangement is proposed in this study.

EXPERIMENTAL PROCEDURES

Materials. DNA modifying enzymes, restriction enzymes, and DNA sequencing kit were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan).

Linker and primer DNAs were purchased from Japan Bio-Service (Tokyo, Japan). *E. coli* JM109 (Takara Shuzo, Kyoto, Japan) was used as a host strain. 25$(OH)D_3$ and 1α,25-$(OH)_2D_3$ were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and 24*R*,25$(OH)_2D_3$ was purchased from Funakoshi Co. Ltd. (Tokyo, Japan). 23*S*,25$(OH)_2D_3$ was kindly given by Dr. Y. Ohyama (Hiroshima University). 24,25,26,27-Tetranor-1α,23$(OH)_2D_3$ and calcitroic acid were kindly given by Sumitomo Pharmaceuticals Co. (Osaka, Japan). NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). [^{3}H]-1α,25$(OH)_2D_3$ was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Vitamin D receptor binding kit containing calf-thymus cytosol was purchased from Yamasa Shoyu (Chiba, Japan). Other chemicals used were of the highest quality commercially available.

Synthesis of 25,26,27-Trinor-23-ene-1α(OH)D_3. Figure 2 outlines the synthesis of 25,26,27-trinor-23-ene-1α$(OH)D_3$.

Vinylmagnesium bromide (1.08 M in THF, 3.25 mL) was added to a stirred solution of aldehyde **1** (*18*) (553 mg) in THF (4 mL) at 0 °C. After being stirred at 0 °C for 2 h, the reaction mixture was quenched by the addition of saturated NH_4Cl, extracted with Et_2O, washed with saturated NaCl, dried over $MgSO_4$, and evaporated in vacuo. Purification of the residue by column chromatography (SiO_2, hexane/AcOEt = 8:1) gave **2** (451 mg, 75%) as a 3:7 epimeric mixture.

Acetic anhydride (185 μL) was added to a mixture of **2** (450 mg), pyridine (264 μL), and 4-(dimethylamino)pyridine (DMAP) (1.6 mg) in CH_2Cl_2 (4 mL) at room temperature.

After being stirred for 14 h, the reaction mixture was diluted with Et_2O, washed with 1 M HCl, saturated $NaHCO_3$, and saturated NaCl. The extract was dried over $MgSO_4$, evaporated in vacuo, and chromatographed (SiO_2, hexane/AcOEt = 12:1) to give **3** (497 mg, 98%) as an epimeric mixture.

To a degassed solution of **3** (104 mg), triethylamine (360 μL), and formic acid (100 μL) in THF (5 mL) was added $PdCl_2(PPh_3)_2$ (17 mg). The mixture was heated at reflux for 2.5 h and extracted with AcOEt. The extract was washed with saturated NaCl, dried over $MgSO_4$, evaporated in vacuo, and chromatographed (SiO_2, hexane/AcOEt = 30:1) to give **4** (84 mg, 95%).

To a stirred solution of **4** (267 mg) in Et_2O (15 mL) at 0 °C was added $LiAlH_4$ (45 mg). After 40 min, the reaction mixture was quenched by the addition of saturated NH_4Cl, diluted with AcOEt, and filtered through Celite. The filtrate was dried over $MgSO_4$, evaporated in vacuo, and chromatographed (SiO_2, hexane/AcOEt = 20:1) to give **5** (180 mg, 99%).

A mixture of **5** (38.9 mg), *N*-methylmorpholine *N*-oxide (45.0 mg), and 4A molecular sieves (80.0 mg) in CH_2Cl_2 (10 mL) was stirred at room temperature for 1 h and then *n*-Pr_4NRuO_4 (6.0 mg) was added. After being stirred at room temperature for 2 h, the mixture was filtered through Celite, evaporated, and chromatographed (SiO_2, hexane/AcOEt = 15:1) to give **6** (34.8 mg, 90%) as a colorless oil.

A solution of **7** (856 mg) in THF (14 mL) was cooled at −78 °C and treated with *n*-BuLi (1.58 M in hexane, 900 μL). The resulting deep red solution was stirred at −78 °C for 5 min, and a solution of **6** (143 mg) in THF (8 mL) was added. After being stirred at −78 °C for 17 h, the reaction mixture was quenched with saturated NH_4Cl, and extracted with CH_2Cl_2. The extract was washed with water and saturated NaCl, dried over $MgSO_4$, evaporated, and chromatographed (SiO_2, hexane/AcOEt = 30:1) to give **8** (180 mg, 47%) as a colorless powder.

To a stirred solution of **8** (72.6 mg) in THF (4 mL) at room temperature was added 1 M *n*-Bu_4NF in THF (750 μL). After being stirred at room temperature for 18 h, the reaction mixture was diluted with CH_2Cl_2, washed with water and saturated NaCl, dried over $MgSO_4$, evaporated in vacuo, and chromatographed (SiO_2, hexane/AcOEt = 4:1) to give **9** (44.2 mg, 99%) as a colorless powder. FT-IR (film) 3334, 1595, 1437, 1296 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ: 0.55 (s, 3H), 0.94 (d, J = 6.6 Hz, 3H), 1.29 (m, 4H), 1.40−2.10 (m, 17H), 2.19 (m, 1H), 2.32 (dd, J = 6.6, 13.2 Hz, 1H), 2.60 (dd, J = 3.0, 13.2 Hz, 1H), 2.83 (dd, J = 3.3, 12.0 Hz, 1H), 4.23 (br s, 1H), 4.43 (br s, 1H), 4.99 (d, J = 11.7 Hz, 2H), 5.02 (br s, 1H), 5.77 (m, 1H), 6.02 (d, J = 11.4 Hz, 1H), 6.38 (d, J = 11.4 Hz, 1H); MS *m/z*: 356 (M^+); HRMS (EI) calcd for $C_{24}H_{36}O_2$ (M^+): 356.2715, found: 356.2695.

Cultivation of the Recombinant E. coli Cells. Recombinant *E. coli* cells were grown in TB medium (*8*) containing 50 μg/mL ampicillin at 26 °C under good aeration. The induction of transcription of human CYP24A1 or rat CYP24A1 cDNA under the tac promoter was initiated by addition of isopropyl-thio-β-D-galactopyranoside (IPTG) at a final concentration of 1 mM when the cell density (O.D. 660) reached 0.5. δ-Aminolevulinic acid was also added at a final concentration of 0.5 mM simultaneously. The recombinant cells were gently shaken at 26 °C for 24 h under good aeration by bubbling air through the culture.

Preparation of Membrane Fraction. Subcellular fractionation was carried out basically according to our previous study (*8*). 100 mM Tris-HCl (pH 7.4) buffer was used for suspension of the membrane fraction.

Measurement of Reduced CO Difference Spectra. The reduced CO-difference spectra were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) (*19*). The absorption-coefficient difference between 445 and 490 nm ($\Delta\epsilon_{445-490}$) = 105 mM^{-1} cm^{-1} was used for the calculation of the P450 hemoprotein concentration, as described previously (*8*).

Measurement of Catalytic Activity of CYP24A1. Activity toward 25(OH)D_3, 1α,25$(OH)_2D_3$, 24*R*,25$(OH)_2D_3$, and 25,26,27-trinor-23-ene-1α (OH)D_3 was measured in the reconstituted system containing a membrane fraction, 0.05 μM of human CYP24A1 or rat CYP24A1, 5.0 μM of adrenodoxin (ADX), 0.5 μM of NADPH-adrenodoxin reductase (ADR), 5.0 μM of the substrate, 0.5 mM of NADPH, 100 mM Tris-HCl pH 7.4 and 1 mM EDTA at 37 °C. The 23*S*,25$(OH)_2D_3$ was added as a substrate at a final concentration of 0.5 μM.

The reaction was initiated by addition of NADPH. Aliquots of the reaction mixture were collected after varying time intervals and extracted with four volumes of chloroform−methanol (3:1). The organic phase was recovered and taken to dryness under vacuum. The resulting residue was solubilized with acetonitrile and applied to HPLC under the following conditions: column, YMC−Pack ODS-AM (4.6 × 300 mm) (YMC Co., Tokyo, Japan); UV detection, 265 nm; flow rate, 1.0 mL/min; column temperature, 40 °C; mobile phase, acetonitrile/water (50:50, v/v) for 5 min followed by a linear gradient of 50−100% acetonitrile aqueous solution per 15 min and 100% acetonitrile for 10 min. The metabolites were also analyzed using a JASCO Finepak SIL-5 column (4.6 × 250 mm; JASCO Co. Tokyo, Japan) under the following conditions: UV detection, 265 nm; flow rate, 1.0 mL/min; column temperature, 40 °C; mobile phase, hexane/methanol/2-propanol (88:2:10).

LC-MS Analysis of the Metabolites. Isolated metabolites from HPLC effluents were subjected to mass spectrometric analysis using a Finnegan mat TSQ-70 with atmospheric pressure chemical ionization (APCI), positive mode. The conditions of LC: column, reverse phase ODS column (6 × 150 mm) (μBondapak C18, Waters); mobile phase, 90% methanol aqueous solution per 25 min; flow-rate, 1.0 mL/min; UV detection, 265 nm.

Binding Assay for Calf-Thymus Vitamin D Receptor. Displacement of [^{3}H]-1α,25$(OH)_2D_3$ from the calf-thymus cytosol receptor (Yamasa Shoyu, Japan) by 1α,25$(OH)_2D_3$ or the novel metabolites of 1α,25$(OH)_2D_3$, P6, was determined as described previously (*20*). Various amounts of 1α,25$(OH)_2D_3$ or P6 in 20 μL of ethanol were added to 500 μL of the calf-thymus cytosol diluted with 50 mM potassium phosphate buffer (pH 7.4) containing 0.3 M KCl, and incubated for 1 h at 20 °C. Next, 34 fmol of [^{3}H]-1α,25-$(OH)_2D_3$ in 25 μL of ethanol was added and the mixture was incubated for 1 h at 20 °C. Then, 200 μL of dextran-charcoal (0.05% dextran T-150, 0.5% Charcoal Decolorizing Neutral) in 50 mM sodium phosphate buffer (pH 7.5), which was freshly prepared and stirred well before addition, was added to separate bound and free [^{3}H]-1α,25$(OH)_2D_3$. The assay tube was shaken with a vortex mixer and centrifuged

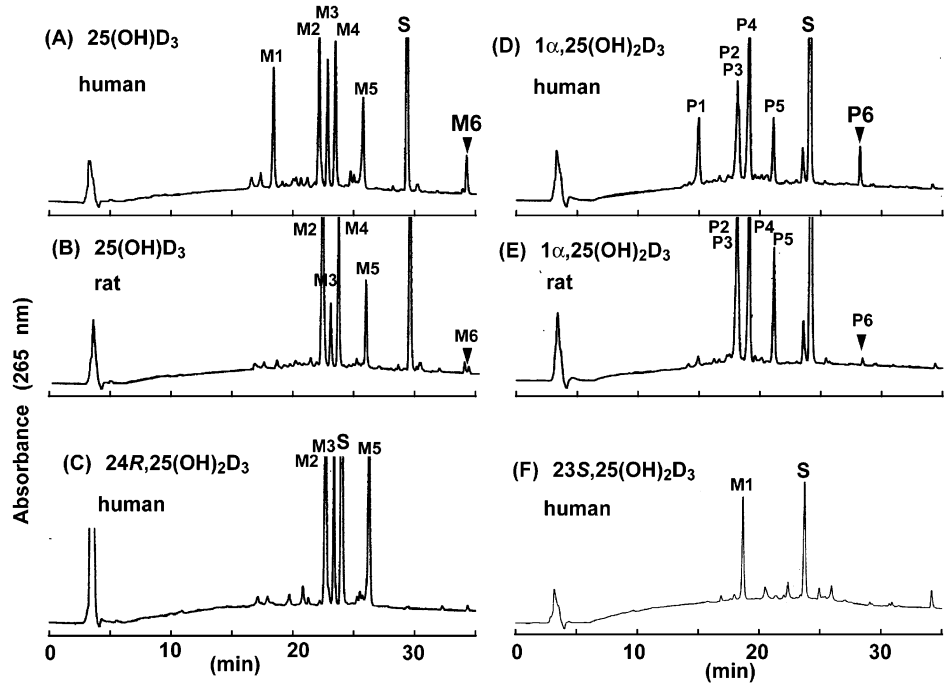

FIGURE 3: HPLC profiles of the metabolites of 25(OH)D_3 by human CYP24A1 (A), and rat CYP24A1 (B), and those of 24*R*,25(OH)$_2D_3$ by human CYP24A1 (C), 1α,25(OH)$_2D_3$ by human CYP24A1 (D), 1α,25(OH)$_2D_3$ by rat CYP24A1 (E), and 23*S*,25(OH)$_2D_3$ by human CYP24A1 (F). The reaction mixture contained the membrane fraction prepared from JM109/pKH24 (human CYP24A1) cells or JM109/pK 24R2 (rat CYP24A1). Following 10 min of incubation with each of the substrates, the reaction mixture was extracted and analyzed by HPLC as described in Experimental Procedures. The peaks designated as M1−5 are 23S,25,26(OH)$_3D_3$ (M1), 24-oxo-23*S*,25(OH)$_2D_3$ (M2), tetranor-23(OH)D_3 (M3), mixture of 24*R*,25(OH)$_2D_3$ and 23*S*,25(OH)$_2D_3$ (M4), and 24-oxo-25(OH)D_3 (M5), respectively. The peaks designated as P1−5 correspond to M1−5 of 25(OH)D_3. The peaks designated as P6 and M6 are the novel metabolites found in this study.

at 1000*g* for 10 min at 4 °C. The radioactivity in the supernatant was measured with a liquid scintillation counter. The ratio B/B_0 was plotted as shown in Figure 8. B and B_0 mean the concentration of [^{3}H]-1α,25(OH)$_2D_3$ bound to VDR in the supernatant, and the concentration of [^{3}H]-1α,25-(OH)$_2D_3$ added in the reaction mixture, respectively.

Other Methods. The concentrations of vitamin D_3 derivatives were estimated by their molar extinction coefficient of 1.80×10^4 M^{-1} cm^{-1} at 264 nm (*21*). Protein concentration was determined by the method of Lowry et al. (*22*), using bovine serum albumin as a standard.

RESULTS

Expression of CYP24A1 in E. coli. The recombinant *E. coli* JM109/pKH24 cells for human CYP24A1 and JM109/pKSN24R2 cells for rat CYP24A1 were lysed and the membrane fraction was prepared. Reduced CO-difference spectra of the membrane fractions from JM109/pKH24 and JM109/pKSN24R2 showed a peak at around 445 nm (data not shown), while the membrane fraction from the control JM109/pKSNdl cells showed no peak, suggesting the production of CYP24A1 hemoprotein. The P450 content in the membrane fraction was calculated to be 102 pmol/mg protein for human CYP24A1 and 74 pmol/mg of protein for rat CYP24A1.

HPLC Analysis of Metabolites. When the metabolites of 25(OH)D_3 by human CYP24A1 were analyzed by reverse phase HPLC, six major peaks were observed as shown in Figure 3A. On the basis of our previous study (*10*), the metabolites M1, M2, and M3 were considered to be 23,25,26(OH)$_3D_3$, 24-oxo-23*S*, 25(OH)$_2D_3$, and 24,25,26,27-tetranor-23(OH)D_3, respectively. The peak M4 contained

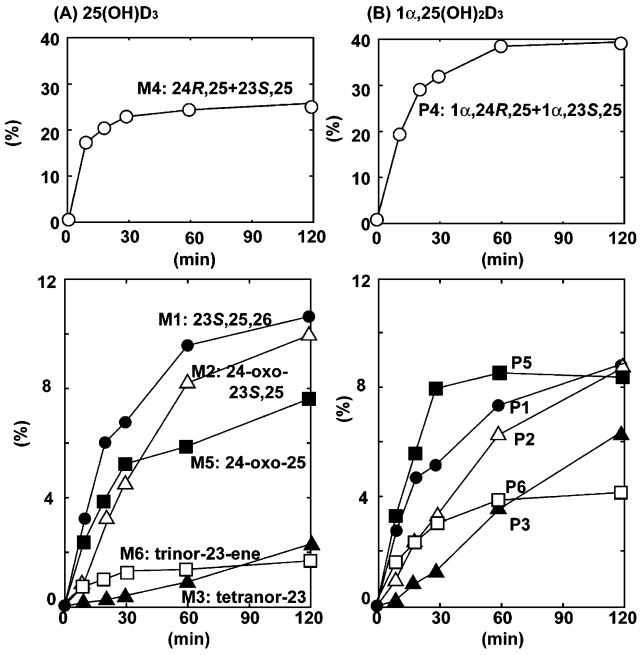

FIGURE 4: Time courses of metabolism of 25(OH)D_3 (A) and 1α,25(OH)$_2D_3$ (B) by human CYP24A1. The metabolites M1-M6 (A) and P1−P6 (B) were designated as follows: M1 and P1 (●), M2 and P2 (△), M3 and P3 (▲), M4 and P4 (○), M5 and P5 (■), M6 and P6 (□).

both 24*R*,25(OH)$_2D_3$ and 23*S*,25(OH)$_2D_3$. The metabolite M5 was considered to be 24-oxo-25(OH)D_3. The metabolite M6 was detected as a novel metabolite in this study. It should be noted that this metabolite is less polar than the substrate 25(OH)D_3. To our knowledge, no reports have been published on the production of a hydrophobic metabolite of

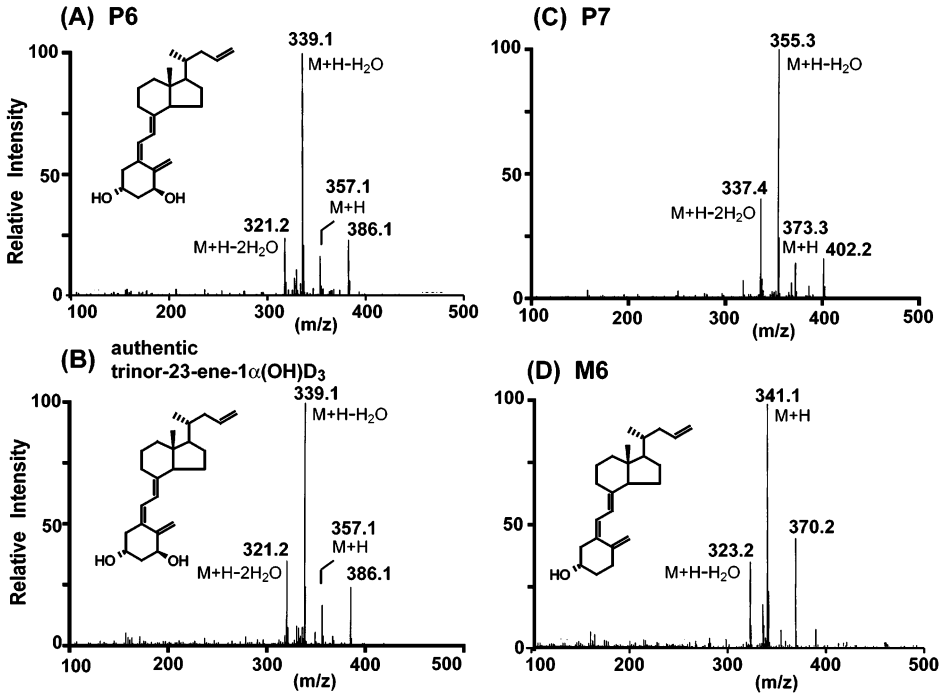

FIGURE 5: Mass spectra of the metabolite P6 (A), the chemically synthesized 25,26,27-trinor-23-ene-1α(OH)D_3 (B), P7 (C), and M6 (D). The metabolites were extracted from the reconstituted system and isolated by HPLC, and then analyzed by mass spectrometry as described in Experimental Procedures.

25(OH)D_3 by CYP24A1. Figure 3B shows the HPLC profile of the metabolites of 25(OH)D_3 by rat CYP24A1. It should be noted that the amounts of 23,25,26$(OH)_3D_3$ and the metabolite M6 were approximately 12 and 15% of those in human CYP24A1, respectively. These results suggest that M6 is closely related with the C-23 hydroxylation pathway. In the metabolism of 24*R*,25$(OH)_2D_3$ and 23*S*,25$(OH)_2D_3$ by human CYP24A1 (Figure 3C,F), the less polar metabolite was not observed, suggesting that M6 was directly converted from 25(OH)D_3. In the metabolism of 1α,25$(OH)_2D_3$ by human CYP24A1, nearly the same metabolic pattern as 25-(OH)D_3 was observed (Figure 3D). Human CYP24A1 converted 1α,25$(OH)_2D_3$ into 1α,23,25,26$(OH)_4D_3$ (P1) and the metabolite P6, but rat CYP24A1 showed a small amount of those metabolites. The peak preceding the peak "S" in (Figure 3D,E) is considered to be 1α,25$(OH)_2$ previtamin D_3 whose content is approximately 3% of 1α,25$(OH)_2$ vitamin D_3 used as a substrate. Figure 4 shows time courses of the metabolites of 25(OH)D_3 and 1α,25$(OH)_2D_3$. It can be seen that the time courses of the novel metabolites are quite similar to those of 23*S*- or 24*R*-hydroxylated metabolites (M4 and P4). These results suggest that the novel metabolites, M6 and P6, are directly converted from 25(OH)-D_3 and 1α,25$(OH)_2D_3$, respectively.

LC-MS Analysis of the Metabolites of 25(OH)D_3 and 1α,25$(OH)_2D_3$ by Human CYP24A1. The metabolite P6 from 1α,25$(OH)_2D_3$ showed a molecular ion at *m*/*z* 357 (M+H), and the fragment ions at 339 (357-H_2O) and 321 (357−2 H_2O) (Figure 5). The ion at 386 (M+H+29), which has been characteristically observed under the LC-MS conditions (*23*), was also observed. On the basis of mass spectrum and HPLC profile, P6 was assumed to be 25,26,27-trinor-23-ene-1α-(OH)D_3 (Figure 5). As expected, the mass spectrum of P6 was identical with that of the chemically synthesized 25,26,27-trinor-23-ene-1α(OH)D_3. In addition, co-chromatography of P6 and the synthesized 25,26,27-trinor-23-ene-1α(OH)D_3 in the HPLC analysis showed a single peak (Figure 6). These results strongly suggest that P6 is 25,26,27-trinor-23-ene-1α(OH)D_3. To examine the further metabolism

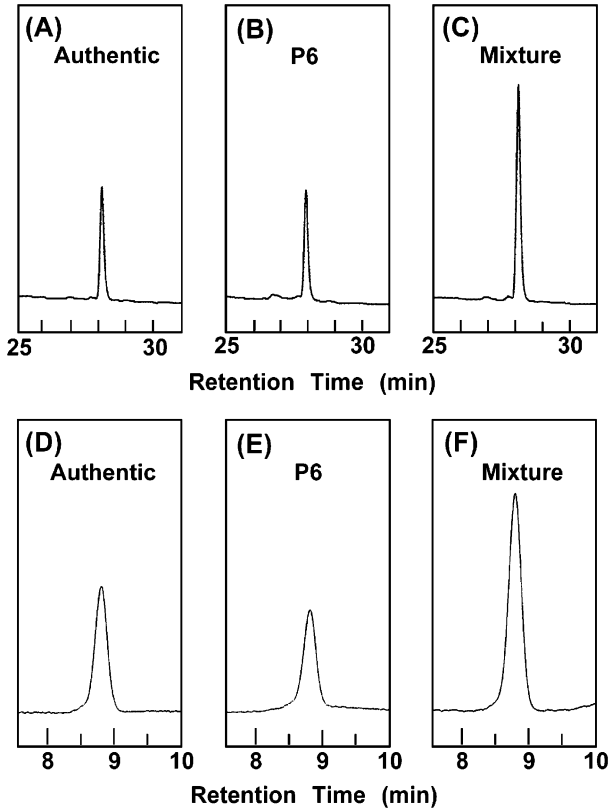

FIGURE 6: Co-chromatography analysis of the metabolite P6 with the chemically synthesized 25,26,27-trinor-23-ene-1α(OH)D_3. 100 pmol of 25,26,27-trinor-23-ene-1α(OH)D_3 (A, D), the metabolite P6 (B, E), and their mixture (C, F) were analyzed by reversed-phase HPLC (A−C) and normal-phase (D−F) HPLC as described in Experimental Procedures.

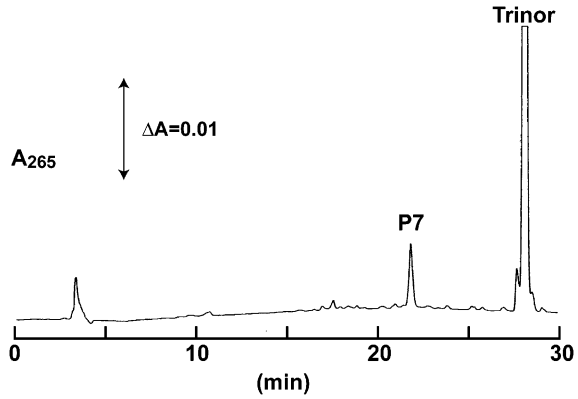

FIGURE 7: HPLC profile of the metabolite P7 of 25,26,27-trinor-23-ene-1α(OH)D_3. Following 30 min of incubation with 5.0 μM of 25,26,27-trinor-23-ene-1α(OH)D_3 designated as Trinor, the reaction mixture containing human CYP24A1 was extracted and analyzed by HPLC as described in Experimental Procedures.

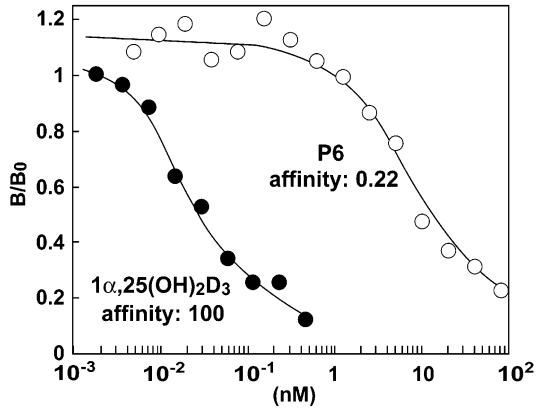

FIGURE 8: Binding assay of the metabolite P6 for calf-thymus vitamin D receptor. The designated concentrations are the final concentrations of P6 (○) and authentic standard of 1α,25$(OH)_2D_3$ (●) in the reaction mixture. *B* and B_0 mean the concentration of [^{3}H]-1α,25$(OH)_2D_3$ bound to VDR, and the concentration of [^{3}H]-1α,25$(OH)_2D_3$ added in the reaction mixture, respectively.

of 25,26,27-trinor-23-ene-1α(OH)D_3 by human CYP24A1, 25,26,27-trinor-23-ene-1α(OH)D_3 was added as the substrate in the reconstituted system. As shown in Figure 7, one major metabolite was observed and designated as P7. The metabolite showed a molecular ion at *m*/*z* 373 (M+H), and the fragment ions at 355 (373-H_2O) and 337 (373-2 H_2O) (Figure 5), suggesting that the metabolite is the monooxygenated compound of 25,26,27-trinor-23-ene-1α(OH)D_3. Although the structure of P7 has not been identified, it is likely that P7 is 25,26,27-trinor-23,24-epoxide-1α(OH)D_3. The metabolite M6 showed a molecular ion at *m*/*z* 341 (M+H) and the fragment ion at 323 (341-H_2O), suggesting that the C_{24}−C_{25} bond of the substrate 25(OH)D_3 was cleaved. These results strongly suggest that both P6 and M6 were produced by human CYP24A1 by a similar mechanism from 1α,25-$(OH)_2D_3$ and 25(OH)D_3, respectively.

Binding Affinity of the Metabolites for Vitamin D Receptor. The calf-thymus VDR binding assay clearly demonstrated that the metabolite P6 had much lower affinity for VDR than 1α,25$(OH)_2D_3$. The concentrations of 1α,25$(OH)_2D_3$ and the metabolite P6 for 50% B/B_0 were 26 pM and 1200 nM, respectively (Figure 8), indicating that the affinity of the metabolite P6 for VDR was approximately 1/460 of 1α,25-$(OH)_2D_3$.

DISCUSSION

Using the *E. coli* expression system, we have revealed that rat CYP24A1 catalyzes the six-step monooxygenation of 1α,25$(OH)_2D_3$ designated as the C-24 hydroxylation pathway (Figure 1) (*10*). In addition to this pathway, human CYP24A1 shows another metabolism of 1α,25(OH)D_3 to 1α,25(OH)-D_3-26,23-lactone in the C-23 hydroxylation pathway (*11*). In this study, we found the novel metabolites 25,26,27-trinor-23-ene-D_3 (M6) and 25,26,27-trinor-23-ene-1α(OH)D_3 (P6). Because the metabolites M6 and P6 with C_{24}−C_{25} bond cleavage were less polar than the corresponding substrates, we missed these essential metabolites in the previous study. The C−C bond cleavage via peroxide adduct including multistep oxidation is often seen in CYPs such as CYP51 (*24*), CYP19 (*25*) and CYP17 (*26*). Both human CYP24A1 and rat CYP24A1 catalyze C_{23}−C_{24} bond cleavage in the conversion from 24-oxo-23(OH) form to 24,25,26,27-tetranor 23(OH) form. This C_{23}−C_{24} bond cleavage is considered to arise via peroxide adduct at the C-24 position. However, the C_{24}−C_{25} bond cleavage observed in this study appeared to occur by a unique reaction mechanism including radical rearrangement (Figure 9), because both M6 and P6 contain no oxygen atoms at the C-24 position. The proposed metabolic pathways and reaction mechanism to yield M6 and P6 were supported by the following results indicated in this study.

(i) The metabolite M6 was not produced from 24,25-$(OH)_2D_3$ nor 23,25$(OH)_2D_3$, but from 25(OH)D_3. In addition, time courses of M6 and P6 production were similar to those for the first metabolites 24,25$(OH)_2D_3$ and 23,25$(OH)_2D_3$. Thus, M6 and P6 are likely to be directly converted from 25(OH)D_3 and 1α,25$(OH)_2D_3$, respectively.

(ii) The metabolites M6 and P6 appear to be closely related with the C-23 hydroxylation pathway, because human CYP24A1 produced much more of these metabolites than rat CYP24A1.

After hydrogen abstraction at the C-23 position of the substrate, a part of the substrate−radical intermediate appeared to be converted into the metabolites M6 and P6, while the major part of them was converted into the corresponding 23-hydroxylated products. If the same reaction occurred toward 24,25$(OH)_2D_3$ or 23,25$(OH)_2D_3$, the hydroxylated product of M6 should have been observed. However, no such metabolites were detected.

The C−C bond cleavage by radical rearrangement has been observed in the oxidation of norcarane by P450cam (CYP101), P450BM-3 (CYP102), CYP2B1 CYP2B4, and CYP2E1 (*27*, *28*). To our knowledge, however, no reports on a reaction against endogenous substrates have been published. The binding affinity of P6 toward VDR was approximately 3-orders less than that of 1α,25$(OH)_2D_3$. On the basis of the reaction mechanism (Figure 9), the ratio of metabolites between the C-23 hydroxylation pathway including the radical rearrangement and the C-24 hydroxylation pathway in human CYP24A1 is estimated to be approximately 1:4, although the ratio is determined in the *E. coli* expression system and may have no bearing as to what happens in humans. On the other hand, the ratio in rat CYP24A1 is estimated to be 1:40. After hydrogen abstraction at the C-23 position, approximately 30% of the resultant substrate−radical intermediate appeared to be converted into

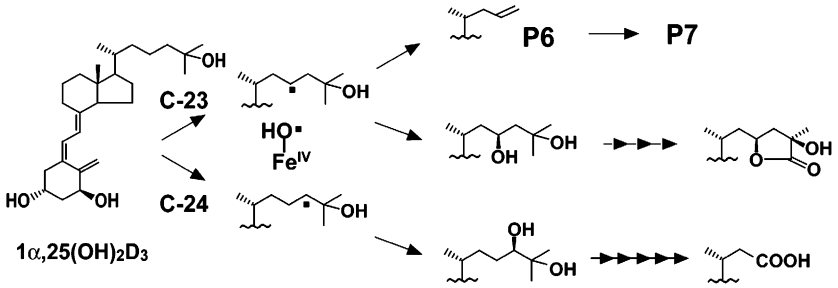

FIGURE 9: Proposed reaction mechanism to yield the metabolites P6 and P7, and metabolic pathways of 1α,25(OH)$_2$D$_3$ by human CYP24A1. After hydrogen abstraction of the C-23 position of 1α,25(OH)$_2$D$_3$, a part of the substrate−radical intermediate was converted into 25,26,27-trinor-23-ene-1α(OH)D$_3$ (P6). The putative structure of P6 was confirmed by HPLC and LC-MS analyses with the chemically synthesized 25,26,27-trinor-23-ene-1α(OH)D$_3$. The metabolite P7 was assumed to be 25,26,27-trinor-23,24-epoxide-1α(OH)D$_3$, although its structure has not been identified yet. The metabolite M6 appeared to be produced from 25(OH)D$_3$ by the same mechanism as P6.

M6 or P6 in both human CYP24A1 and rat CYP24A1 (Figure 9). Generally, the oxenoid iron of P450 abstracts hydrogen from the carbon atom of the substrate, and immediately the hydroxide radical rebinds to the carbon of the substrate. In the case of norcarane, only 0.3 to 2.0% of the substrate−radical intermediate is converted to the metabolite with C−C bond cleavage (*27*, *28*). Thus, the conversion ratio (approximately 30%) appears surprisingly high. Together with our previous studies, we have revealed a complicated metabolism toward 25(OH)D$_3$ and 1α,25-(OH)$_2$D$_3$ by human CYP24A1. Twelve metabolites are produced from 1α,25(OH)$_2$D$_3$ by this enzyme as shown in Figures 1 and 9.

It is noteworthy that M6 and P6 were detected in the human CYP24A1-dependent metabolism, but only trace amounts of them were detected in the rat CYP24A1-dependent one. Most of the first reaction by rat CYP24A1 is hydroxylation at the C-24 position, suggesting that the C-24 atom of 1α,25(OH)$_2$ is significantly closer to heme iron than the C-23 atom. On the other hand, the hydrogen atom at the C-23 position of 1α,25(OH)$_2$ appears to be close enough to the heme-iron of human CYP24A1 to be abstracted. A small difference in the binding mode of 1α,25-(OH)$_2$ in the substrate-binding pocket may result in the difference in metabolic pathways of 1α,25(OH)$_2$ between rat CYP24A1 and human CYP24A1.

As stated earlier, vitamin D analogues are potentially useful for clinical treatments of type I rickets, osteoporosis, renal osteodystrophy, psoriasis, leukemia, and breast cancer (*1*). The metabolism of vitamin D analogues in target tissues such as the kidneys, small intestine, and bones is pharmacologically essential as reported by Komuro et al. (*15*). The major metabolic enzyme of the vitamin D analogues in these tissues appears to be CYP24A1 (*9*, *12*). Recently, we revealed the $C_{24}-C_{25}$ cleavage of 26,26,26,27,27,27-hexafluoro-1α,25(OH)$_2$D$_3$ (*12*). Thus, the $C_{24}-C_{25}$ cleavage would generally be expected in the metabolism of vitamin D$_3$ analogues. Because a human kidney specimen is not easily obtained, an in vitro system containing human CYP24A1 will be required for the prediction of the drug metabolism in the human body.

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