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In vitro and in vivo glucuronidation of 24,25-dihydroxyvitamin D_3☆

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Abstract

Glucuronidation of 24,25-dihydroxyvitamin D_3 has been investigated in in vitro and in vivo experiments. Three positional isomers of 24,25-dihydroxyvitamin D_3 monoglucuronide were synthesized from 24,25-dihydroxyprovitamin D_3 derivatives with Koenigs-Knorr reaction and used as standard samples. In the presence of the rat liver microsomal fraction and uridine-5′-diphosphoglucuronic acid, 24,25-dihydroxyvitamin D_3 gave 3- and 24-glucuronides as the main products in almost equal amounts, but only a small amount of the corresponding 25-glucuronide was obtained. 24,25-Dihydroxyvitamin D_3 monoglucuronide was deconjugated with rat intestine homogenate, which indicated the entero-hepatic circulation of 24,25-dihydroxyvitamin D_3. After the administration of 24,25-dihydroxyvitamin D_3 to rats, its 3- and 24-glucuronides were identified from the bile as inferred from the in vitro experiment. However, the in vivo glucuronidation occurred at the 24-position in preference to the 3-position, and the corresponding 25-glucuronide was not detected. These glucuronides were identified in comparison with standard samples based on their chromatographic behavior during high-performance liquid chromatography and data obtained from liquid chromatography-electrospray ionization-mass spectrometry, which was helpful in identifying these compounds.

Keywords: 24,25-Dihydroxyvitamin D_3; Monoglucuronide; Synthesis; Rat liver microsome; Rat bile; LC-MS

1. Introduction

(24*R*)-24,25-Dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] is one of the major metabolites of vitamin D_3, which causes a marked increase in bone volume and mechanical strength in animals without hypercalcemia at pharmacological doses [1,2]. Based on these criteria, the metabolite is being examined as an anti-osteoporosis medicine, and much interest is focused on the metabolism of 24,25$(OH)_2D_3$. 24,25$(OH)_2D_3$ is reported to be oxidized on its side chain at the C23 or C24 position by vitamin D 24-hydroxylase 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] [3], but conjugates of 24,25$(OH)_2D_3$, such as glucuronide or sulfate, still remain poorly understood.

Recently, 23,25$(OH)_2$-24-oxo-D_3 23-glucuronide was reported as a biliary metabolite obtained from dogs dosed with 24,25$(OH)_2D_3$, but a conjugate of 24,25$(OH)_2D_3$ has not been obtained [4]. On the other hand, in a previous paper in this series, we reported that 25-hydroxyvitamin D_3 3-glucuronide [25(OH)$D_3$3G] and 25(OH)$D_3$25G were obtained as biliary metabolites of rats dosed with 25(OH)D_3 per os (p.o.) [5], in which glucuronidation was observed not only at the *sec*-hydroxy group (3G) but also at the *tert*-hydroxy one (25G) in a ratio of 5:2. This glucuronidation was also observed during an in vitro study using rat liver microsomal fraction as an enzyme source [6]. It is inferred from our data that 24,25$(OH)_2D_3$ is also converted to its glucuronide.

In the present paper,† we first synthesized three positional isomers of 24,25$(OH)_2D_3$ monoglucuronide as standard samples. Second, in vitro glucuronidation of 24,25$(OH)_2D_3$ was investigated, and the formed monoglucuronides were identified in comparison with the standard synthetic samples based on their chromatographic behavior during high-performance liquid chromatography (HPLC) and data obtained from liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). Their structures were also confirmed by enzymic hydrolysis using β-glucuronidase. In addition, the entero-hepatic circulation of 24,25$(OH)_2D_3$ was also investigated. Third, 24,25$(OH)_2D_3$ was administered to rats, and 24,25$(OH)_2D_3$ monoglucuronides excreted into the bile were identified by similar methods.

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2. Experimental

2.1. Materials and apparatus

24,25$(OH)_2D_3$ was obtained from Duphar (Amsterdam, The Netherlands). (20*S*)-22-Phenylsulfonyl-23,24-dinorchola-5,7-dien-3β-ol (**1**) [8,9] and (24*R*)-24-(*tert*-butyldimethylsilyloxy)cholesta-5,7-diene-3β,25-diol (**2**) [10] were synthesized from ergosterol in our laboratories.

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) without correction. Optical rotation was measured on a Horiba SEPA-300 polarimeter (Tokyo, Japan) for solutions in $CHCl_3$ at 24°C, and $[\alpha]_D$ values are given in units of g/dl. UV spectra (in EtOH) were obtained on a Hitachi U-2000 spectrophotometer (Tokyo). ^{1}H-NMR spectra were obtained with a Jeol JNM-EX-270 (270 MHz) spectrometer (Tokyo). $CDCl_3$ was used as the solvent with tetramethylsilane as internal standard. The abbreviations used are s = singlet, d = doublet, dd = doublet of doublets, and m = multiplet. Mass spectra [electron impact (EI) or fast-atom bombardment (FAB)] were determined with Jeol JMS-SX-102A and Jeol JMS-DX-303 spectrometers, respectively. Column and flash column chromatographies were carried out with Silica gel 60 (70–230 mesh; E. Merck, Darmstadt, Germany) and Wakogel FC-40 (20–40 mesh; Wako, Osaka, Japan), respectively. Preparative (prep.) thin-layer chromatography (TLC) was conducted with 0.5 mm pre-coated Silica gel 60 F_{254} (Merck). Isolute C18 (EC) cartridges (500 mg: International Sorbent Tech., Hengoed, UK) were obtained from Uniflex (Tokyo), and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) [11] was prepared in our laboratories. 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 the enzymic hydrolysis.

2.2. Syntheses of 24,25$(OH)_2D_3$ monoglucuronides

2.2.1. (20S)-3β-(tert-Butyldimethylsilyloxy)-22-phenylsulfonyl-23,24-dinorchola-5,7-diene (3)

A mixture of **1** (363 mg, 0.799 mmol), *tert*-butyldimethylsilyl chloride (240 mg, 1.59 mmol) and imidazole (220 mg, 3.23 mmol) in *N*,*N*-dimethylformamide (4.0 ml) was stirred at room temperature for 1 h. The resulting solution was diluted with AcOEt, washed (H_2O) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by column chromatography (37 x 1.8 cm i.d.)($CHCl_3$) to give compound **3** (443 mg, 97.6%) as a colorless solid. ^{1}H-NMR δ: 0.06 [6H, s, $Si(Me)_2$], 0.59 (3H, s, H-18), 0.89 (9H, s, Si-*tert*-Bu), 0.91 (3H, s, H-19), 1.23 (3H, d, J = 6.3 Hz, H-21), 2.82–3.18 (2H, m, H-22), 3.56 (1H, m, H-3α), 5.36, 5.52 (1H each, m, H-6, 7), 7.54–7.93 (5H, m, H-Ph).

2.2.2. (20S,22ξ,24R)-3β-(tert-Butyldimethylsilyloxy)-22-phenylsulfonylcholesta-5,7-diene-24,25-diol (4)

BuLi (2.5 M solution in hexane; 2.1 ml) was added dropwise to a solution of **3** (375 mg, 0.660 mmol) and (2*R*)-2,3-dihydroxy-3-methylbutan-1-yl *p*-toluenesulfonate [12] (362 mg, 1.32 mmol) in tetrahydrofuran (THF) (10 ml) at −50°C. The resulting mixture was stirred under argon gas at −30°C for 2 h and quenched with saturated aq. NH_4Cl. The mixture was extracted with AcOEt, and the organic layer was washed (brine) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by flash column chromatography (30 x 1.0 cm i.d.) [hexane/AcOEt (1:1 v/v)] to give compound **4** (epimers at C22; total 369 mg, 83.4%) as a colorless foam. ^{1}H-NMR of the main epimer (less polar) δ: 0.06 [6H, s, $Si(Me)_2$], 0.37 (3H, s, H-18), 0.88 (9H, s, Si-*tert*-Bu), 1.03 (3H, d, J = 6.6 Hz, H-21), 1.21, 1.27 (3H each, s, H-26, 27), 3.50–3.60 (2H, m, H-3α, 22), 3.78 (1H, m, H-24), 5.32, 5.51 (1H each, m, H-6, 7), 7.56–7.91 (5H, m, H-Ph).

2.2.3. (24R)-3β-(tert-Butyldimethylsilyloxy)cholesta-5,7-diene-24,25-diol (5)

Five percent Na-Hg (7.0 g) and Na_2HPO_4 (1.5 g) were added to a solution of **4** (264 mg, 0.394 mmol) in THF/MeOH (40:3 v/v, 8.6 ml), and then the mixture was stirred under N_2 gas at room temperature for 3.5 h. The resulting suspension was poured into chilled 0.05 M sodium phosphate buffer (pH 7.3), and the insoluble material was removed by filtration. The filtrate was extracted with AcOEt, and the organic layer was washed (brine) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by flash column chromatography (30 x 1.0 cm i.d.) [hexane/AcOEt (3:2 v/v)] to give compound **5** (154 mg, 73.8%) as a colorless solid. ^{1}H-NMR δ: 0.07 [6H, s, $Si(Me)_2$], 0.62 (3H, s, H-18), 0.90 (9H, s, Si-*tert*-Bu), 0.94 (3H, s, H-19), 0.96 (3H, d, J = 6.5 Hz, H-21), 1.17, 1.21 (3H each, s, H-26, 27), 3.33 (1H, m, H-24), 3.57 (1H, m, H-3α), 5.38, 5.55 (1H each, m, H-6, 7).

2.2.4. (24R)-3β-(tert-Butyldimethylsilyloxy)-25-hydroxycholesta-5,7-dien-24-yl Acetate (6)

A solution of **5** (50.5 mg, 95.3 μmol) in pyridine/Ac_2O (2:1 v/v, 1.5 ml) was stirred at room temperature for 4 h. The resulting solution was poured into chilled H_2O and extracted with AcOEt. The organic layer was washed (chilled 5% HCl, 5% $NaHCO_3$, and brine) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by column chromatography (30 x 1.0 cm i.d.) [$CHCl_3$/MeOH (100:1 v/v)] to give compound **6** (50.1 mg, 92.0%) as colorless needles (from hexane-CH_2Cl_2). mp 151.5–152.5°C. $[\alpha]_D$ −42.2° (c = 0.102). ^{1}H-NMR δ: 0.06 [6H, s, $Si(Me)_2$], 0.61 (3H, s, H-18), 0.89 (9H, s, Si-*tert*-Bu), 0.93 (3H, s, H-19), 0.94 (3H, d, J = 6.9 Hz, H-21),

1.20 (6H, s, H-26, 27), 2.11 (3H, s, Ac), 3.57 (1H, m, H-3α), 4.78 (1H, dd, J = 3.0, 6.9 Hz, H-24), 5.39, 5.55 (1H each, m, H-6, 7).

*2.2.5. (24R)-3β,25-Dihydroxycholesta-5,7-dien-24-yl Acetate **(7)***

A solution of **6** (50.0 mg, 87.4 μmol) and tetrabutylammonium fluoride (TBAF) (1.0 mmol) in THF (1.5 ml) was stirred at room temperature for 2 h. The resulting solution was diluted with AcOEt, washed (H_2O) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by flash column chromatography (30 x 1.0 cm i.d.) [$CHCl_3$/MeOH (50:1 v/v)] to give compound **7** (33.3 mg, 83.2%) as a colorless semi-solid. ^{1}H-NMR δ: 0.62 (3H, s, H-18), 0.94 (3H, s, H-19), 0.95 (3H, d, J = 4.6 Hz, H-21), 1.20 (6H, s, H-26, 27), 2.11 (3H, s, Ac), 3.63 (1H, m, H-3α), 4.78 (1H, dd, J = 3.0, 6.9 Hz, H-24), 5.39, 5.57 (1H each, m, H-6, 7).

*2.2.6. (24R)-24-(tert-Butyldimethylsilyloxy)-25-hydroxycholesta-5,7-dien-3β-yl Acetate **(8)***

A solution of **2** (230 mg, 0.434 mmol) in pyridine/Ac_2O (2:1 v/v, 6.0 ml) was stirred at room temperature for 2.5 h. The resulting solution was poured into chilled H_2O and extracted with AcOEt. The organic layer was washed (chilled 5% HCl, 5% $NaHCO_3$, and brine) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by column chromatography [hexane/AcOEt (10:1 v/v)] to give compound **8** (213 mg, 85.8%) as a colorless semi-solid. ^{1}H-NMR δ: 0.10 [3H each, s, Si(Me)$_2$], 0.62 (3H, s, H-18), 0.92 (9H, s, Si-*tert*-Bu), 0.95 (3H, s, H-19), 1.15 (6H, s, H-26, 27), 2.04 (3H, s, Ac), 3.40 (1H, m, H-24), 4.70 (1H, m, H-3α), 5.40, 5.56 (1H each, m, H-6, 7).

*2.2.7. (24R)-24,25-Dihydroxycholesta-5,7-dien-3β-yl acetate **(9)***

A solution of **8** (208 mg, 0.364 mol) and TBAF (3.5 mmol) in THF (3.5 ml) was stirred at room temperature for 30 min. The resulting solution was diluted with AcOEt, washed (H_2O) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by column chromatography (30 x 1.8 cm i.d.) [hexane/AcOEt (1:1 v/v)] to give compound **9** (155 mg, 93.1%) as a colorless semi-solid. ^{1}H-NMR δ: 0.63 (3H, s, H-18), 0.95 (3H, s, H-19), 0.96 (3H, d, J = 5.9 Hz, H-21), 1.17, 1.22 (3H each, s, H-26, 27), 2.04 (3H, s, Ac), 3.34 (1H, m, H-24), 4.70 (1H, m, H-3α), 5.39, 5.57 (1H each, m, H-6, 7).

2.2.8. Koengs-Knorr reaction of compound **7**

Freshly prepared methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (Br-sugar) (119 mg, 0.300 mmol) and Ag_2CO_3 (86.2 mg, 0.313 mmol) were added to a solution of **7** (33.0 mg, 72.1 μmol) in $CHCl_3$ (2.0 ml), and the reaction mixture was stirred under argon gas at room temperature for 2.5 h. The resulting mixture was filtered, the filtrate was evaporated under reduced pressure and the crude product thus obtained was purified by flash column chromatography (30 x 1.0 cm i.d.) [hexane/AcOEt (1:1 v/v)] to give methyl {[(24*R*)-24-acetoxycholesta-5,7-dien-25-ol-3β-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}uronate (**10**, 26.3 mg, 47.1%) as a colorless semi-solid. ^{1}H-NMR δ: 0.60 (3H, s, H-18′), 0.91 (3H, s, H-19′), 0.94 (3H, d, J = 6.6 Hz, H-21′), 1.20 (6H, s, H-26′, 27′), 2.02, 2.05, 2.11 (6H, 3H, 3H, s each, 4 x Ac), 3.62 (1H, m, H-3′α), 3.75 (3H, s, COOMe), 4.02 (1H, d, J = 9.2 Hz, H-5), 4.67 (1H, d, J = 7.9 Hz, H-1), 4.78 (1H, dd, J = 3.0, 6.6 Hz, H-24′), 4.98, 5.24 (1H, 2H, m each, H-2, 3, 4), 5.38, 5.56 (1H each, m, H-6′, 7′).

Diglucuronide acetate methyl ester (**11**) was not separated from decomposed compounds of Br-sugar by the previous flash column chromatography; the crude fraction containing compound **11** was subjected to prep. TLC [20 x 20 cm, hexane/AcOEt (1:1 v/v), developed two times]. The zone corresponding to *Rf ca*. 0.6 was extracted [$CHCl_3$/MeOH (5:1 v/v)] to yield compound **11** (14.5 mg, 18.4%) as a colorless semi-solid. ^{1}H-NMR δ: 0.60 (3H, s, H-18′), 0.91 (3H, s, H-19′), 0.93 (3H, d, J = 7.3 Hz, H-21′), 1.18, 1.23 (3H each, s, H-26′, 27′), 2.01, 2.02, 2.05 (21H, s each, 7 x Ac), 3.62 (1H, m, H-3′α), 3.74, 3.75 (3H each, s, COOMe), 4.00, 4.03 (1H, d, J = 9.6 Hz, 2 x H-5), 4.67, 4.76 (1H, d, J = 7.6, 7.6 Hz, 2 x H-1), 4.85 (1H, m, H-24′), 4.99, 5.23 (2H, 4H, m each, 2 x H-2, 3, 4), 5.38, 5.56 (1H each, m, H-6′, 7′).

2.2.9. Koengs-Knorr reaction of compound **9**

Freshly prepared Br-sugar (417 mg, 1.05 mmol) and Ag_2CO_3 (305 mg, 1.10 mmol) were added to a solution of **9** (95.7 mg, 0.209 mmol) in $CHCl_3$ (4.0 ml), and the reaction mixture was stirred under argon gas at room temperature for 5 h. The resulting mixture was filtered, the filtrate was evaporated under reduced pressure and the crude product thus obtained was purified by flash column chromatography (33 x 1.0 cm i.d.) [hexane-AcOEt (3:2 v/v)] to give methyl {[(24*R*)-3β-acetoxycholesta-5,7-dien-24-ol-25-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}uronate (**13**, 76.5 mg, 47.3%) as a colorless amorphous substance (from Hexane-CH_2Cl_2). mp 180.5–182.5°C. $[\alpha]_D$ −38.9° (c = 0.099). ^{1}H-NMR δ: 0.62 (3H, s, H-18′), 0.94 (3H, d, J = 5.9 Hz, H-21′), 0.95 (3H, s, H-19′), 1.16, 1.21 (3H each, s, H-26′, 27′), 2.02, 2.03, 2.04 (3H, 3H, 6H, s each, 4 x Ac), 3.36 (1H, m, H-24′), 3.75 (3H, s, COOMe), 4.03 (1H, d, J = 9.6 Hz, H-5), 4.71 (1H, m, H-3′α), 4.76 (1H, d, J = 7.9 Hz, H-1), 4.98, 5.25 (1H, 2H, m each, H-2, 3, 4), 5.40, 5.56 (1H each, m, H-6′, 7′). Methyl {[(24*R*)-3β-acetoxycholesta-5,7-dien-25-ol-24-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}-uronate (**12**, 43.3 mg, 26.7%) was obtained from hexane/AcOEt (1:1 v/v) fraction as colorless needles (from hexane-CH_2Cl_2). mp 163.5–165°C. $[\alpha]_D$ −44.2° (c = 0.103). ^{1}H-NMR δ: 0.63 (3H, s, H-18′), 0.94 (3H, d, J = 8.6 Hz,

H-21′), 0.95 (3H, s, H-19′), 1.14, 1.16 (6H, s, H-26′, 27′), 2.02, 2.03, 2.05 (3H, 3H, 6H, s each, 4 x Ac), 3.38 (1H, m, H-24′), 3.75 (3H, s, COOMe), 4.06 (1H, d, J = 9.6 Hz, H-5), 4.64 (1H, d, J = 7.6 Hz, H-1), 4.71 (1H, m, H-3′α), 5.07, 5.24 (1H, 2H, m each, H-2, 3, 4), 5.41, 5.57 (1H each, m, H-6′, 7′).

*2.2.10. Methyl {[(5Z,7E)-(3S,24R)-24-acetoxy-25-hydroxy-9,10-secocholesta-5,7,10 (19)-trien-3β-yl]-2,3,4-tri-O-acetyl-β-D-glucopyranosid}uronate **(14)***

A solution of **10** (32.6 mg, 42.1 μmol) in Et_2O (400 ml) was intermittently irradiated (for 10, 5 and 4 s) with a 400 W high-pressure mercury lamp through a Vycor filter at 0°C with argon gas bubbling through the solution. After removal of the solvents under reduced pressure, the obtained residue was dissolved in EtOH (20 ml) and stored in the dark at 65 for 10 h and room temperature for 15 h under argon gas. The solvent was evaporated off, and the crude product thus obtained was purified by prep. TLC [20 x 20 cm, hexane/AcOEt (3:2 v/v); developed two times]. The zone corresponding to *Rf ca*. 0.7 was extracted [$CHCl_3$/MeOH (5:1 v/v)] to yield compound **14** (5.2 mg, 15.8%) as a colorless oil. UV λ_{max} nm: 264.0, λ_{min} nm: 228.0. ^{1}H-NMR δ: 0.53 (3H, s, H-18′), 0.92 (3H, d, J = 6.3 Hz, H-21′), 1.20 (6H, s, H-26′, 27′), 1.95, 2.016, 2.021 (3H, 6H, 3H, s each, 4 x Ac), 3.75 (3H, s, COOMe), 3.95 (1H, m, H-3′), 4.03 (1H, d, J = 9.2 Hz, H-5), 4.67 (1H, d, J = 7.6 Hz, H-1), 4.77 (1H, m, H-24′), 4.79, 5.03 (1H each, brs, H-19′), 5.01, 5.24 (1H, 2H, m each, H-2, 3, 4), 6.00, 6.17 (1H each, d, J = 10.9 Hz, H-6′, 7′). FAB-MS *m/z*: 797 $[M+Na]^+$ (2.84%), 43 (100%).

*2.2.11. (5Z,7E)-(3S,24R)-24,25-Dihydroxy-9,10-secocholesta-5,7,10 (19)-trien-3β-yl β-D-glucopyranuronic acid **(15)***

A solution of 0.1 M NaOH-MeOH (0.3 ml) was added to a solution of **14** (3.10 mg, 4.00 μmol) in MeOH (0.4 ml), and the mixture was stirred at room temperature for 24 h. The mixture was diluted with H_2O and neutralized with 5% HCl under ice cooling. After the addition of NaCl, the mixture was extracted with THF. The organic layer was washed (brine) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by prep. TLC [10 x 20 cm, $CHCl_3$/MeOH/H_2O (70:30:4 v/v/v)]. The zone corresponding to *Rf ca*. 0.3 was extracted [$CHCl_3$/MeOH/H_2O (70:30:4 v/v/v)] to yield compound **15** (1.95 mg, 84.0%) as a colorless semi-solid. UV λ_{max} nm: 264.0, λ_{min} nm: 228.0. FAB-MS *m/z*: 591 $[M-H]^-$ (25.3%), 12 (100%).

*2.2.12. Methyl {[(5Z,7E)-(3S,24R)-3-acetoxy-25-hydroxy-9,10-secocholesta-5,7,10 (19)-trien-24-yl]-2,3,4-tri-O-acetyl-β-D-glucopyranosid}uronate **(16)***

A solution of **12** (31.3 mg, 40.4 μmol) in Et_2O/CH_2Cl_2 (400:1 v/v, 401 ml) was intermittently irradiated (for 10, 8 and 5 s) with a 400 W high-pressure mercury lamp through a Vycor filter at 0°C with argon gas bubbling through the solution. After removal of the solvents under reduced pressure, the obtained residue was dissolved in EtOH (20 ml) and stored in the dark at 60°C for 14 h and room temperature for 13.5 h under argon gas. The solvent was evaporated off, and the crude product thus obtained was purified by prep. TLC [20 x 20 cm, hexane/AcOEt (2:1 v/v); developed four times]. The zone corresponding to *Rf ca*. 0.6 was extracted [$CHCl_3$-MeOH (5:1 v/v)] to yield compound **16** (4.30 mg, 13.7%) as a colorless oil. UV λ_{max} nm: 264.0, λ_{min} nm: 227.0. ^{1}H-NMR δ: 0.55 (3H, s, H-18′), 0.91 (3H, d, J = 5.9 Hz, H-21′), 1.14, 1.15 (3H each, s, H-26′, 27′), 2.027, 2.033, 2.04, 2.07 (3H each, s, 4 x Ac), 3.53 (1H, m, H-24′), 3.75 (3H, s, COOMe), 4.05 (1H, d, J = 9.6 Hz, H-5), 4.64 (1H, d, J = 7.9 Hz, H-1), 4.85 (1H, d, J = 1.5 Hz, H-19′), 4.94 (1H, m, H-3′), 5.07 (1H, brs, H-19′), 5.27 (1H, 2H, m each, H-2, 3, 4), 6.04, 6.21 (1H each, d, J = 11.2 Hz, H-6′, 7′). FAB-MS *m/z*: 797 $[M+Na]^+$ (1.75%), 774 $[M]^+$, 43 (100%).

*2.2.13. (5Z,7E)-(3S,24R)-3,25-Dihydroxy-9,10-secocholesta-5,7,10 (19)-trien-24-yl β-D-glucopyranuronic acid **(17)***

A solution of 0.1 M KOH-MeOH (0.3 ml) was added to a solution of **16** (2.06 mg, 2.66 μmol) in MeOH (0.3 ml), and the mixture was stirred at room temperature for 14 h. The mixture was diluted with H_2O and neutralized with 5% HCl under ice-cooling. After the addition of NaCl, the mixture was extracted with THF. The organic layer was washed (brine) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by prep. TLC [8 x 20 cm, $CHCl_3$/MeOH/H_2O (70:30:4 v/v/v)]. The zone corresponding to *Rf ca*. 0.4 was extracted [$CHCl_3$/MeOH/H_2O (70:30:4 v/v/v)] to yield compound **17** (1.32 mg, 83.8%) as a colorless semi-solid. UV λ_{max} nm: 264.0, λ_{min} nm: 227.0. FAB-MS *m/z*: 591 $[M-H]^-$ (12.5%), 153 (100%).

*2.2.14. Methyl {[(5Z,7E)-(3S,24R)-3-acetoxy-24-hydroxy-9,10-secocholesta-5,7,10 (19)-trien-25-yl]-2,3,4-tri-O-acetyl-β-D-glucopyranosid}uronate **(18)***

A solution of **13** (33.5 mg, 43.3 μmol) in Et_2O (400 ml) was intermittently irradiated (for 10, 5 and 3 s) with a 400 W high-pressure mercury lamp through a Vycor filter at 0°C with argon gas bubbling through the solution. After removal of the solvents under reduced pressure, the obtained residue was dissolved in EtOH (25 ml) and stored in the dark at 60°C for 16 h and room temperature for 29 h under argon gas. The solvent was evaporated off, and the crude product thus obtained was purified by prep. TLC [20 x 20 cm, hexane/AcOEt (3:2 v/v); developed three times]. The zone corresponding to *Rf ca.* 0.5 was extracted [$CHCl_3$/MeOH (5:1 v/v)] to yield compound **18** (5.45 mg, 16.3%) as a colorless oil. UV λ_{max} nm: 264.5, λ_{min} nm: 227.0. ^{1}H-NMR δ: 0.55 (3H, s, H-18′), 0.92 (3H, d, J = 5.6 Hz, H-21′), 1.16, 1.21 (3H each, s, H-26′, 27′), 2.02, 2.04 (12H, s each,

4 x Ac), 3.35 (1H, m, H-24′), 3.75 (3H, s, COOMe), 4.02 (1H, d, J = 9.6 Hz, H-5), 4.75 (1H, d, J = 9.2 Hz, H-1), 4.84 (1H, d, J = 1.5 Hz, H-19′), 4.94 (1H, m, H-3′), 5.06 (1H, brs, H-19′), 4.99, 5.24 (1H, 2H, m each, H-2, 3, 4), 6.02, 6.21 (1H each, d, J = 11.2 Hz, H-6′, 7′). FAB-MS *m/z*: 797 $[M+Na]^+$ (1.75%), 774 $[M]^+$ (0.84%), 43 (100%).

*2.2.15. (5Z,7E)-(3S,24R)-3,24-Dihydroxy-9,10-secocholesta-5,7,10 (19)-trien-25-yl β-D-glucopyranuronic acid **(19)***

A solution of 0.1 M KOH-MeOH (0.3 ml) was added to a solution of **18** (2.00 mg, 2.58 μmol) in MeOH (0.3 ml), and the mixture was stirred at room temperature for 16 h. The mixture was diluted with H_2O and neutralized with 5% HCl under ice-cooling. After the addition of NaCl, the mixture was extracted with THF. The organic layer was washed (brine) and then dried over anhydrous Na_2SO_4. After removal of the solvents under reduced pressure, the crude product thus obtained was purified by prep. TLC [10 x 20 cm, $CHCl_3$/MeOH/H_2O (70:30:4 v/v/v)]. The zone corresponding to *Rf ca*. 0.4 was extracted [$CHCl_3$/MeOH/H_2O (70:30:4 v/v/v)] to yield compound **19** (1.26 mg, 82.4%) as a colorless semi-solid. UV λ_{max} nm: 264.0, λ_{min} nm: 227.0. FAB-MS *m/z*: 591 $[M-H]^-$ (12.8%), 153 (100%).

2.3. HPLC analysis

HPLC was performed on a Shimadzu LC-10AT chromatograph (Kyoto) equipped with a Shimadzu SPD-10A UV (265 nm) or a Shimadzu SPD-M6A photodiode array UV (200–340 nm) detector. J'sphere ODS-H80 (4 μm, 150 x 4.6 mm i.d.) (YMC, Kyoto) column was used at a flow rate of 1 ml/min under ambient conditions. The pH of the mobile phase containing $AcONH_4$ or $NaClO_4$ was adjusted with AcOH or $HClO_4$, respectively.

2.4. LC-MS analysis

LC-MS was performed using a Finnigan MAT LCQ (San Jose, CA, USA) connected to a JASCO PU-980 (Tokyo) chromatograph, and ESI was used. The spray needle voltage was 5 kV, and the heated capillary temperature was set at 200°C. The sheath gas and auxiliary gas flow rate was 70 and 20 units, respectively. The relative collision energy for MS^n was 20%. The capillary voltage was 1 or −1 V, and the tube lens offset was 10 or −10 V in the positive- or negative-ion mode, respectively. Develosil ODS HG-5 (5 μm, 150 x 2.0 mm i.d.) and YMC-Pack Pro C18 (5 μm, 150 x 3.0 mm i.d.) columns were used at a flow rate of 0.25 and 0.4 ml/min, respectively, at 30°C.

2.5. Methylation

Freshly prepared ether solution of diazomethane (1 ml) was added to the solution of glucuronides [*ca*. 100 ng in MeOH (50 μl)]. The mixture was stored at room temperature for 1 h and the solvent was evaporated.

2.6. Incubation procedure for in vitro glucuronidation

The liver microsomal fraction from Wistar strain rats (female, *ca*. 140 g, 7 w, Japan S.L.C., Hamamatsu, Japan) was prepared as previously reported [6]. The assay medium containing 24,25$(OH)_2D_3$ [10 μg in EtOH (10 μl)], uridine-5′-diphosphoglucuronic acid [4 μM in 0.1 M Tris-HCl buffer (Tris buffer; pH 7.2; 0.2 ml)], $MgCl_2$ [10 μmol in Tris buffer (50 μl)], Triton X-100 [0.5 μl in Tris buffer (50 μl)], ethylenediaminetetraacetic acid.2Na [20 nmol in Tris buffer (50 μl)], microsomal fraction [2 mg protein in Tris buffer containing 0.25 M sucrose (60 μl)] and Tris buffer in a total volume of 1.0 ml. The mixture was incubated in air at 37°C for 6 h. The reaction was stopped by cooling on ice.

2.7. Separation of the formed monoglucuronides from incubation specimens

An incubation specimen was mixed with EtOH (1 ml) and subjected to centrifugation at 1500*g* for 10 min. The obtained precipitate was re-suspended with Tris buffer (1 ml) and EtOH (1 ml) followed by centrifugation as above. The combined supernatant was 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 PHP-LH-20 column chromatography (20 x 6 mm i.d.). After washing with 90% EtOH (5 ml) and 0.1 M AcOH in 90% EtOH (5 ml), the glucuronides 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 evaporated under N_2 gas stream. The residue was re-dissolved in EtOH and stored at −20°C until use.

2.8. Enzymic hydrolysis of monoglucuronides

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 separately pre-incubated 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 x 3), and the organic layer was evaporated under N_2 gas stream. The residue was dissolved in EtOH and subjected to HPLC, and 24,25$(OH)_2D_3$ was identified in comparison with an authentic sample [MeOH/H_2O (5:1 v/v), t_R 6.4 min].

2.9. Deconjugation of monoglucuronides with rat intestine homogenate

A female Wistar strain rat (140 g, 7 w, Japan S.L.C.) was starved for 15 h before being killed. The intestine was homogenized in ice-cold acetate buffer followed by centrifugation at 1500*g* for 10 min. The supernatant was diluted with the buffer to a concentration of 10 mg protein/ml and used as an enzyme solution (1 ml). 24,25$(OH)_2D_3$3G [300 ng in EtOH (40 μl)] was added to the solution and then incubated at 37°C for 3 h. The reaction was stopped by the addition of ice-cold MeOH (1 ml), and the mixture was centrifuged at 2800*g* for 10 min. The supernatant was diluted with H_2O (2 ml) and then applied to an Isolute C18 cartridge. After washing with H_2O (4 ml) and 30% MeOH (3 ml), the remaining glucuronide was eluted with 70% MeOH (3 ml), and then the formed 24,25$(OH)_2D_3$ was eluted with MeOH (3 ml). The obtained glucuronide fraction was subjected to PHP-LH-20 column chromatography and then treated as described in the in vitro experimental section. The solvent of the corresponding fraction was evaporated under N_2 gas stream, and a part of the obtained residue was subjected to HPLC and compared with an authentic sample.

2.10. Bile samples from rats

Wistar strain rats (male: *ca*. 170 g, female: *ca*. 140 g, 7 w, Japan S.L.C.) were used. The rats were anaesthetized with diethylether, and the bile duct was cannulated with a polyethylene tube (SP 31) (Natsume, Tokyo, Japan) for the collection of bile. All animals were starved for 15 h prior to the administration of 24,25$(OH)_2D_3$. A suspension of 24,25$(OH)_2D_3$ (0.5 mg) in dimethylsulfoxide (0.1 ml) with saline (0.7 ml) and Tween 80 (0.2 ml) was orally given to each rat, and the bile was collected over a period of 24 h following the administration.

2.11. Separation of the excreted monoglucuronides from rat bile

A 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 followed by PHP-LH-20 column chromatography as described in the in vitro experimental section. The obtained fraction containing 24,25$(OH)_2D_3$ monoglucuronides was subjected to prep. HPLC [MeCN/0.5% $AcONH_4$ (pH 5.0) (1:2 v/v), 24,25$(OH)_2D_3$-3G; t_R 8.1–9.1 min, -24G; t_R 15.2–16.2 min, -25G; t_R 13.2–14.4 min]. After dilution with H_2O, each obtained fraction was applied to an Isolute C18 cartridge in the manner previously described to remove any inorganic salts, and then the solvent was evaporated under N_2 gas stream. Each fraction was further purified by prep. HPLC [MeCN/2% $NaClO_4$ (pH 3.0) (2:3 v/v), 24,25$(OH)_2D_3$-3G; t_R 9.2–10.6 min, -24G; t_R 13.2–14.6 min, -25G; t_R 14.9–16.0 min]. After neutralization with 2% $NaHCO_3$ and dilution with H_2O, each obtained fraction was treated as previously described. The EtOH eluate from an Isolute C18 cartridge was evaporated under N_2 gas stream, and a part of the residue was subjected to HPLC, LC-MS and enzymic hydrolysis.

3. Results

3.1. Syntheses of 24,25$(OH)_2D_3$ monoglucuronides

Three positional isomers of 24,25$(OH)_2D_3$ monoglucuronide (**15**, **17**, **19**) were synthesized as shown in Fig. 1. In order to synthesize these glucuronides selectively, the starting materials (**1**, **2**) were converted to 24,25$(OH)_2$proD_3 3- and 24-acetate (**9**, **7**), respectively. The former was subjected to the Koenigs-Knorr reaction using Ag_2CO_3 and Br-sugar [13], and two isomers of monoglucuronide acetate methyl ester (G') (**12**, **13**) were obtained in a ratio of 3:5. The reacted position was confirmed by the usual acetylation using Ac_2O and pyridine. Although compound **13** easily gave the respective acetate, which was confirmed by TLC and ^{1}H-NMR (H-24, δ 4.85 ppm), the reaction did not work on the *tert*-hydroxy group of compound **12**; therefore compounds **12** and **13** were confirmed as 24G′ and 25G′, respectively. Both compounds were subjected to photochemical reaction followed by alkaline hydrolysis to give the desired 24,25$(OH)_2D_3$-24G (**17**) and -25G (**19**) with a total yield of 2.4% and 5.1%, respectively.

On the other hand, 24,25$(OH)_2D_3$ 3G (**15**) was synthesized from compound **7**. The Koenigs-Knorr reaction was carried out with the compound, and 3G' (**10**) was obtained together with 3,25-diG' (**11**) in a ratio of 3:1. The photochemical reaction followed by alkaline hydrolysis gave 24,25$(OH)_2D_3$ 3G (**15**) with a total yield of 2.9%.

All the compounds synthesized here exhibited satisfactory spectral data. Furthermore, the structures of glucuronides were confirmed by enzymic hydrolysis using β-glucuronidase.

3.2. Characterization of 24,25$(OH)_2D_3$ monoglucuronides formed in incubation specimen

The incubation specimen was pre-treated according to the procedure shown in Fig. 2 and then subjected to UV-HPLC analysis. As a consequence, two major peaks were observed, which were identified as 24,25$(OH)_2D_3$-3G and -24G in comparison with standard synthetic samples based on their chromatographic behavior during HPLC using three solvent systems [t_R of 24,25$(OH)_2D_3$ -3G, -24G, and -25G; MeCN/2% $NaClO_4$ (pH 3.0) (2:3 v/v), 10.2, 15.4 and, 16.9 min, MeCN/0.5% $AcONH_4$ (pH 5.0) (1:2 v/v), 8.1, 14.9, and 13.8 min, MeOH/2% $NaClO_4$ (pH 3.0) (7:3 v/v), 16.0, 24.8, and 22.3 min]. Furthermore, photodiode array UV detection of these two metabolites showed the characteristic

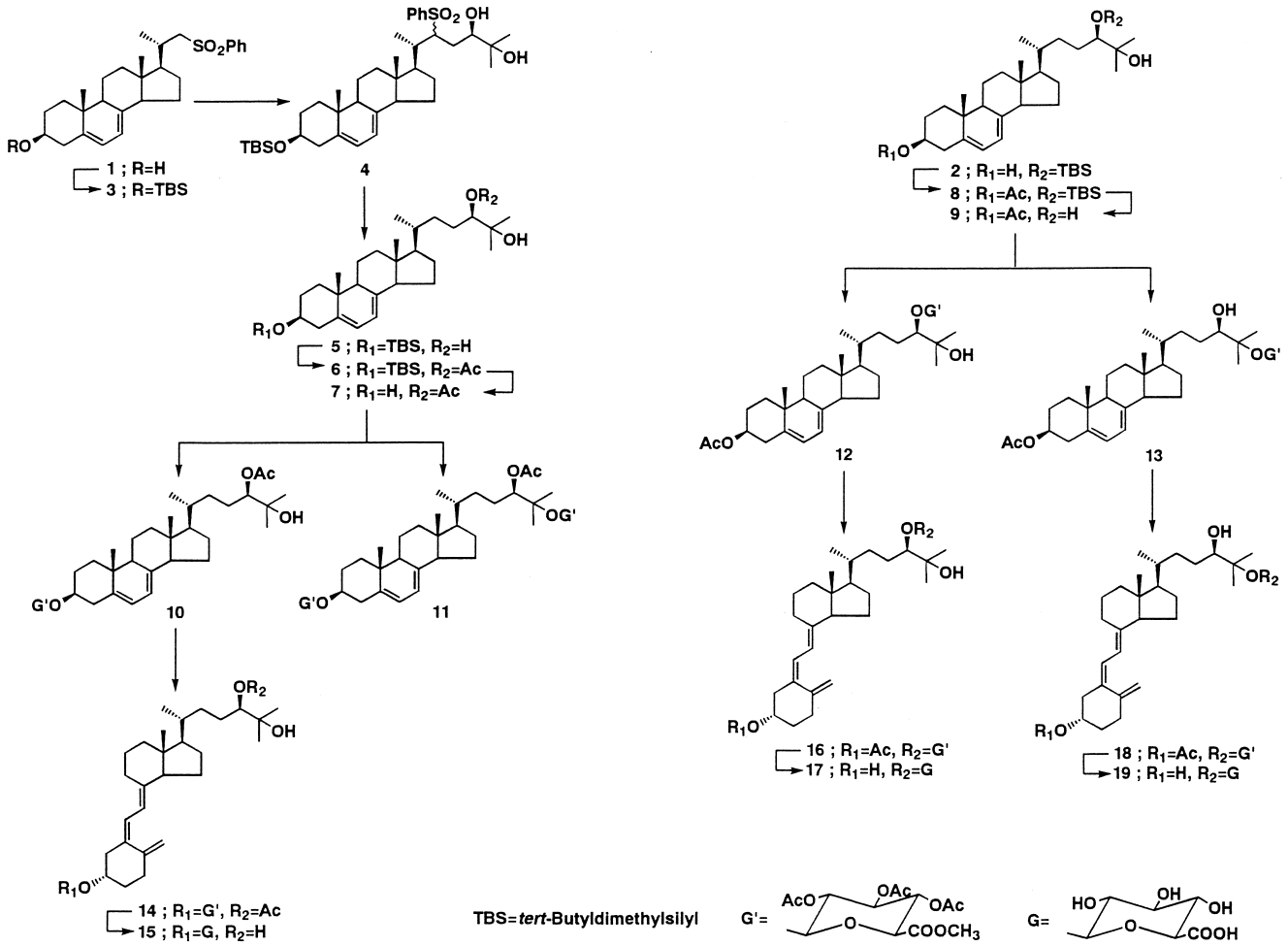

Fig. 1. Syntheses of $24,25(OH)_2D_3$ monoglucuronides.

UV absorbance (λ_{min} *ca.* 230 nm, λ_{max} *ca.* 268 nm) of the vitamin D structure. The recovery rates of $24,25(OH)_2D_3$ monoglucuronides during the pre-treatment were almost equal in three positional isomers [$n = 2$, $24,25(OH)_2D_3$-3G; 68.7%, -24G; 66.5% and -25G; 71.9%], and the conversion rates of these glucuronides from the substrate were about 7.0% and 6.5% for $24,25(OH)_2D_3$-3G and -24G, respectively. The peak corresponding to $24,25(OH)_2D_3$ 25G was also observed in the HPLC chromatogram, but photodiode array UV detection could not be carried out due to the shortage of the sample. These data prompted us to use LC-MS for the identification of the compound.

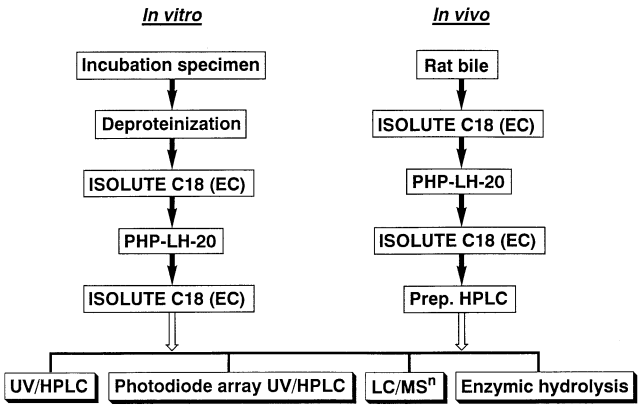

Fig. 2. Procedure for characterization and determination of monoglucuronides in in vitro and in vivo experiments.

In the previous communication [7], we used LC-atmospheric pressure chemical ionization (APCI)-MS for the characterization of $24,25(OH)_2D_3$ monoglucuronide formed from in vitro experiments, but it was performed by only selected ion monitoring (SIM) in the negative-ion mode, because of the low sensitivity of the used instrument (Hitachi M-1000H). In order to make the identification of monoglucuronides more reliable, the pre-treated sample was subjected to an ion-trap LC-ESI-MS^n [Develosil ODS HG-5, MeCN/10 mM $AcONH_4$ (1:2 v/v)] in the positive- and negative-ion modes. As a consequence, the peaks corresponding to $24,25(OH)_2D_3$-3G, -24G, and -25G were observed in the LC-MS chromatogram in both the positive- and negative-ion modes (Fig. 3), and the mass spectrum of each glucuronide was identical with that of an authentic

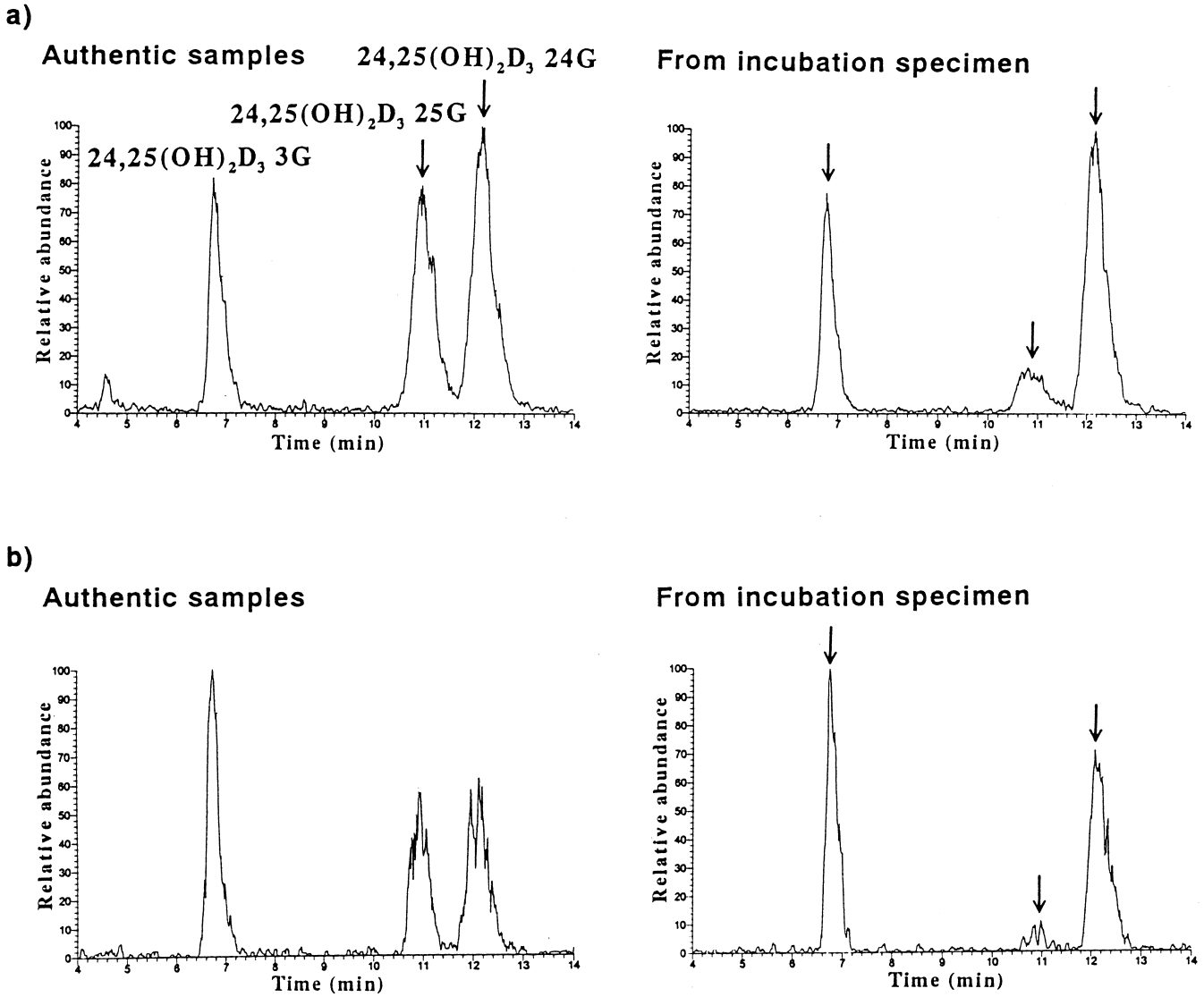

Fig. 3. LC-ESI-MS chromatograms of $24,25(OH)_2D_3$ monoglucuronides. a) Positive-ion mode. b) Negative-ion mode. Conditions: column, Develosil ODS HG-5, mobile phase, MeCN/10 mM $AcONH_4$ (1:2 v/v), detection, *m/z* 610 and 591 were monitored in a) and b), respectively, after *m/z* 500–700 was scanned.

sample. However, only a molecular-related ion in both the positive-ion (*m/z* 610; $[M+NH_4]^+$) and negative-ion modes (*m/z* 591; $[M\text{-}H]^-$) was obtained, and any fragment ion was not observed in the spectrum. Therefore, we attempted LC-MS^2 analysis using the above ion as a precursor ion. Although any fragmentation was not observed in the negative-ion mode, the following characteristic fragment ions were detected in the positive-ion mode, and its product ion mass spectrum of each glucuronide was identical with that of the authentic sample (Fig. 4a and b): *m/z* 593 $[M+H]^+$, 575 $[593–H_2O]^+$, 557 $[593–2H_2O]^+$, 417 $[genin+H]^+$, 399 $[417–H_2O]^+$, 381 $[417–2H_2O]^+$, and 363 $[417–3H_2O]^+$. Furthermore, the formed $24,25(OH)_2D_3$ 25G was converted to the methyl ester with diazomethane which gave a molecular-related ion (*m/z* 665, $[M+AcO]^-$) in LC-MS [YMC-Pack Pro C18, MeOH/10 mM $AcONH_4$ (4:1 v/v), t_R 8.3 min] operating in the negative-ion mode. Compared with $24,25(OH)_2D_3$ 25G, the intensity of a molecular-related ion of the methyl ester was increased to about 4 times, which was very useful in identifying a small amount of glucuronide. These data showed that $24,25(OH)_2D_3$ 25G was formed in in vitro experiments together with $24,25(OH)_2D_3$-3G and -24G.

In addition to these data, the structure of the formed $24,25(OH)_2D_3$ 25G was confirmed by the enzymic hydrolysis using β-glucuronidase. The hydrolyzed product gave a peak corresponding to $24,25(OH)_2D_3$ on the reversed-phase HPLC, which was confirmed by co-chromatography with an authentic sample.

3.3. Deconjugation of glucuronide in rat intestine

The entero-hepatic circulation of $24,25(OH)_2D_3$ was investigated using $24,25(OH)_2D_3$ 3G as a model compound.

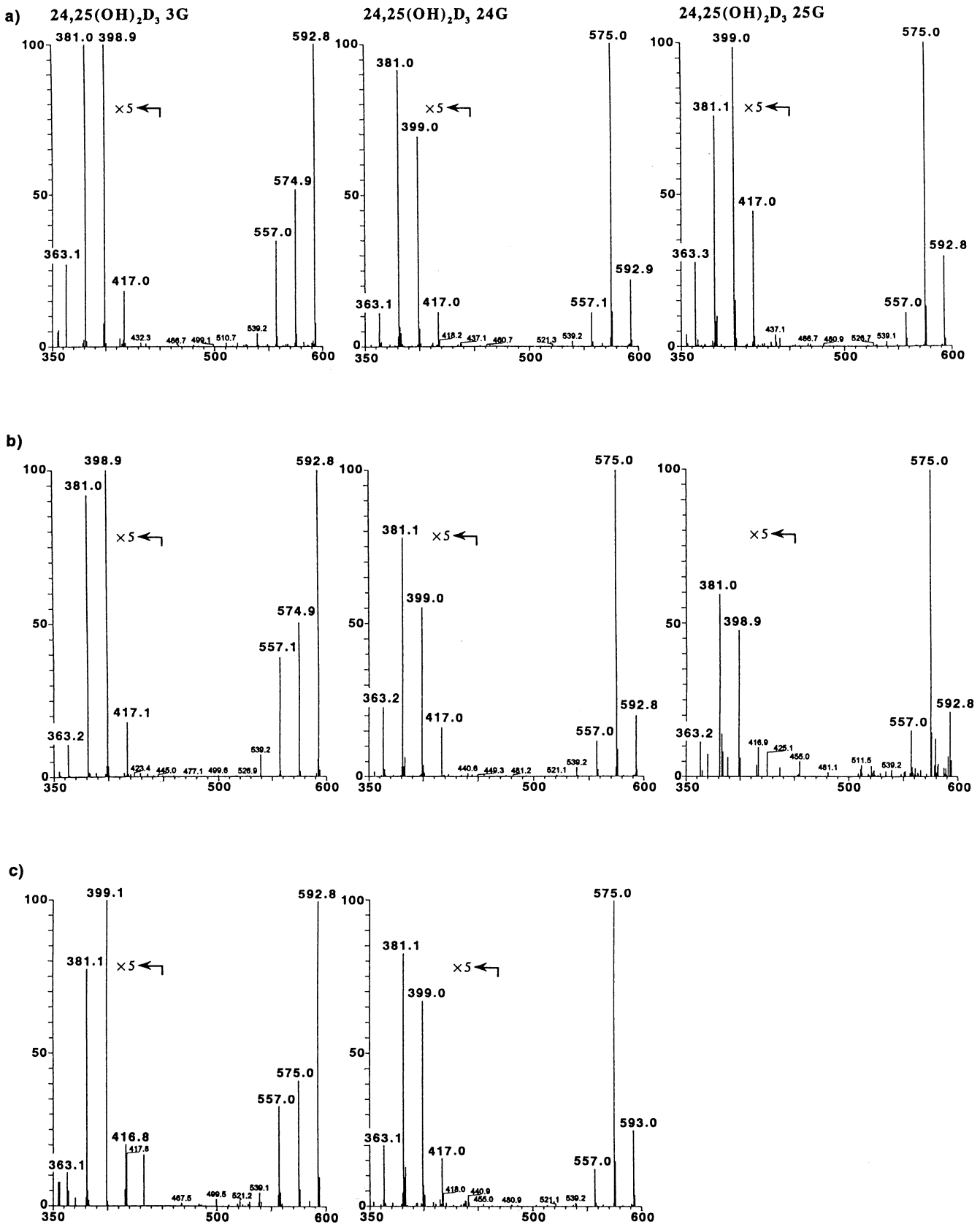

Fig. 4. Product ion mass spectra of 24,25$(OH)_2D_3$ monoglucuronides. a) Authentic sample. b) From incubation specimen. c) From rat bile. Vertical line: relative abundance. Horizontal line: *m/z*. Conditions: precursor ion, *m/z* 610, relative collision energy, 20%, scan range, *m/z* 300–650, chromatographic conditions, the same as in Fig. 3.

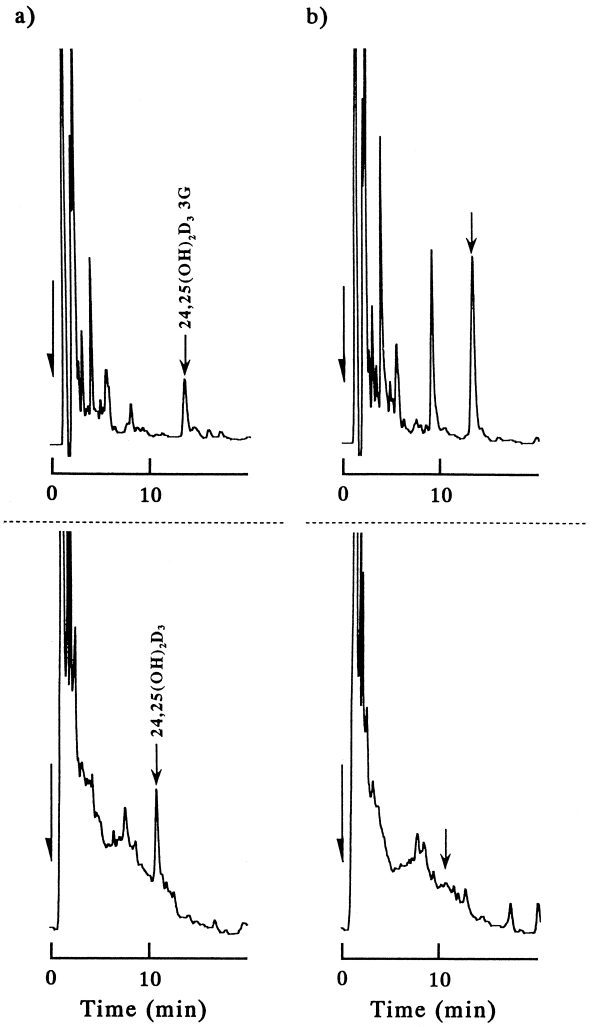

Fig. 5. Chromatograms of $24,25(OH)_2D_3$ 3G and its hydrolyzed product. a) Incubation with rat intestine homogenate. b) Incubation with boiled rat intestine homogenate. Upper: remaining substrate. Lower: hydrolyzed product. Conditions: column, J'sphere ODS-H80, mobile phase, upper: MeCN/0.5% $AcONH_4$ (pH 5.0) (4:9 v/v), lower: MeCN/H_2O (3:2 v/v).

The glucuronide was incubated with rat intestine homogenate, and the remaining substrate and the formed product were monitored with UV-HPLC (Fig. 5). Compared with a case of boiled intestine (inactivated enzyme), 68% of the substrate disappeared and $24,25(OH)_2D_3$ was produced with a conversion rate of 17%. These results suggested that a part of the glucuronides of $24,25(OH)_2D_3$ was deconjugated by the enzyme from bacteria in the intestine and formed $24,25(OH)_2D_3$ is probably reabsorbed and further oxidized to other metabolites.

3.4. Characterization of 24,25(OH)$_2$D$_3$ monoglucuronide in rat bile

The bile specimen was subjected to solid phase extraction and anion exchange chromatography followed by two successive prep. HPLC (Fig. 2). The obtained fractions corresponding to three positional isomers of $24,25(OH)_2D_3$ monoglucuronide were analyzed using HPLC and LC-MS and subjected to enzymic hydrolysis. First, the chromatographic behavior of the putative in vivo glucuronides were compared with that of synthetic standards [MeOH/2% $NaClO_4$ (pH 3.0) (3:1 v/v), $24,25(OH)_2D_3$ -3G; t_R 7.2 min, -24G; t_R 10.6 min, and -25G; 9.7 min]. As a consequence, although $24,25(OH)_2D_3$ 25G was not detected, the peaks corresponding to $24,25(OH)_2D_3$-3G and -24G were observed clearly. These two metabolites were separately treated with β-glucuronidase, and the disappearance of them and the formation of $24,25(OH)_2D_3$ were confirmed by HPLC. Furthermore, the LC-MS^n spectra of the metabolites, which were obtained in the same conditions of the in vitro experiments, were agreed with those of synthetic standards, that is, the product ion mass spectra derived from the ion at *m/z* 610 showed the following characteristic ions: *m/z* 593, 575, 557, 417, 399, 381, and 363 (Fig. 4c). These results led to the conclusions that the two metabolites were $24,25(OH)_2D_3$-3G and -24G, and the corresponding 25G was not formed. Although precise quantitative determination was not performed, *ca*. 30 ng of $24,25(OH)_2D_3$ 3G and 120 ng of $24,25(OH)_2D_3$ 24G were obtained from 1 ml of bile (Table 1).

Table 1
Formation ratio of $24,25(OH)_2D_3$ monoglucuronide

	$24,25(OH)_2D_3$ 3G	$24,25(OH)_2D_3$ 24G	$24,25(OH)_2D_3$ 25G
In vitro	1[a] (7.0%)[b]	0.9 (6.5%)	$\ll$0.1 (−)[c]
In vivo	1 (30 ng/ml)[d]	4.1 (122 ng/ml)	Not detected

[a] The value of 3G was taken as 1.
[b] Conversion rate (mean of two animals).
[c] The value was too small to calculate.
[d] Yield from 1 ml of bile (mean of three animals).

4. Discussion

It is well known that $24,25(OH)_2D_3$ is metabolized to $25(OH)$-24-oxo-D_3 and then to $23,25(OH)_2$-24-oxo-D_3 [3], but conjugates of $24,25(OH)_2D_3$ have been poorly investigated. From this point of view, we examined in vitro glucuronidation of $24,25(OH)_2D_3$ using rat liver microsomal fraction as an enzyme source. LC-ESI-MS^n was very useful for the characterization of the formed $24,25(OH)_2D_3$ monoglucuronide. Because a glucuronide has an anionic functional group, it can be detected in the negative-ion mode with strong intensity, but the positive-ion mass spectrometry has a greater tendency to produce fragment ions which afford more structural information [14]. Based on this point, the determination of their structures was performed with LC-MS^2 operating in the positive-ion mode, and the fragment ions that occurred from the loss of water or sugar molecules were observed as expected. In consequence, the glucuronidation mainly occurs at the 3- and 24-positions of

24,25(OH)$_2$D$_3$ in almost equal amounts, and a very small amount of 24,25(OH)$_2$D$_3$ 25G was also formed.

As already described in the introductory section, the monoglucuronide of 23,25(OH)$_2$-24-oxo-D$_3$ was reported as a biliary metabolite obtained from dogs dosed with 24,25(OH)$_2$D$_3$, but that of 24,25(OH)$_2$D$_3$ has not been obtained [4]. However, we proved that 24,25(OH)$_2$D$_3$ monoglucuronide was formed in vitro. It is supposed that one possible reason for this discrepancy is the entero-hepatic circulation of 24,25(OH)$_2$D$_3$. Shimoyamada et al. collected bile from the gall bladder of dogs given 24,25(OH)$_2$D$_3$ at 504 h after treatment [4]. It is inferred that the formed 24,25(OH)$_2$D$_3$ monoglucuronide is deconjugated in the intestine as shown in our experiment (Fig. 5), and then the oxidation of the 24,25(OH)$_2$D$_3$ side chain followed by the glucuronidation of the oxidized metabolite occurs. To obtain more information, an in vivo study using rats was performed.

We have now shown that 24,25(OH)$_2$D$_3$-3G and -24G are excreted in rat bile after oral administration of 24,25(OH)$_2$D$_3$ and the glucuronidation occurs at the 24-position in preference to the 3-position. Based on these results, it is inferred that the glucuronidation occurs on the side chain more easily than on the A-ring. As mentioned previously, in the in vivo study using 25(OH)D$_3$, the glucuronidation occurred at 25-position with a large proportion, although it is *tert*-hydroxy group. However, it was not observed on 24,25(OH)$_2$D$_3$. These results demonstrated that in the presence of a *sec*-hydroxy group on the side chain, the glucuronidation at 25-position hardly occurs. Although the formation ratio of 3G and 24G is different between in vivo and in vitro experiments (Table 1), it is obvious that the following metabolic pathway of 24,25(OH)$_2$D$_3$ exits, that is, intact 24,25(OH)$_2$D$_3$ is conjugated as a glucuronide and then excreted into the bile.

The presence of other conjugated vitamin D metabolites, such as 23,25(OH)$_2$-24-oxo-D$_3$ 23-glucuronide, in rat bile should also be considered. Their identification and characterization using LC-MS^n is now in progress in our laboratories, details of which will be reported in the future.

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