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Characterization of new conjugated metabolites in bile of rats administered 24,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3

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Abstract

The characterization of new conjugated vitamin D metabolites in rat bile was performed using HPLC, liquid chromatography/tandem mass spectrometry combined derivatization, and GC-MS. After the administration of 24,25-dihydroxyvitamin D_3 to rats, 23,25-dihydroxy-24-oxovitamin D_3 23-glucuronide, 3-epi-24,25-dihydroxyvitamin D_3 24-glucuronide, and 24,25-dihydroxyvitamin D_3 3-sulfate were obtained as new biliary metabolites together with 24,25-dihydroxyvitamin D_3 3- and 24-glucuronides. The above metabolites, except 24,25-dihydroxyvitamin D_3 3-glucuronide, were obtained from rats dosed with 25-hydroxyvitamin D_3. 23,25-Dihydroxyvitamin D_3 23-glucuronide was also obtained from the bile of rats administered 25-hydroxyvitamin D_3 in addition to its 3-glucuronide, 25-glucuronide, and 3-sulfate. Thus, it was found that 24,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 were directly conjugated as glucuronide and sulfate, whereas at the C-23 position, they were hydroxylated and then conjugated. Furthermore, we found that the C-3 epimerization acts as one of the important pathways in vitamin D metabolism.

Keywords: 24,25-Dihydroxyvitamin D_3; 25-Hydroxyvitamin D_3; Rat bile; Conjugated metabolite; Liquid chromatography tandem mass spectrometry; 3-Epi-24,25-dihydroxyvitamin D_3 24-glucuronide

1. Introduction

In the vitamin D-supplemented state, 25-hydroxyvitamin D_3 [25(OH)D_3], a major circulating metabolite, is metabolized to various side-chain dihydroxylated metabolites. Among these metabolites, (24*R*)-24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] and (23*S*)-23,25-dihydroxyvitamin D_3 [23,25$(OH)_2D_3$] are reported to be formed by the same enzyme, 25-hydroxyvitamin D-24-hydroxylase (CYP24) [1]. The physiological roles of the side-chain dihydroxylated metabolites still remain poorly understood. For example, 24,25$(OH)_2D_3$ at pharmacological doses was reported to cause a marked increase in bone volume [2] and mechanical strength [3] in animals without hypercalcemia, and it is expected to be an anti-osteoporotic drug. On the other hand, it has been argued that 24,25$(OH)_2D_3$ is a catabolic metabolite to be excreted [4]. A study of the metabolism of vitamin D is expected to be helpful in understanding the physiological role of the metabolites and in developing new drugs.

24,25$(OH)_2D_3$ is reported to be further oxidized on its side-chain at the 23- or 24-position by CYP24 to give metabolites such as 25-hydroxy-24-oxovitamin D_3 [25(OH)-24-oxo-D_3] and (23*S*)-23,25-dihydroxy-24-oxovitamin D_3 [23,25$(OH)_2$-24-oxo-D_3] [1]. Thus, the phase I reactions in vitamin D metabolism, particularly oxidation of the side-chain, have been investigated thoroughly, but little is known regarding the conjugates of vitamin D metabolites, such as the glucuronides or sulfates [5].

In previous papers in this series, we reported that monoglucuronides of 24,25$(OH)_2D_3$ [6] or 25(OH)D_3 [7] were obtained as biliary metabolites of rats dosed with 24,25$(OH)_2D_3$ or 25(OH)D_3, respectively, per os (p.o.). On the contrary, Shimoyamada et al. reported that in dogs dosed with 24,25$(OH)_2D_3$, 23,25$(OH)_2$-24-oxo-D_3 23-glucuronide (23G) was excreted into the bile [8]. These data

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indicate that the dosed compounds are either directly conjugated as glucuronides or oxidized and then conjugated.

In the present paper and in a preliminary communication [9], we studied other conjugated vitamin D metabolites in rat bile after the administration of $24,25(OH)_2D_3$ or $25(OH)D_3$, and new metabolites (peaks A, B, C, and D in Fig. 1) were isolated. The new metabolites were identified using HPLC with photodiode array UV detection, liquid chromatography (LC)/multistage tandem mass spectrometry (MS^n, $n = 2$ or 3), and gas chromatography/MS (GC/MS).

2. Materials and methods

2.1. Materials

$24,25(OH)_2D_3$ and $25(OH)D_3$ were obtained from Duphar (Amsterdam, The Netherlands) and Wako (Osaka, Japan), respectively. (24*S*)-24,25-Dihydroxyvitamin D_3 [24*S*,$25(OH)_2D_3$] and $23,25(OH)_2$-24-oxo-D_3 were donated by Kureha Chemical Co. (Tokyo, Japan). $24,25(OH)_2D_3$ G [$24,25(OH)_2D_3$-3G, -24G, and -25G], $25(OH)D_3$ 3-sulfate (3S), 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) were the same as those used in previous papers [6,10]. Isolute C18 (EC) cartridges (500 mg; International Sorbent Tech., Hengoed, UK) were obtained from Uniflex (Tokyo). β-Glucuronidase, originating from *Escherichia coli* (Type IX-A), was obtained from Sigma (St. Louis, MO, USA). An acetate buffer [0.1 M AcONa-AcOH (pH 5.0)] was used for enzymic hydrolysis. All other reagents and solvents were of analytical grade.

2.2. HPLC analysis

HPLC was performed on a Shimadzu (Kyoto, Japan) LC-10AT chromatograph equipped with a Shimadzu SPD-10A UV detector (265 nm) or a Shimadzu SPD-M6A photodiode array UV (200–340 nm) detector. A J'sphere ODS H-80 [4 μm, 150 × 4.6 mm inner diameter (i.d.); YMC, Kyoto)] and a Develosil 60-5 (5 μm, 250 × 4.6 mm i.d.; Nomura Chemical, Seto, Japan) were used in reversed-phase and normal-phase HPLC, respectively, at a flow rate of 1 ml/min at 30°C. The pH of the mobile phase containing $AcONH_4$ or $NaClO_4$ was adjusted with AcOH or $HClO_4$, respectively.

The photodiode array UV detection of each compound was performed using a J'sphere ODS H-80 column and the following mobile phases: peaks A, B, and D, MeCN/2% $NaClO_4$ (pH 3.0) (2:3 v/v); peak C, MeCN/2% $NaClO_4$ (pH 3.0) (7:13 v/v).

2.3. Enzymic hydrolysis of glucuronide

The glucuronide [ca. 25 ng in EtOH (20 μl)] dissolved in acetate buffer (0.88 ml) and β-glucuronidase (ca. 1500 Fishman units) in acetate buffer (0.1 ml) were preincubated separately at 37°C for 20 min. The two solutions were mixed and incubated at 37°C for 3 h. The reaction mixture was extracted with AcOEt (1 ml × 3), and the organic layer was evaporated under a N_2 gas stream. The residue was dissolved in EtOH and subjected to HPLC.

2.4. Acidic solvolysis of sulfate

The sulfate (ca. 25 ng) was dissolved in AcOEt (1 ml) containing 1 μl of 0.5 M H_2SO_4. This solution was stored at room temperature for 1 h, washed with H_2O, and the solvent was evaporated. The residue was dissolved in EtOH and subjected to HPLC.

2.5. Derivatization for LC/MS^n analysis

2.5.1. Reaction with PTAD

Steroids (ca. 100 ng) were dissolved in AcOEt (50 μl) containing PTAD (5 μg) and kept at room temperature for 1.5 h. MeOH (50 μl) was added to decompose excess reagent, and the solvent was evaporated under a N_2 gas stream.

2.5.2. Acetylation

Steroids (ca. 100 ng) were dissolved in pyridine/Ac_2O (2:1 v/v, 50 μl) and stored at room temperature for 1.5 h. MeOH (50 μl) was added to the mixture, and the solvent was evaporated under a N_2 gas stream.

2.6. LC/MS analysis

LC/MS was performed using a Finnigan MAT (San Jose, CA, USA) LCQ connected to a JASCO (Tokyo) PU-980 chromatograph, and the following conditions were used. MS/MS was performed in the trap with helium gas as the collision gas, and the relative collision energy was set at 20%.

2.7. Electrospray ionization (ESI)

The spray needle voltage was 5 kV, and the heated capillary temperature, the sheath gas flow rate, and the auxiliary gas flow rate were set at 200°C, 70 units, and 20 units, respectively. The capillary voltage was 1 or −1 V, and the tube lens offset was 10 or −10 V in the positive or the negative-ion mode, respectively. A Develosil ODS HG-5 (5 μm, 150 × 2.0 mm i.d.) and a YMC Pack Pro C18 (5 μm, 150 × 3.0 mm i.d.) columns were used at a flow rate of 0.3 and 0.4 ml/min, respectively, at 30°C. For the ESI-MS analysis of each compound, the following columns and mobile phases were used: $24,25(OH)_2D_3$ 24G, peaks A, B, and D, Develosil ODS HG-5 and MeCN/10 mM $AcONH_4$ (1:2 v/v); peak C, Develosil ODS HG-5 and MeCN/10 mM $AcONH_4$ (2:3 v/v); $25(OH)D_3$ 3S-PTAD adduct, YMC Pack Pro C18 and MeOH/10 mM $AcONH_4$

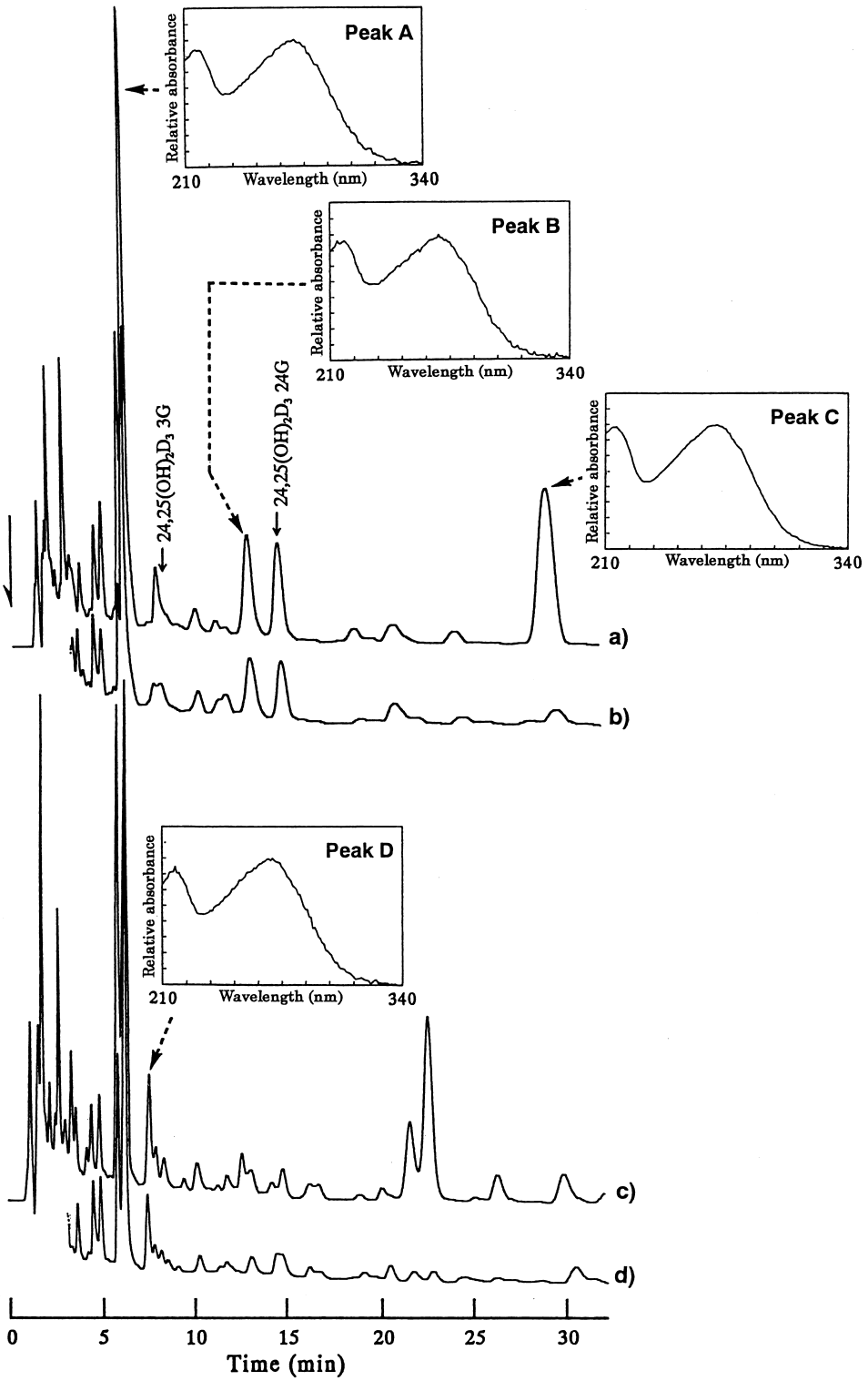

Fig. 1. Representative chromatograms and UV spectra of conjugated metabolites from rat bile. (a), Female rat administered $24,25(OH)_2D_3$; (b), male rat administered $24,25(OH)_2D_3$; (c), female rat administered $25(OH)D_3$; (d), male rat administered $25(OH)D_3$. Conditions: column, J'sphere ODS-H80; mobile phase, MeCN/0.5% $AcONH_4$ (pH 5.0) (1:2 v/v).

(1:1 v/v); peaks A-, B-, and D-PTAD adducts, YMC Pack Pro C18 and MeOH/10 mM $AcONH_4$ (7:3 v/v); peak C-PTAD adduct, Develosil ODS HG-5 and MeCN/10 mM $AcONH_4$ (2:3 v/v); peaks A- and B-acetates, YMC Pack Pro C18 and MeCN/10 mM $AcONH_4$ (3:1 v/v); peak C-acetate, Develosil ODS HG-5 and MeCN/10 mM $AcONH_4$ (2:3

v/v); peak D-acetate, YMC Pack pro C18 and MeOH/10 mM $AcONH_4$ (13:2 v/v).

2.8. Atmospheric pressure chemical ionization (APCI)

The source current was 5 μA, and the heated capillary temperature and the sheath gas flow rate were set at 225°C and 80 units, respectively, with a vaporizer temperature of 350°C. The capillary voltage and the tube lens offset were 1 and 10 V, respectively. A J'sphere ODS H-80 column was used at a flow rate of 1 ml/min at 30°C. MeOH/H_2O (3:1 v/v) was used as the mobile phase.

2.9. Derivatization for GC/MS analysis: trimethylsilylation

The genin of metabolites obtained by enzymic hydrolysis was purified by preparative (prep.) HPLC. The solvent was evaporated, and the residue was dissolved in pyridine (50 μl). *N,O*-bis(trimethylsilyl)trifluoroacetamide, containing 2% trimethylsilyl chloride (50 μl), was added to this solution and stored at 60°C for 1 h. The solvent and excess reagent were evaporated, the residue was dissolved in hexane (100 μl), and an aliquot was subjected to GC/MS.

2.10. GC/MS analysis

GC/MS was performed with a Finnigan MAT GCQ, using EI ionization. A Rtx-5MS capillary column (25 m × 0.25 mm i.d., 0.25 μm df) was inserted into the ion source through a heated (275°C) transfer line. The temperature programmable injector was used and set at 60°C for the first minute and then programmed successively at a rate of 180°C/min–250°C, where it was held for 30 min. The flow rate of the helium carrier gas was set at 40 cm/s. The column oven temperature was maintained at 60°C for 5 min and then successively programmed at a rate of 40°C/min to 290°C, where it was held for 30 min. The ionization energy was set at 70 eV.

2.11. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) of genin of peak B

^{1}H-NMR spectra of the genin of peak B was obtained with a JEOL (Tokyo) alpha (600 MHz) spectrometer at 27°C, and $CDCl_3$ was used as the solvent.

2.12. Separation of the conjugated vitamin D metabolites from rat bile

Bile was collected from Wistar strain rats (male, ca. 170 g; female, ca. 140 g, 7 w, Japan S.L.C., Hamamatsu, Japan) dosed with 24,25$(OH)_2D_3$ (0.5 mg) or 25$(OH)D_3$ (0.5 mg) as described in a previous paper [6]. The bile specimen (2 ml) was diluted with 0.5 M sodium phosphate buffer (pH 7.0; 40 ml) and passed through an Isolute C18 cartridge. After washing with H_2O (5 ml), the steroids were eluted with EtOH (5 ml), and H_2O (0.56 ml) was added to the eluate. The entire sample was applied to a column (20 × 6 mm i.d.) of PHP-LH-20. After washing with 90% EtOH (5 ml) and 0.1 M AcOH in 90% EtOH (5 ml), the conjugated metabolites were eluted with 0.1 M $AcONH_4$ in 90% EtOH (5 ml). The eluate was diluted with H_2O (7 ml) and then applied to an Isolute C18 cartridge to remove $AcONH_4$. After washing with H_2O (5 ml), the desired compounds were eluted with EtOH (5 ml), which was subsequently evaporated under a N_2 gas stream.

2.13. Analysis of bile samples from rats dosed with 24,25$(OH)_2D_3$

The above residue was redissolved in EtOH and subjected to prep. HPLC [J'sphere ODS-H80, MeCN/0.5% $AcONH_4$ (pH 5.0) (1:2 v/v); peak A, retention time (t_R) 5.5–6.8 min; peak B, t_R 12.5–14.0 min; peak C, t_R 27.9–30.1 min]. After dilution with H_2O, each fraction obtained was applied to an Isolute C18 cartridge in the manner described above to remove any inorganic salts and then evaporated. Each fraction was further purified by HPLC [J'sphere ODS-H80, MeCN/2% $NaClO_4$ (pH 3.0) (2:3 v/v); peak A, t_R 8.3–9.3 min; peak B, t_R 13.1–14.3 min; MeCN/2% $NaClO_4$ (pH 3.0) (7:13 v/v); peak C, t_R 14.6–16.1 min]. After neutralization with 2% $NaHCO_3$ and dilution with H_2O, each fraction obtained was treated as described above. The EtOH eluate was evaporated, and part of the resulting residue was subjected to HPLC and LC/MS. After hydrolysis, the residue obtained was subjected to GC/MS.

2.14. Analysis of bile samples from rats dosed with 25(OH)D_3

The above residue was redissolved in EtOH and subjected to prep. HPLC [J'sphere ODS-H80, MeCN/0.5% $AcONH_4$ (pH 5.0) (1:1 v/v); 25$(OH)D_3$ 3S, t_R 8.3–9.3 min; MeCN/0.5% $AcONH_4$ (pH 5.0) (1:2 v/v); 24,25$(OH)_2D_3$ 24G, t_R 14.8–15.9 min; peak A, t_R 5.5–6.8 min; peak B, t_R 12.5–13.7 min; peak C, t_R 27.9–29.7 min; peak D, t_R 7.3–8.2 min]. After dilution with H_2O, each fraction obtained was applied to an Isolute C18 cartridge in the manner described above to remove any inorganic salts and then evaporated. Each fraction was further purified by HPLC [J'sphere ODS-H80, MeCN/2% $NaClO_4$ (pH 3.0) (1:1 v/v); 25$(OH)D_3$ 3S, t_R 6.9–7.7 min; MeCN/2% $NaClO_4$ (pH 3.0) (2:3 v/v); 24,25$(OH)_2D_3$ 24G, t_R 14.5–15.5 min; peak A, t_R 8.3–9.3 min; peak B, t_R 13.1–14.3 min; peak D, t_R 9.1–9.8 min; MeCN/2% $NaClO_4$ (pH 3.0) (7:13 v/v); peak C, t_R 14.6–16.1 min]. After neutralization with 2% $NaHCO_3$ and dilution with H_2O, each fraction obtained was treated as described previously. The EtOH eluate was evaporated, and part of the resulting residue was subjected to HPLC and

LC/MS. After hydrolysis, the residue obtained was subjected to GC/MS.

2.15. Synthesis of 3-epi-24,25(OH)$_2$D$_3$

2.15.1. General

Optical rotations were measured with a JASCO DIP-140 polarimeter. Infrared (IR) spectra were obtained using a JASCO FT/IR-5300 spectrophotometer. ^{1}H-NMR spectra were recorded on a VARIAN (Palo Alto, CA, U.S.A.) Gemini-300 (300 MHz) or a JEOL FX-200 (200 MHz) spectrometers using $CDCl_3$ as a solvent. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane or are calibrated from $CHCl_3$. The abbreviations used are: s, singlet; d, doublet; t, triplet; hept, heptet; m, multiplet; and br, broad. HRMS were recorded on a JEOL JMS-AX-500 instruments. All reactions were carried out under an atmosphere of Ar. All extracts were dried over $MgSO_4$ and evaporated under reduced pressure with a rotary evaporator. Chromatographic purification was carried out with Merck (Darmstadt, Germany) silica gel 60 (70–230 mesh).

2.15.2. (5S)-5,6-Epoxy-1-(4-methoxybenzyl)oxy-2-hexyne (2)

BuLi in hexane (1.56 M, 12.2 ml) was added to a stirred solution of 4-methoxybenzyl propargyl ether (3.86 g, 18.5 mmol) in tetrahydrofuran (THF; 58 ml) at −78°C, and the mixture was stirred at −78°C for 1 h. (*S*)-(+)-Epichlorohydrin (**1**) (0.9 ml, 11.6 mmol) and $BF_3 \cdot Et_2O$ (2.1 ml, 18.5 mmol) were added, and the mixture was stirred at −78°C for 1.5 h. The reaction was quenched with saturated NaCl, and the mixture was extracted with Et_2O. The extract was washed with H_2O, dried, and the solvent was evaporated. The resulting residue was dissolved in THF (58 ml) and treated with 60% NaH dispersed in oil (740 mg, 18.5 mmol) at −10°C. After being stirred at −10°C for 1 h, the reaction mixture was diluted with Et_2O, washed with H_2O, dried, evaporated, and chromatographed. Elution with hexane/Et_2O (3:1 v/v) gave **2** (2.50 g, 92%) as a colorless oil: $[\alpha]_D^{24}$ +12.5° (*c* 2.37, $CHCl_3$); IR (neat) 1612, 1513, 1248, 1071 cm^{-1}; ^{1}H-NMR δ: 2.50–2.90 (m, 4H), 3.11 (m, 1H), 3.80 (s, 3H), 4.13 (t, J = 2.1 Hz, 2H), 4.51 (s, 2H), 6.87 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H).

2.15.3. (5R)-1-(4-Methoxybenzyl)oxynona-8-en-2-yn-5-ol (3)

Allylmagnesium chloride in THF (0.41 M, 38.4 ml) was added dropwise to a stirred solution of **2** (1.48 g, 6.32 mmol) in THF (45 ml) at −78°C, and the mixture was allowed to warm to room temperature. After 40 min, the reaction was quenched with saturated NH_4Cl, and the mixture was extracted with AcOEt. The extract was washed with H_2O, dried, and the solvent was evaporated to give **3** (1.60 g) as a pale yellow oil, which was used for the next reaction without purification. The pure sample was obtained as a colorless oil by column chromatography [hexane/AcOEt (2:1 v/v)] : $[\alpha]_D^{23}$ −1.2° (*c* 2.49, $CHCl_3$); IR (neat) 3543, 1616, 1088 cm^{-1}; ^{1}H-NMR δ: 1.64 (q, J = 7.5 Hz, 2H), 1.73 (br s, 1H), 2.06 (br s, 1H), 2.11–2.27 (m, 2H), 2.34–2.53 (m, 2H), 3.80 (s, 3H), 4.14 (t, J = 2.1 Hz, 2H), 4.51 (s, 2H), 4.99 (dd, J = 1.5, 10.2 Hz, 1H), 5.06 (dd, J = 1.5, 16.8 Hz, 1H), 5.83 (ddt, J = 10.2, 16.8, 6.9 Hz, 1H), 6.87 (d, J = 8.7 Hz, 2H), 7.27 (d, J = 8.7 Hz, 2H).

2.15.4. (5R)-5-(t-butyldimethylsilyl)oxy-1-(4-methoxybenzyl)oxynona-8-en-2-yne (4)

A mixture of crude **3** (1.60 g), *t*-butyldimethylsilyl chloride (1.43 g, 9.48 mmol), and imidazole (1.29 g, 19.0 mmol) in *N*,*N*-dimethylformamide (DMF; 10 ml) was stirred at room temperature for 3 h. The reaction mixture was diluted with AcOEt, washed with H_2O, dried, evaporated, and chromatographed. Elution with AcOEt-hexane (20:1 v/v) gave **4** (2.03 g, 82% from **2**) as a colorless oil: $[\alpha]_D^{24}$ +20.3° (*c* 1.63, $CHCl_3$); IR (neat) 1612, 1513, 1300, 1250, 1176, 1082 cm^{-1}; ^{1}H-NMR δ: 0.08 (s, 3H), 0.09 (s, 3H), 0.90 (s, 9H), 1.57–1.81 (m, 2H), 2.04–2.22 (m, 2H), 2.37–2.42 (m, 2H), 3.78–3.84 (m, 1H), 3.81 (s, 3H), 4.13 (t, J = 2.4 Hz, 2H), 4.52 (s, 1H), 4.97 (dd, J = 1.8, 10.2 Hz, 1H), 5.03 (dd, J = 1.8, 17.1 Hz, 1H), 5.84 (ddt, J = 10.2, 17.1, 6.6 Hz, 1H), 6.88 (d, J = 8.7 Hz, 2H), 7.28 (d, J = 8.7 Hz, 2H).

2.15.5. (5R)-5-(t-butyldimethylsilyl)oxynona-8-en-2-yn-1-ol (5)

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ; 971 mg, 4.27 mmol) was added to a stirred solution of **4** (793 mg, 2.04 mmol) in CH_2Cl_2/H_2O (20:1 v/v, 10.5 ml) at room temperature. After 3 h, the reaction mixture was diluted with CH_2Cl_2 and filtered through Celite. The filtrate was washed with saturated $NaHCO_3$, dried, and the solvent was evaporated. The resulting residue was dissolved in MeOH (8 ml) and cooled in an ice bath. $NaBH_4$ (38.4 mg, 1.01 mmol) was added to this solution, and the mixture was stirred at 0°C for 10 min. The reaction mixture was diluted with CH_2Cl_2, washed with saturated NaCl, dried, evaporated, and chromatographed. Elution with hexane/AcOEt (20:1 v/v) gave **5** (429 mg, 79%) as a colorless oil: $[\alpha]_D^{22}$ +21.2° (*c* 2.8, $CHCl_3$); ^{1}H-NMR δ: 0.06 (s, 3H), 0.08 (s, 3H), 0.89 (s, 9H), 1.52–1.76 (m, 3H), 1.98–2.22 (m, 2H), 2.34–2.38 (m, 2H), 3.80 (ddt, J = 4.5, 6.6, 12.3 Hz, 1H), 4.25 (dt, J = 6.3, 2.1 Hz, 2H), 4.96 (dd, J = 2.1, 10.2 Hz, 1H), 5.03 (dd, J = 2.1, 17.1 Hz, 1H), 5.82 (ddt, J = 10.2, 17.1, 6.6 Hz, 1H).

2.15.6. (2Z)-(5R)-5-(t-Butyldimethylsilyl)oxy-3-iodonona-2,8-dien-1-ol (6)

Seventy percent $[(MeOCH_2CH_2O)_2AlH_2]Na$ in toluene (2.24 g, 7.22 mmol) was dissolved in Et_2O (20 ml), and the resulting solution was cooled in an ice bath. Compound **5** (807 mg, 3.01 mmol) in Et_2O (10 ml) was added to this solution at 0°C, and the mixture was stirred at room temperature for 6 h. The reaction mixture was treated with

AcOEt (0.42 ml) with cooling in an ice bath to destroy excess hydride and then cooled to −50°C. After addition of I_2 (1.15 g, 4.53 mmol), the mixture was stirred at −50°C for 10 min and then allowed to warm to room temperature. After 1.5 h, the reaction was quenched by the addition of 2% $Na_2S_2O_3$, and the mixture was extracted with Et_2O. The extract was washed with H_2O and saturated $NaHCO_3$, dried, evaporated, and chromatographed. Elution with hexane/Et_2O (10:1 v/v) gave **6** (773 mg, 65%) as a pale yellow oil: $[\alpha]_D^{24}$ −3.9° (*c* 2.37, $CHCl_3$); IR (neat) 3338, 1643, 1463, 1365, 1254, 1029 cm^{-1}; ^{1}H-NMR δ: 0.06 (s, 3H), 0.07 (s, 3H), 0.87 (s, 9H), 1.44–1.68 (m, 3H), 2.10 (br q, *J* = 8.1 Hz, 2H), 2.58 (dd, *J* = 5.7, 14.1 Hz, 1H), 2.67 (dd, *J* = 6.6, 14.1 Hz, 1H), 3.96 (q, *J* = 5.7 Hz, 1H), 4.18 (br d, *J* = 4.5 Hz, 2H), 4.61 (dd, *J* = 1.8, 9.3 Hz, 1H), 5.03 (dd, *J* = 1.8, 15.6 Hz, 1H), 5.80 (ddt, *J* = 9.3, 15.6, 3.0 Hz, 1H), 5.89 (t, *J* = 5.7 Hz, 1H).

*2.15.7. (2Z)-(5R)-2-[5-(t-Butyldimethylsilyl)oxy-2-methylenecyclohexylidene]ethanol (**7**)*

After Ar gas was introduced into a solution of **6** (181 mg, 0.457 mmol) in MeCN (25 ml) for 15 min, $Pd(PPh_3)_4$ (53 mg, 45.9 μmol) and triethylamine (77 μl, 0.552 mmol) were added, and the resulting mixture was heated at reflux for 4 h. The solvent was removed, followed by purification by column chromatography. Elution with hexane/Et_2O (2:1 v/v) gave **7** (82 mg, 67%) as a pale yellow oil: $[\alpha]_D^{24}$ −40.6° (*c* 2.79, $CHCl_3$); IR (neat) 3344, 1639, 1466, 1371, 1254, 1095, 1011 cm^{-1}; ^{1}H-NMR δ: 0.05 (s, 3H), 0.06 (s, 3H), 0.88 (s, 9H), 1.47 (br s, 1H), 1.52–1.64 (m, 1H), 1.82–1.92 (m, 1H), 2.07 (td, *J* = 1.5, 13.5 Hz, 1H), 2.20 (dd, *J* = 8.7, 13.5 Hz, 1H), 2.37 (dd, *J* = 5.4, 13.2 Hz, 1H), 2.41 (dd, *J* = 5.4, 13.2 Hz, 1H), 3.83 (hept, *J* = 3.9 Hz, 1H), 4.10–4.30 (m, 2H), 5.44 (t, *J* = 6.9 Hz, 1H).

*2.15.8. (2Z)-(5R)-2-[5-(t-Butyldimethylsilyl)oxy-2-methylenecyclohexylidene]-1-chloroethane (**8**)*

Dimethyl sulfide (100 μl, 1.61 mmol) was added to an ice-cooled solution of *N*-chlorosuccinimide (181 mg, 1.36 mmol) in CH_2Cl_2 (4.4 ml), and the mixture was stirred at 0°C for 40 min. Part of the resulting mixture (1.12 ml) was added to a solution of **7** (47 mg, 0.175 mmol) in CH_2Cl_2 (0.57 ml) at −20°C, and the reaction mixture was allowed to warm to room temperature. After being stirred for 1 h, the reaction mixture was diluted with hexane, washed with H_2O and saturated NaCl, dried, and the solvent was evaporated. The residue was taken up with AcOEt/hexane (10:1 v/v) and filtered through silica gel. Evaporation of the filtrate gave **8** (44 mg) as a pale yellow oil, which was used for the next reaction without further purification: ^{1}H-NMR δ: 0.06 (s, 6H), 0.83 (s, 9H), 1.56–1.69 (m, 1H), 1.82–1.92 (m, 1H), 1.82–1.95 (m, 1H), 2.39 (br s, 1H), 2.43 (br s, 1H), 3.86 (q, *J* = 3.9 Hz, 1H), 4.19 (dd, *J* = 6.3, 8.0 Hz, 2H), 4.84 (s, 1H), 5.02 (s, 1H), 5.46 (t, *J* = 7.8 Hz, 1H).

*2.15.9. (2Z)-(5R)-[2-[5-(t-Butyldimethylsilyl)oxy-2-methylenecyclohexylidene]ethyl]diphenylphosphine oxide (**9**)*

BuLi in hexane (1.53 M, 447 μl) was added to an ice-cooled solution of diphenylphosphine (119 μl, 0.690 mmol) in THF (2.3 ml), and the mixture was stirred at 0°C for 10 min. The mixture was cooled to −50°C, and a solution of crude **8** (44 mg) in THF (1 ml) was added. After being stirred at −50°C for 1 h, the reaction was quenched by the addition of H_2O; the mixture was allowed to warm to room temperature, and then $CHCl_3$ (6.9 ml) and 30% H_2O_2 (5.1 ml) were added. After being stirred for 30 min, the mixture was diluted with $CHCl_3$, washed with saturated $Na_2S_2O_3$ and H_2O, dried, evaporated, and chromatographed. Elution with AcOEt/hexane (2:1 v/v) gave **9** (56 mg, 69% from **7**) as a colorless crystalline solid: $[\alpha]_D^{24}$ −20.7° (*c* 1.20, $CHCl_3$); IR (neat) 1631, 1254, 1190, 1095 cm^{-1}; ^{1}H-NMR δ: 0.00 (s, 6H), 0.84 (s, 9H), 1.41–1.82 (m, 4H), 2.05–2.40 (m, 3H), 3.20 (dddd, *J* = 1.5, 6.3, 15.3, 21.3 Hz, 1H), 3.36 (dt, *J* = 9.3, 15.3 Hz, 1H), 3.52 (hept, *J* = 3.9 Hz, 1H), 4.68 (s, 1H), 4.92 (s, 1H), 5.35 (td, *J* = 6.9, 7.8 Hz, 1H), 7.41–7.54 (m, 6H), 7.65–7.75 (m, 4H); HRMS calcd for $C_{27}H_{37}O_2SiP$ [M^+]: 452.2307, found 452.2301.

*2.15.10. (5Z,7E)-(3R,24R)-3,24,25-Tri(t-butyldimethylsilyl)oxy-9,10-secocholesta-5,7,10(19)-triene (**11**)*

A solution of **9** (53 mg, 0.117 mmol) in THF (1.5 ml) was cooled to −78°C and treated with 1.52 M BuLi in hexane (80 μl, 0.119 mmol). The resulting deep red solution was stirred at −78°C for 20 min, and a solution of (1*R*,3a*R*,7a*R*)-1-[(1*R*,4*R*)-4,5-di(*t*-butyldimethylsilyl)oxy-1,5-dimethylhexyl]-7a-methyl-octahydro-4*H*-inden-4-one (**10**) [11] (13 mg, 24.8 μmol) in THF (1.2 ml) was added. After being stirred at −78°C for 1 h, the reaction mixture was allowed to warm to −50°C, quenched with saturated NH_4Cl, and extracted with AcOEt. The extract was washed with H_2O and saturated NaCl, dried, evaporated, and chromatographed. Elution with hexane/AcOEt (30:1 v/v) gave **11** (19 mg, 99%) as a colorless viscous oil: $[\alpha]_D^{20}$ −6.0° (*c* 0.975, $CHCl_3$); IR (neat) 1466, 1369, 1252, 1165, 1093, 1041 cm^{-1}; ^{1}H-NMR δ: 0.03, 0.05, 0.06, 0.07, 0.08 (each s, 18H), 0.54 (s, 3H), 0.85 (s, 9H), 0.88 (s, 9H), 0.89 (s, 9H), 0.92 (d, *J* = 5.4 Hz), 1.11 (s, 3H), 1.18 (s, 3H), [1.26–1.35 (m), 1.41–1.56 (m), 1.61–1.68 (m), 20H], 1.89–2.48 (m, 7H), 2.83 (dd, *J* = 0.3, 15.3 Hz, 1H), 3.22 (br s, 1H), 3.78 (m, 1H), 4.82 (s, 1H), 5.03 (s, 1H), 6.05 (d, *J* = 11.1 Hz, 1H), 6.18 (d, *J* = 11.1 Hz, 1H).

*2.15.11. (5Z,7E)-(3R,24R)-9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol [3-epi-24,25(OH)$_2$D$_3$] (**12**)*

Bu_4NF in THF (1 M, 105 μl) was added to a stirred solution of **11** (16 mg, 21.1 μmol) in THF (0.5 ml) at room temperature. After being stirred at −78°C for 4 days, the reaction mixture was diluted with AcOEt, washed with H_2O and saturated NaCl, dried, evaporated, and chromatographed. Elution with AcOEt/hexane (5:1 v/v) gave **12** (7 mg, 79%) as a colorless crystalline solid: $[\alpha]_D^{23}$ +29.3° (*c* 0.345, $CHCl_3$); IR

(neat) 3367, 1643, 1442, 1377, 1219, 1157, 1051 cm^{-1}; ^{1}H-NMR δ: 0.56 (s, 3H), 0.94 (d, J = 6.3 Hz, 3H), 1.17 (s, 3H), 1.22 (s, 3H), 1.12–1.74 (m, 17H), 1.89–2.02 (m, 7H), 2.10–2.20 (m, 2H), 2.27 (dd, J = 9.0, 12.9 Hz, 1H), 2.41 (dt, J = 6.0, 13.5 Hz, 1H), 2.58 (dd, J = 3.9, 12.9 Hz, 1H), 2.68 (br t, J = 7.8 Hz, 1H), 2.82 (dd, J = 3.3, 12.0 Hz, 1H), 3.33 (dd, J = 6.6, 4.8 Hz, 1H), 3.89 (br tt, J = 4.0, 8.4 Hz, 1H), 4.84 (d, J = 2.1 Hz, 1H), 5.06 (br s, 1H), 6.04 (d, J = 11.1 Hz, 1H), 6.24 (d, J = 11.1 Hz, 1H); HRMS calcd for $C_{27}H_{44}O_3$ [M^+]: 416.3290, found 416.3270.

3. Results

3.1. Search for conjugated vitamin D metabolites in rat bile

The bile specimens were subjected to solid phase extraction, followed by anion exchange chromatography, and then UV-HPLC. Four new major peaks were observed in the chromatogram from female rats administered 24,25$(OH)_2D_3$ (Fig. 1a), and one of them was 24,25$(OH)_2D_3$ 24G, which has previously been reported [6]. Fractions containing the other three peaks, which we called peaks A, B, and C, were collected and further purified by prep. HPLC. The obtained fractions were subjected to photodiode array UV-HPLC. As a result, all three fractions showed typical UV absorption spectra (λ_{max}: 268 nm, λ_{min}: 230 nm) of the vitamin D-triene structure. Peak C was scarcely observed in the chromatogram from male rats (Fig. 1b). Incidentally, 24,25$(OH)_2D_3$ 3G was also isolated by a previously described method [6], and its elution position is shown by an arrow in Fig. 1.

On the other hand, a new metabolite showing the typical UV absorption spectra of vitamin D was obtained from rats dosed with 25$(OH)D_3$, and called peak D (Fig. 1c and d). Furthermore, fractions having the same t_R as authentic 25$(OH)D_3$ 3S and 24,25$(OH)_2D_3$ 24G, and peaks A, B, and C as above were also collected. Two major peaks in Fig. 1c (t_R 20–24 min) were not vitamin D compound as determined by their UV spectra.

3.2. Identification of the peak A metabolite as 23,25$(OH)_2$-24-oxo-D_3 23G

Peak A completely disappeared and a new single peak, which co-eluted with 23,25$(OH)_2$-24-oxo-D_3 [J'sphere ODS H-80, MeCN/H_2O (3:2 v/v), t_R 7.8 min; Develosil 60–5, hexane/*i*-PrOH (22:3 v/v), t_R 9.2 min], emerged after treatment with β-glucuronidase. Furthermore, GC/MS data for the trimethylsilyl (TMS)-derivatized genin and 23,25$(OH)_2$-24-oxo-D_3 completely agreed: t_R (21.4 min), *m/z* 646 $[M]^+$, 541 $[M-TMSOH-CH_3]^+$, 487 [cleavage of C-23–24 bond]$^+$, 397 $[487-TMSOH]^+$, 307 [487–2TMSOH]$^+$, and 131 $[C(CH_3)_2OTMS]^+$ (Fig. 2). In the LC/ESI-MS mass spectra of the intact peak A (t_R 5.1 min), the base ion peak was observed at *m/z* 624 $[M+NH_4]^+$ and 605 $[M-H]^-$ in the positive- and the negative-ion modes, respectively. These data indicated that peak A was the monoglucuronide of 23,25$(OH)_2$-24-oxo-D_3. The MS/MS, using the ion at *m/z* 624 as a precursor ion, showed a satisfactory product ion mass spectrum and supported the above structure: *m/z* 607 $[M+H]^+$, 589 $[607-H_2O]^+$, 431 [genin+H]$^+$, 413 $[431-H_2O]^+$, and 395 $[431-2H_2O]^+$, but the conjugation position (3, 23 or 25) could not be determined. We have reported that the PTAD adducts of conjugated vitamin D metabolites are cleaved at the C-6–7 bond of the vitamin D skeleton and provide a product ion with conjugation position indicated by a positive-ESI-MS/MS/MS [10]. That is, in the case where the conjugation position is at the A-ring, a characteristic product ion at *m/z* 474 for the glucuronide or 378 for the sulfate is observed, whereas in the case where conjugation is at the side-chain, only a corresponding ion at *m/z* 298 is observed. Thus, peak A was converted to the PTAD adduct and subjected to LC/ESI-MS/MS/MS using *m/z* 799 $[M+NH_4]^+$ and *m/z* 782 $[M+H]^+$ as precursor ions with 20% relative collision energy. The product ion mass spectrum of the peak A derivative (t_R 4.5 min) showed *m/z* 298, but not 474. This result indicated that peak A had a glucuronic acid at the side-chain, but there were still two possible conjugation positions, that is, at the 23- or 25-positions. The mass spectrum of acetylated peak A (t_R 3.9 min) afforded evidence to indicate the conjugation position. The spectrum showed the molecular-related ions at *m/z* 792 $[M+NH_4]^+$ and 773 $[M-H]^-$ in the positive- and the negative-ion modes, respectively, indicating the presence of four reactive *sec*- and one non-reactive *tert*-hydroxy groups in peak A. These results showed that peak A was 23,25$(OH)_2$-24-oxo-D_3 23G.

3.3. Identification of the peak B metabolite as 3-epi-24,25$(OH)_2D_3$ 24G

All of the ESI-MS^n spectral data for peak B completely agreed with those of 24,25$(OH)_2D_3$-24G or -25G, but its t_R (10.6 min) was different from those of the two glucuronides. Enzymic hydrolysis of the peak B metabolite with β-glucuronidase gave a new peak, which eluted closely with 24,25$(OH)_2D_3$ [J'sphere ODS H-80, MeCN/H_2O (3:2 v/v), the genin of peak B t_R 10.7 min, 24,25$(OH)_2D_3$ t_R 10.9 min; Develosil 60–5, hexane/*i*-PrOH (22:3 v/v), the genin of peak B t_R 13.0 min, 24,25$(OH)_2D_3$ t_R 12.9 min]. These data indicated that the metabolite was a monoglucuronide of dihydroxyvitamin D_3. Furthermore, the GC/MS mass spectrum of the TMS-genin derivative completely agreed with that of TMS-24,25$(OH)_2D_3$ (Fig. 2), but their t_R values were much different [the genin of peak B derivative t_R 21.9 min; 24,25$(OH)_2D_3$ derivative t_R 19.5 min]. Based on these results, we guessed that the genin of peak B was a stereoisomer of 24,25$(OH)_2D_3$, such as 24*S*,25$(OH)_2D_3$, which was obtained from incubation of 24,25$(OH)_2D_3$ with the chick kidney homogenate [12]. However, TMS-24*S*,25$(OH)_2D_3$

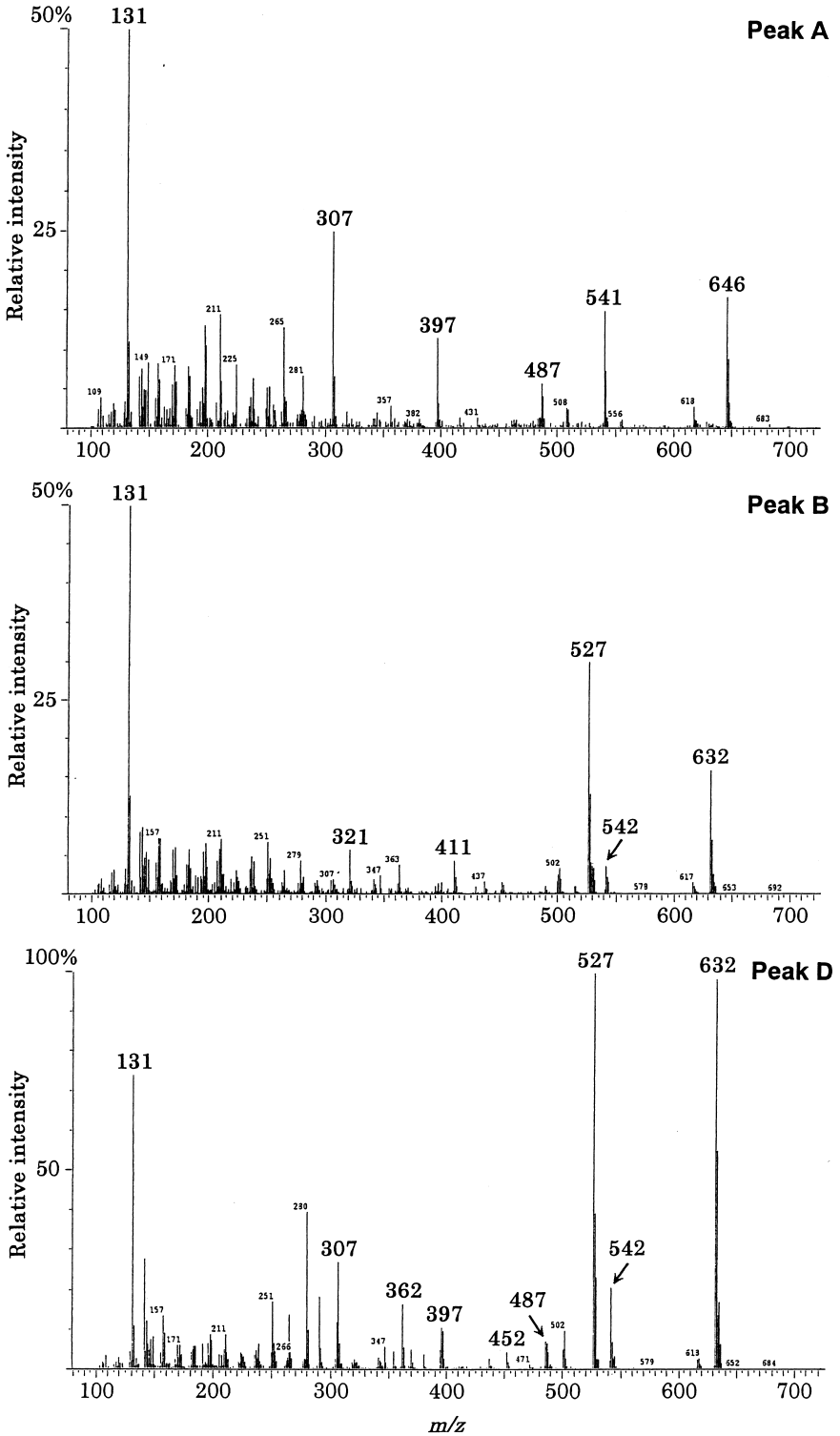

Fig. 2. GC/MS spectra of TMS derivatives of pyro-genins of peaks A, B, and D.

(t_R 19.7 min) and the TMS-genin of peak B did not co-migrate.

It is well-known that the reaction of vitamin D compounds with PTAD gives 2-epimers, 6*S*- and 6*R*-adducts, because the reagent attacks at the *s-cis* diene of vitamin D from the β- and α-sides, respectively [13]. It is also obvious that the 6*S*-adduct forms preferentially to the 6*R*-one in the case of compounds with no hydroxy group at the 1α-position. The PTAD adduct of the genin of the peak B metabolite showed two equivalent peaks (t_R 5.7 and 6.2 min) in

LC/APCI-MS monitoring at *m/z* 574 $[M+H-H_2O]^+$, which were much different from the data from 24,25(OH)$_2$D$_3$ [t_R 5.0 (6*R*) and 7.4 min (6*S*) with a ratio of ca. 1:5] (Fig. 3). 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 seven rats in the manner described above, and ^{1}H-NMR spectral data suggested that the genin was 3-epi-24,25(OH)$_2$D$_3$. That is, except for the chemical shift of a proton at the 3-position [the genin, 3.89 ppm; 24,25(OH)$_2$D$_3$, 3.94 ppm], a clear difference between the two compounds could not be observed. Although the A-ring of 24,25(OH)$_2$D$_3$ exists essentially in the α-chair form, that of its 3-epimer is inferred to exist in the β-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)$_2$D$_3$ and the genin in the reaction with PTAD was caused by the different conformations.

To identify the genin more reliably, we synthesized 3-epi-24,25(OH)$_2$D$_3$ based on the Lythgoe coupling [14] of the A-ring fragment **9** with the C/D-ring fragment **10** (Fig. 4). The synthesis of the A-ring fragment **9** followed a method we had previously developed [15,16]. Reaction of *S*-(+)-epichlorohydrin (**1**) with lithium acetylide of *p*-methoxybenzyl propargyl ether followed by base-treatment gave epoxide **2**. Sequential epoxide-opening of **2** with allylmagnesium chloride and silylation gave silyl ether **4**, which was then subjected to oxidative deprotection to give alcohol **5**. Hydroalumination of **5** with sodium bismethoxyethoxyaluminum hydride, followed by treatment of the resulting alkenylaluminum complex with iodine, gave (*Z*)-iodoalkene **6**, which, upon palladium-catalyzed cyclization, afforded dienol **7**. The dienol **7** was then converted to the required A-ring fragment **9** through chlorination, reaction of **8** with lithium diphenylphosphide, and oxidation. According to the Hoffman La Roche procedure [14], the A-ring fragment **9** was allowed to react with the C/D-ring fragment **10** to give triene **11** almost quantitatively. Finally, desilylation of **11** with tetrabutylammonium fluoride furnished 3-epi-24,25(OH)$_2$D$_3$ (**12**). The ^{1}H-NMR spectrum of the synthetic 3-epi-24,25(OH)$_2$D$_3$ gave a broad triplet of triplets peak of H-3 at 3.89 ppm, which agreed with that of the genin of the biliary metabolite. Furthermore, the behavior of the synthetic compound and the genin in LC/APCI-MS after derivatization with PTAD and in GC/MS completely agreed. From these results, the genin was identified as 3-epi-24,25(OH)$_2$D$_3$.

On the other hand, the conjugation position was determined from the ESI-MS^n spectral data for the PTAD (t_R 7.2 min) and the acetylated (t_R 5.7 min) derivatives as above. As a result, peak B was identified as 3-epi-24,25(OH)$_2$D$_3$ 24G.

3.4. Identification of the peak C metabolite as 24,25(OH)$_2$D$_3$ 3S

Peak C hardly changed when treated with β-glucuronidase. This metabolite was surmised not to be a glucuronide but to have an acidic functional group, such as a sulfuric group, because it was retained on an anion exchange gel, PHP-LH-20. In LC/ESI-MS, the peak C metabolite (t_R 4.8 min) gave an ion only at *m/z* 495 $[M-H]^-$ in the negative-ion mode, which indicated that its molecular weight was larger than 24,25(OH)$_2$D$_3$ by 80 mass units. Based on these data, we inferred that this compound was a sulfate of 24,25(OH)$_2$D$_3$. The genin of peak C obtained by solvolysis with sulfuric acid co-migrated with 24,25(OH)$_2$D$_3$ on the reversed- and normal-phase HPLC. Incidentally, we have reported that sulfates of vitamin D metabolites are poorly hydrolyzed by treatment with sulfatase [17], and peak C showed the same. The structure of peak C was also analyzed by LC/ESI-MS^n combined with some derivatization. After acetylation (t_R 12.6 min), the molecular weight of the compound increased by 42–538, which indicated that this metabolite had one *pri*- or *sec*-hydroxy group. The PTAD adduct of peak C (t_R 3.1 min) showed molecular-related ions at *m/z* 689 $[M+NH_4]^+$ and 670 $[M-H]^-$ in the positive- and the negative-ion modes, respectively. Its product ion mass spectrum, using *m/z* 689 and then 654 $[M+H-H_2O]^+$ as precursor ions, showed an ion at *m/z* 378, which indicates that the sulfated position was a 3-hydroxy group. In addition, the following characteristic fragment ions were observed: *m/z* 636 $[654-H_2O]^+$, 574 $[654-SO_3]^+$. These results showed that peak C was 24,25(OH)$_2$D$_3$ 3S.

3.5. Identification of the peak D metabolite as 23,25(OH)$_2$D$_3$ 23G

The results from LC/ESI-MS/MS (t_R 6.5 min) and enzymic hydrolysis indicated that peak D was a monoglucuronide of a dihydroxyvitamin D$_3$. The GC/MS spectrum of the TMS-genin derivatives (t_R 16.1 min) showed a series of fragment ions at *m/z* 487 [cleavage of C-23–24 bond]$^+$, 397 [487−TMSOH]$^+$, and 307 [487–2TMSOH]$^+$, which are peculiar to compounds having a hydroxy group at the 23-position [18], together with the following ions: *m/z* 632 $[M]^+$, 542 [M-TMSOH]$^+$, 527 [542-CH_3]$^+$, 452 [632–2TMSOH]$^+$, 362 [632–3TMSOH]$^+$, and 131 $[(CH_3)_2COTMS]^+$ (Fig. 2). On the basis of these data and the reported metabolic pathway of 25(OH)D$_3$, the genin of peak D was identified as 23,25(OH)$_2$D$_3$. The conjugation position was determined to be at the 23-position by LC/ESI-MS^n after derivatization [PTAD-adduct (t_R 6.3 min) and acetate (t_R 6.8 min)] as above. From the results stated above, peak D was identified as 23,25(OH)$_2$D$_3$ 23G.

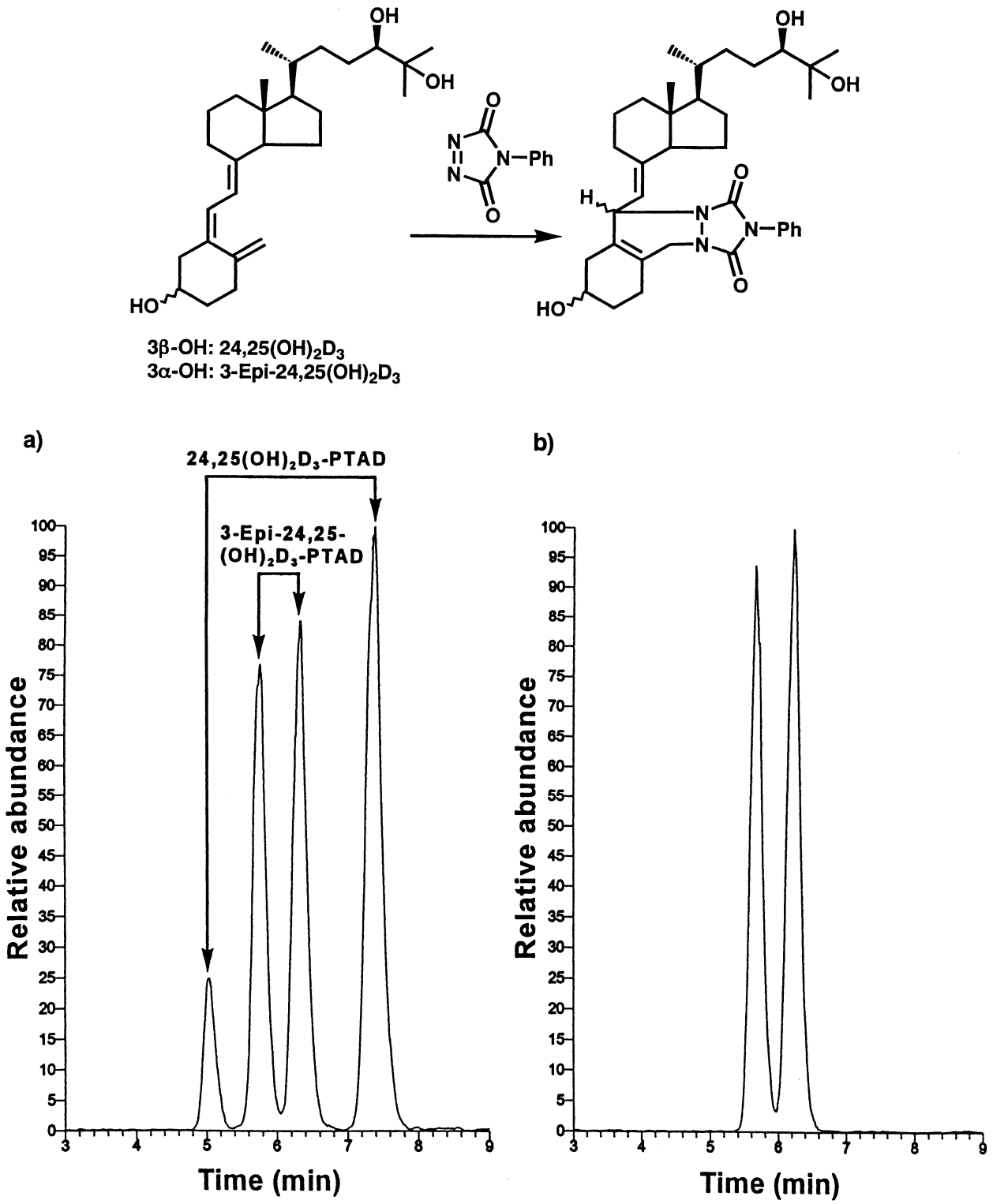

Fig. 3. LC/APCI-MS chromatograms of PTAD adducts of 24,25$(OH)_2D_3$, 3-epi-24,25$(OH)_2D_3$ and genin of peak B. (a), PTAD adducts of 24,25$(OH)_2D_3$ and 3-epi-24,25$(OH)_2D_3$; (b), PTAD adduct of genin of peak B. Monitoring ion: *m/z* 574 $[M+H-H_2O]^+$.

3.6. Identification of 25(OH)D_3 3S and other metabolites

25$(OH)D_3$ 3S in bile from rats dosed with 25$(OH)D_3$ was identified by comparison with the standard sample based on their chromatographic behavior and data obtained from LC/ESI-MS^n. The putative 25$(OH)D_3$ 3S was co-eluted with an authentic sample [J'sphere ODS H-80, MeOH/2% $NaClO_4$ (pH 3.0) (4:1 v/v), t_R 9.8 min], and its PTAD adduct (t_R 7.7 min) also gave the same product ions as the authentic sample in positive-ESI-MS/MS/MS:

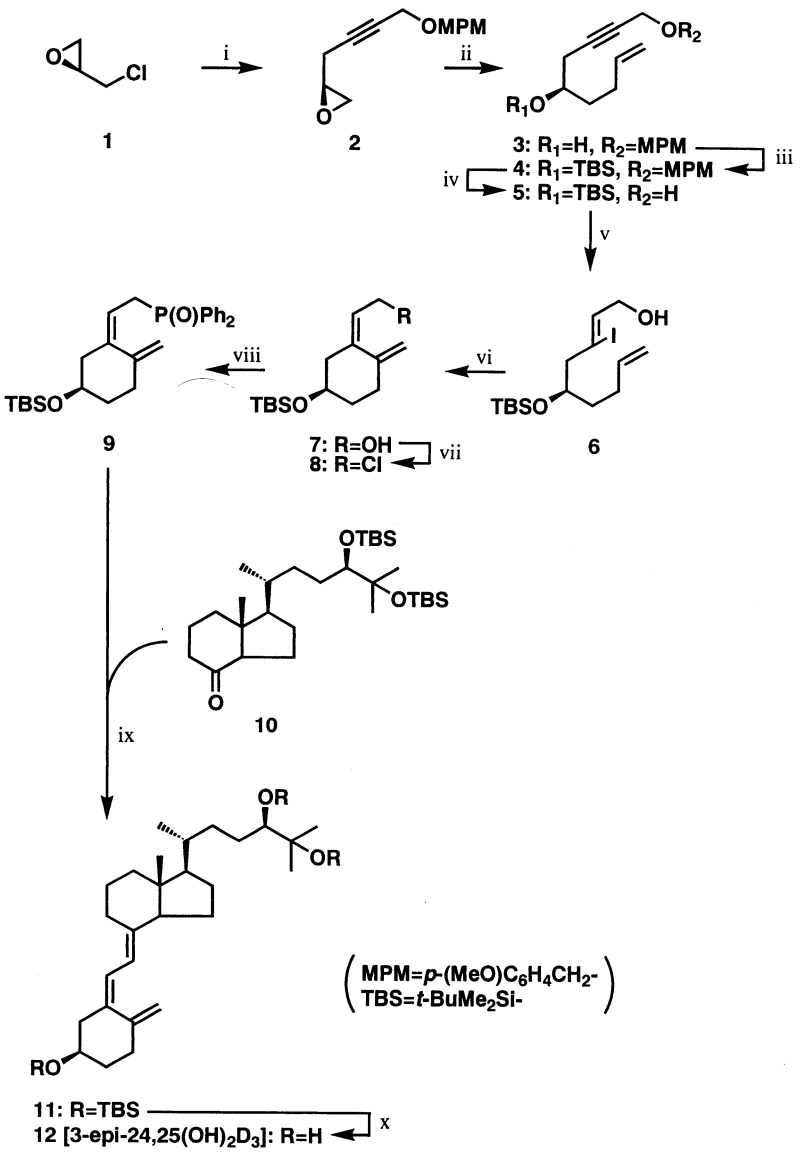

Fig. 4. Synthesis of 3-epi-24,25$(OH)_2D_3$. Reagents (yield): i, $CH{\equiv}CCH_2OMPM$, BuLi, $BF_3{\cdot}Et_2O$ then NaH (92%); ii, $CH_2{=}CHCH_2MgCl$; iii, TBSCl, imidazole (82% from **2**); iv, DDQ then $NaBH_4$ (79%); v, $[(MeOCH_2CH_2O)_2AlH_2]Na$ then I_2 (65%); vi, $Pd(PPh_3)_4$, Et_3N (67%); vii, *N*-chlorosuccinimide, Me_2S; viii, Ph_2PH, BuLi then H_2O_2 (69% from **7**); ix, BuLi (99%); x, Bu_4NF (79%).

m/z 638 $[M+H-H_2O]^+$ and 378 [cleavage of C-6–7 bond]$^+$.

The other metabolites [24,25$(OH)_2D_3$-3G, -24G, 25$(OH)D_3$-3G, and -25G] were also identified by comparison with standard samples as previously reported [6,7].

4. Discussion

It has been accepted that vitamin D compounds are excreted after successive oxidation of the side-chain, which leads to the formation of polar compounds, such as C-23 carboxylic acid [1,19]. On the contrary, a few reports suggested that vitamin D metabolites were excreted into the bile as conjugates [20], but a detailed investigation, including the determination of their structures, had not been done. We have identified the monoglucuronides of 24,25$(OH)_2D_3$ [6] and 25$(OH)D_3$ [7] from rat bile dosed with each genin, but there are many unsolved questions, such as the relationship between side-chain oxidation and the phase II reaction. In these respects, we studied the conjugated metabolites formed after the administration of 24,25$(OH)_2D_3$ or 25$(OH)D_3$ and found the metabolic pathway shown in Fig. 5. The structures of these metabolites were determined using LC/MS^n and GC/MS, and the former method combined with the derivatization reported previously [10] was very useful in determining the conjugation position. Incidentally, monoglucuronides of 25$(OH)D_3$ and 24,25$(OH)_2D_3$ were isolated by a method we had previously reported [6,7]. The approximate yields of each compound are also shown in

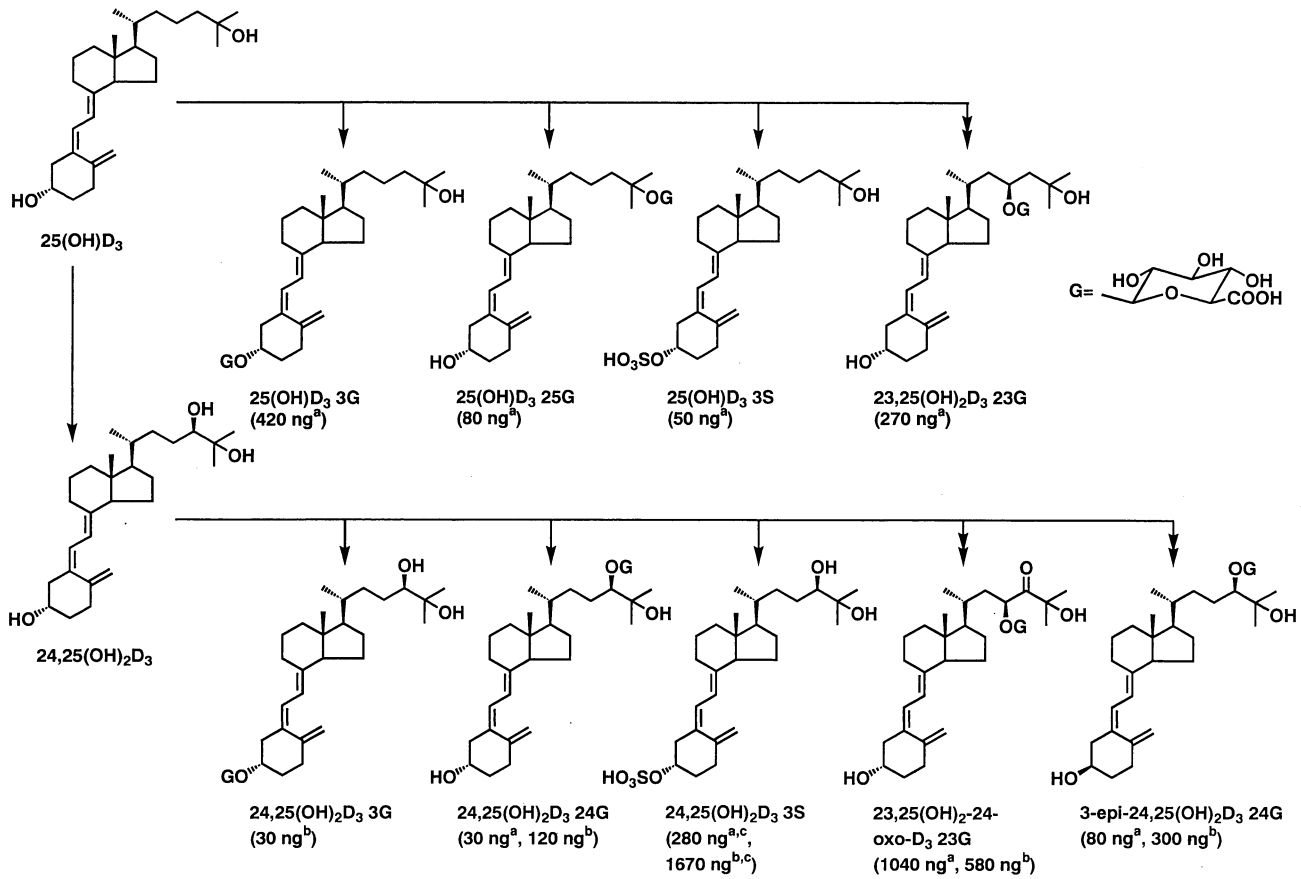

Fig. 5. Metabolic pathways of $25(OH)D_3$ and $24,25(OH)_2D_3$. Approximate yield from 1 ml of bile from rats administered $25(OH)D_3$ (a) and $24,25(OH)_2D_3$ (b) (mean of 2 or more animals); (c), from only female rats.

Fig. 5, which were determined by comparison with known amounts of the synthetic standards co-eluted or eluted closely in HPLC. Glucuronidation of dihydroxylated metabolites occurred mainly at the *sec*-hydroxy group on the side-chain; on the other hand, sulfation was observed only at the 3-hydroxy group.

As mentioned in the introduction, $24,25(OH)_2D_3$ was reported to be metabolized to $23,25(OH)_2$-24-oxo-D_3 23G and then excreted into the bile in dogs [8]. The present study showed that a similar metabolism occurred in rats; moreover, the yield of $23,25(OH)_2$-24-oxo-D_3 23G was several times that of $24,25(OH)_2D_3$-3G or -24G reported previously [6]. We also searched for the monoglucuronide of 25(OH)-24-oxo-D_3, the intermediate of $23,25(OH)_2$-24-oxo-D_3 from $24,25(OH)_2D_3$, in the bile using LC/MS (monitoring: *m/z* 590 $[M-H]^-$), but this metabolite could not be found.

The genin of peak B was identified as 3-epi-$24,25(OH)_2D_3$ by comparison with a synthetic standard in ^{1}H-NMR and various MS spectral data. Epimerization of the 3-hydroxy group of vitamin D metabolites has been reported for 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] incubated in several cell lines, such as human keratinocytes [21], colon adenocarcinoma [22], and rat osteosarcoma [23]. Recently, 3-epi-$1,25(OH)_2D_3$ was isolated from the serum of rats treated with $1,25(OH)_2D_3$ [24]. To our knowledge, the present paper is the first to report the isolation and characterization of a 3α-hydroxyvitamin D metabolite with no hydroxy group at the 1α-position. In the case of $1,25(OH)_2D_3$, it has been reported that the reaction proceeds from 3β toward 3α unidirectionally and is enzymatic, but not inhibited by a P450 inhibitor, such as ketoconazole [21,23]. In other steroids, 3-epi-digoxigenin is known as a metabolite of digoxin and is formed via the 3-oxo form by oxidoreductase [25]. Although the mechanism of the formation of 3-epi-$24,25(OH)_2D_3$ is not clear, this epimerization is one of the important pathways in vitamin D metabolism because the yield of 3-epi-$24,25(OH)_2D_3$ 24G was much larger than that of $24,25(OH)_2D_3$-3G or -24G [6].

From the bile of rats administered $25(OH)D_3$, $24,25(OH)_2D_3$ 24G, $23,25(OH)_2$-24-oxo-D_3 23G, and 3-epi-$24,25(OH)_2D_3$ 24G were obtained together with $25(OH)D_3$-3G and -25G. It is not surprising that the above metabolites were formed because $25(OH)D_3$ metabolized to $24,25(OH)_2D_3$ in the kidney. We did not search for $24,25(OH)_2D_3$ 3G in this bile because its amount was inferred to be very small. We also isolated $23,25(OH)_2D_3$ 23G

from the bile, in a slightly smaller yield than that of $25(OH)D_3$ 3G but about ten times that of $24,25(OH)_2D_3$ 24G. Under physiological conditions, the plasma levels of 23-hydroxylated metabolites, such as $23,25(OH)_2D_3$ and $23,25(OH)_2$-24-oxo-D_3, were much lower than that of their precursors, $25(OH)D_3$ or $24,25(OH)_2D_3$, but the yields of their glucuronides were much larger than estimated from their plasma levels when large amounts of vitamin D metabolites were dosed. These results demonstrate that the 23-hydroxylated metabolites are more easily conjugated with glucuronic acid and more promptly excreted than their precursors. That is, hydroxylation at the 23-position is inferred to play an important role in the excretion of vitamin D.

It should be noted that a large amount of $24,25(OH)_2D_3$ 3S was isolated from the bile of female rats dosed with either $24,25(OH)_2D_3$ or $25(OH)D_3$. $25(OH)D_3$ 3S also existed in bile after the administration of $25(OH)D_3$. These data indicated that vitamin D metabolites were usually excreted as sulfates as well. However, the yield of $25(OH)D_3$ 3S was smaller than that of $24,25(OH)_2D_3$ 3S, even in the rats dosed with $25(OH)D_3$. This result and the fact that $25(OH)D_3$ 3S exists in human plasma with an almost equal amount of $25(OH)D_3$ [26] suggest that $25(OH)D_3$ 3S is a storage or circulating form of vitamin D_3 rather than an excretory form. On the contrary, the sulfation of $24,25(OH)_2D_3$ can be considered to be one of the major excretory routes of vitamin D_3. However, a sex difference was observed with the sulfation of $24,25(OH)_2D_3$ such that it almost selectively occurred in female rats. Concerning this, Matsui and Watanabe reported that hydroxysteroid sulfotransferase activity in female rats was higher than that in male rats [27]. On the other hand, $25(OH)D_3$ 3S was obtained in almost equal amounts from male and female rats.

In conclusion, we demonstrated that, in rats, $25(OH)D_3$ and $24,25(OH)_2D_3$ were oxidized on the side-chain to form the 23-hydroxy metabolites and then conjugated as glucuronides, while the parent compounds were directly converted to glucuronides or sulfates. We also found a new metabolic pathway in which the 3-hydroxy group of $24,25(OH)_2D_3$ was epimerized, and this serves as one of the important pathways in vitamin D metabolism.

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