Syntheses and Enzymatic Hydrolysis of 25-Hydroxyvitamin D Monoglucuronides

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25-Hydroxyvitamin D_3 (D_2) 3- and 25-monoglucuronides were synthesized from the corresponding provitamin D or its derivatives with the Koenigs–Knorr reaction using silver carbonate as a catalyst, followed by photochemical reaction, thermal isomerization and then alkali hydrolysis. The obtained glucuronides were subjected to enzymatic hydrolysis using β-glucuronidase, and substrate specificities were found in the examined enzymes originating from different sources.

Key words 25-hydroxyvitamin D_3; 25-hydroxyvitamin D_2; monoglucuronide; Koenigs–Knorr reaction; β-glucuronidase; substrate specificity

It is now widely accepted that both vitamin D_2 (D_2) and vitamin D_3 (D_3) are 25-hydroxylated in the liver as the first step in their conversion to 1,25-dihydroxylated compounds which are the active metabolites in the intestine and bone.[1] Despite recent intensive investigation of D metabolism, conjugates of D metabolites still remain poorly understood. The following are the representative data previously known. Axelson reported that 25-hydroxyvitamin D_3 [25(OH)D_3] 3-sulfate is a major circulating form of D_3 in man,[2] which was also confirmed by us.[3] Some investigators clarified the existence of glucuronides (G) of D and related compounds in mammalian bile, but their structures were not always identified due to the absence of authentic specimens.[4,5] These data prompted us to synthesize the 3G and 25G of 25(OH)D_3 and 25(OH)D_2. The substrate specificities for these positionally isomeric derivatives were found in enzymatic hydrolysis using β-glucuronidase originating from different sources.

Fürst *et al.* reported that glucosylation of 25(OH)D_3 gave the 25-glucoside in addition to 3-glucoside.[6] These data prompted us to submit the substrate having unprotected 3- and 25-hydroxy groups to glucuronidation reaction to prepare 25(OH)D_3-3G and -25G (**6b**, **7b**: Chart 1). Introduction of a glucuronyl residue into 25-hydroxy-7-dehydrocholesterol [25-hydroxyprovitamin D_3 (**2**)], which was prepared from 3β-hydroxy-5-cholenic acid (**1**),[7] was undertaken using the Koenigs–Knorr reaction with silver carbonate as a catalyst. Condensation of **2** with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (Br-sugar) in anhydrous chloroform proceeded readily to afford two positional isomers [3- or 25-

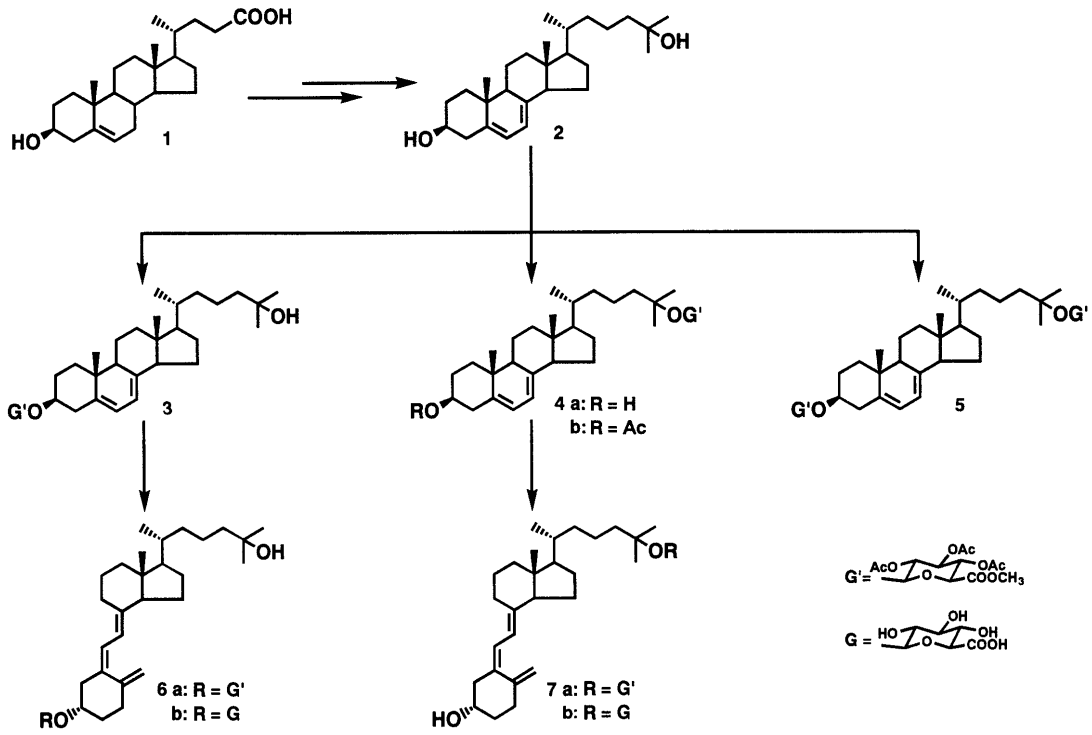

Chart 1

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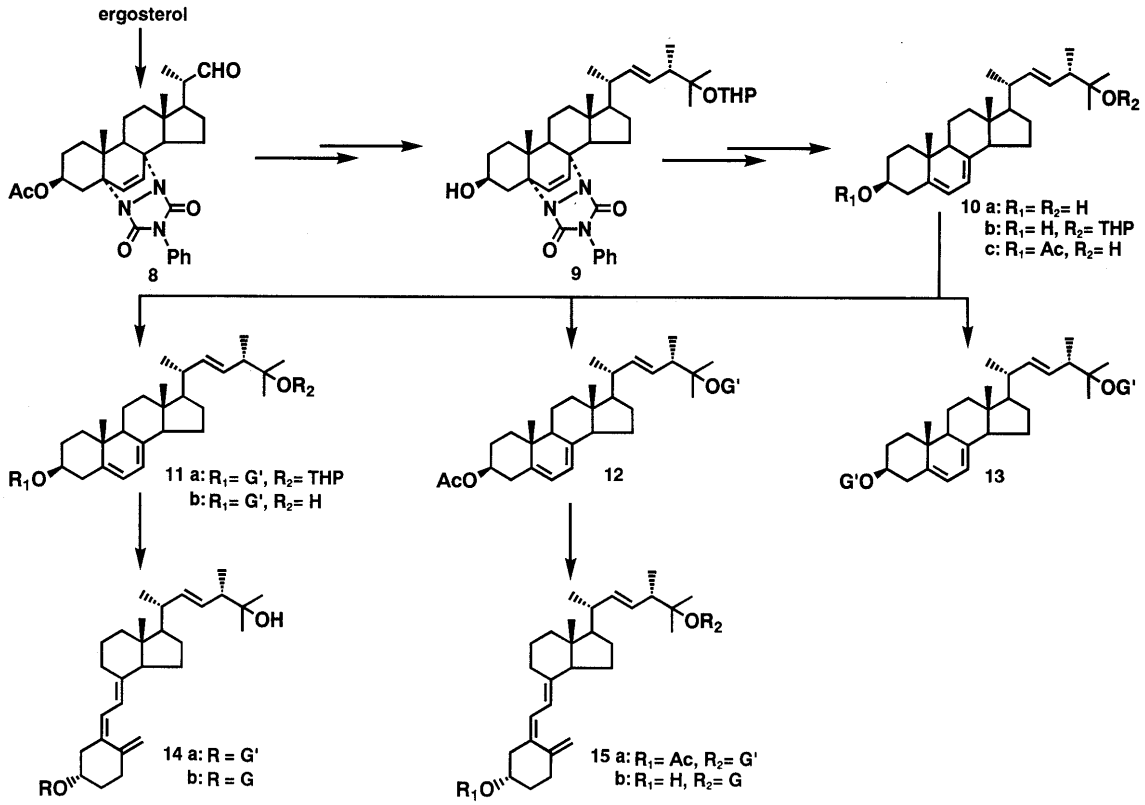

Chart 2

monoglucuronide acetate-methyl ester (G′) (**3**, **4a**)] and one diglucuronide acetate-methyl ester (diG′) (**5**) in yields of 51.5%, 12.9% and 1.66%, respectively. These were separated by flash chromatography, and recrystallization of the crude products provided **3**, **4a** and **5** in the pure state, respectively.

Inspection of ^{1}H-NMR spectra of all these compounds revealed the formation of a β-glucuronoside linkage. The anomeric proton of the sugar moiety appeared at *ca.* 4.7 ppm as a doublet (J=*ca.* 8 Hz), indicating a *trans*-diaxial relationship to the vicinal 2′-proton. The positions of the G′ residue in **3** and **4a** were confirmed by the following evidence. In ^{1}H-NMR spectra, the peaks corresponding to 26- and 27-methyl residues of **3** were observed as a singlet (6H) at the same ppm (1.22) as those of the substrate (**2**), whereas those of **4a** were observed as two singlets (each 3H) at 1.18 and 1.21 ppm, respectively. Furthermore, **4a** was easily acetylated to yield the 3-acetate (**4b**), in which the 3α-proton signal appeared at 4.70 ppm as a multiplet. These data unequivocally established the positions of the introduced G′ as 3 (**3**) and 25 (**4a**), respectively. FAB-MS and the ^{1}H-NMR spectra of **5** showed a quasi-molecular ion at 1031 [$(M-H)^-$] and two singlets (each 3H) that correspond to carboxymethyl ester residues of sugar moieties, respectively. These data clarified the structure of **5** as 3,25-diG′. Irradiation of **3** or **4a** with a high-pressure mercury lamp (400 W, Vycor filter) followed by thermal isomerization gave a mixture from which the 25(OH)D$_3$G′ [**6a** (20.8%), **7a** (9.6%)] was separated by preparative (prep.) TLC. Treatment of **6a** or **7a** with methanolic sodium hydroxide or potassium hydroxide followed by prep. TLC gave the desired 3G (**6b**) or 25G (**7b**) in yields of 84.6% and 69%, respectively. All the novel compounds (**3**—**7**) exhibited satisfactory spectral and/or analytical data (Chart 1).

Our next effort was directed at the preparation of 25(OH)D$_2$-3G and -25G (**14b**, **15b**: Chart 2). Glucuronidation of 25-hydroxyergosterol [25-hydroxyprovitamin D$_2$ (**10a**)], which was prepared from ergosterol,[8,9] was undertaken as described above. Although 3G′ (**11b**) and 3,25-diG′ (**13**) were obtained in yields of 29.4% and 25.2%, respectively, the corresponding 25G′ was not obtained. These data prompted us to use the 25-tetrahydropyranyl ether (THP) (**10b**) and the 3-acetate (**10c**) as the substrate for the preparation of 3G′ (**11b**) and 25G′ (**12**), respectively. These substrates were also prepared from ergosterol as shown in Chart 2. Glucuronidation of **10b** followed by treatment with pyridinium *p*-toluenesulfonate (PPTS) gave the desired 3G′ (**11b**), which was identical with that obtained by glucuronidation of **10a**. Glucuronidation of **10c** also gave the desired 25G′ (**12**) in a yield of 31.8%. Compounds **11b** and **12** were submitted to photochemical reaction, thermal isomerization and then alkali hydrolysis as described above to give the desired compounds **14b** and **15b** in yields of 10.6% and 10.5%, respectively. All the novel compounds (**9**, **10b**, **c**, **11**—**15**) exhibited satisfactory spectral and/or analytical data (Chart 2).

To obtain further evidence of the structure of monoglucuronides, **6b**, **7b**, **14b** and **15b** were subjected to enzymatic hydrolysis using β-glucuronidase originating from bovine liver and the liberated 25(OH)D was characterized by HPLC. Although 25(OH)D$_3$3G (**6b**), 25(OH)D$_2$-3G (**14b**) and -25G (**15b**) liberated fairly large amounts of genin, only a small amount was detected from 25(OH)D$_3$25G (**7b**). These data prompted us to examine the substrate specificities of this enzyme together with that

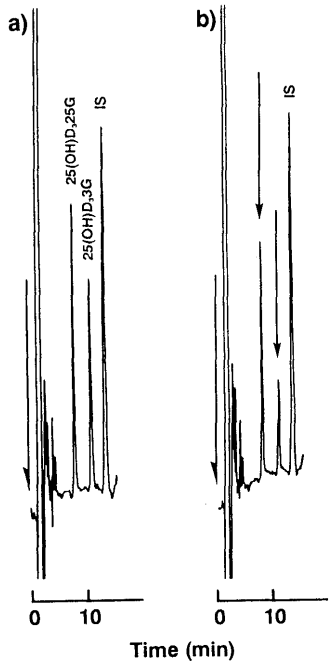

Fig. 1. Chromatograms of Remaining Substrate in Incubation Medium

a) Extract from incubation medium containing denatured enzyme and substrate. b) Remaining 25(OH)D_3G in incubation medium containing enzyme from bovine liver. Conditions: column, J'sphere ODS-M80; mobile phase, MeCN–0.5% $AcONH_4$ (pH 5.0) (2:3).

Table 1. Enzymatic Hydrolysis of Positionally Isomeric Pair of 25(OH)DG

	Remaining substrate		Hydrolyzed product
	3G	25G	25(OH)D
Bovine liver[a]			
25(OH)D_3G[b]	47.1±14.2[c]	80.3± 6.0	34.7±10.4
25(OH)D_2G	38.5±10.6	42.6± 9.4	66.7±11.3
E. coli			
25(OH)D_3G	24.7± 5.1	n.d.[d]	69.2± 5.2
25(OH)D_2G	44.0±14.6	n.d.	84.8±10.2
Helix pomatia			
25(OH)D_3G	83.8± 2.7	45.8± 3.3	24.1± 1.7
25(OH)D_2G	90.5± 2.5	6.9± 4.5	54.8± 6.5
Patella vulgata			
25(OH)D_3G	72.8± 4.2	35.6± 5.9	31.0± 4.9
25(OH)D_2G	84.1± 9.0	39.9±17.4	44.9±12.6

a) Enzyme source. *b*) Substrate. *c*) Mean±S.D. (%, $n=6$). *d*) Not detectable.

originating from *Escherichia coli* (*E. coli*), *Helix pomatia* or *Patella vulgata*. The positionally isomeric pair of substrates (**6b**, **7b**; **14b**, **15b**) was incubated with the enzyme in the same tube, the remaining substrates (Fig. 1) and the liberated genin were measured by HPLC.[10] The enzymes originating from sources other than bovine liver preferably hydrolyzed the 25G isomer as shown in Table 1.

The availability of these authentic specimens should assist the characterization and determination of these glucuronides in biological fluids. Studies on the metabolism of D and substrate specificities of β-glucuronidase are being conducted in these laboratories, and the details will be reported elsewhere.

Materials and Methods

All melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) without correction. Spectral data were obtained as follows: ^{1}H-NMR spectra with a JEOL JNM-EX 270 (270 MHz) spectrometer (Tokyo, Japan), using tetramethylsilane (TMS) as an internal standard (IS). The following abbreviations are used; s=singlet, d=doublet, dd=double doublet, q=quartet, br=broad, m=multiplet; MS with a Hitachi M-80 (electron ionization; EI) (Tokyo), a JEOL JMS DX-303 (FAB) and a JASCO AutoSpec EQ (electrospray ionization; ESI) (Tokyo); UV spectra (in EtOH) with a Hitachi U-2000. Optical rotation with a JASCO DIP-181. Column and flash 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. All air-sensitive reactions were carried out under argon or nitrogen. The phrase "dried and evaporated" indicates drying with Na_2SO_4 followed by evaporation of the solvents under reduced pressure. TLC and prep. TLC (20×20 cm) were conducted with 0.25 and 0.5 mm pre-coated Silica gel $60F_{254}$, respectively. HPLC was performed on a Shimadzu LC-6A chromatograph (Kyoto) equipped with a Shimadzu SPD-10A or -6AV UV detector (265 nm) at a flow rate of 1 ml/min under ambient conditions unless otherwise stated. The following columns were used: reversed phase, J'sphere ODS-M80, J'sphere ODS-H80 (YMC, Kyoto), Develosil ODS-5 (Nomura, Seto, Japan) (each 5 μm, 15×0.46 cm i.d.), normal phase, Develosil 60-5 (5 μm, 25×0.46 cm i.d.). The prep. HPLC was done with Develosil ODS-5 column (5 μm, 15×1.0 cm i.d., Nomura) at a flow rate of 4.7 ml/min. The pH of the mobile phase containing $AcONH_4$ or $NaClO_4$ was adjusted with AcOH or $HClO_4$, respectively. Ergosterol and D_3 were obtained from Tokyo Kasei (Tokyo). 25(OH)D_3 and 3β-hydroxy-5-cholenic acid were generously donated by Teikoku Hormone Mfg. (Tokyo). β-Glucuronidase originating from bovine liver (type B-1), *E. coli* (type IX-A), *Helix pomatia* (type H-1) and *Patella vulgata* (type L-II) were obtained from Sigma (St. Louis, MO, U.S.A.). 25(OH)D_2,[9] IS and 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MBOTAD)[10] used for the determination of substrate specificities were synthesized in these laboratories. An acetate buffer [0.1 M AcONa–AcOH (pH 5.0)] was used for the enzymatic reaction.

Methyl(cholesta-5,7-dien-25-ol-3β-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (3), Methyl(cholesta-5,7-dien-3β-ol-25-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (4a), Methyl(cholesta-5,7-dien-3β,25-diyl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (5) Freshly prepared Ag_2CO_3 (660 mg, 2.39 mmol) and Br-sugar (350 mg, 0.88 mmol) were added to a solution of **2**[7] (235 mg, 0.59 mmol) in anhydrous $CHCl_3$ (23 ml), and the reaction mixture was stirred at room temperature. After 1 h, an additional amount of Ag_2CO_3 (290 mg, 1.05 mmol) was added to the mixture and stirred for 1 d. The reaction mixture was filtered, the filtrate was evaporated *in vacuo* and the crude product was purified by flash chromatography [35×1.0 cm i.d., CH_2Cl_2–AcOEt (40:1)] to yield **3** [TLC: CH_2Cl_2–AcOEt (6:1), *Rf* 0.54; 217 mg, 51.5%] as colorless needles (from Et_2O). mp 159—162 °C. $[\alpha]_D^{17}$ −40.5° ($c=0.10$, $CHCl_3$). *Anal.* Calcd for $C_{40}H_{60}O_{11}$: C, 67.01; H, 8.44. Found: C, 66.72; H, 8.38. ^{1}H-NMR ($CDCl_3$) δ: 0.61 (3H, s, H-18), 0.92 (3H, s, H-19), 0.96 (3H, d, $J=6.6$ Hz, H-21), 1.22 (6H, s, H-26, 27), 2.02 (6H, s, 2×OAc), 2.05 (3H, s, OAc), 3.63 (1H, m, H-3α), 3.75 (3H, s, $COOCH_3$), 4.03 (1H, d, $J=9.6$ Hz, H-5′), 4.67 (1H, d, $J=7.9$ Hz, H-1′), 4.95—5.02, 5.16—5.30 (total 3H, m, 3×CHOAc), 5.38, 5.57 (1H each, m, H-7, 6). EI-MS *m/z*: 716 (M^+).

Compound **4a** [TLC: CH_2Cl_2–AcOEt (6:1), *Rf* 0.42; 54.2 mg, 12.9%] was obtained from the above fraction as a colorless amorphous substance (from MeOH). mp 174—176 °C. $[\alpha]_D^{19}$ −74.6° ($c=0.10$, $CHCl_3$). *Anal.* Calcd for $C_{40}H_{60}O_{11}\cdot 1/2H_2O$: C, 66.18; H, 8.47. Found: C, 65.97; H, 8.58. ^{1}H-NMR ($CDCl_3$) δ: 0.61 (3H, s, H-18), 0.92 (3H, s, H-19), 0.93 (3H, d, $J=6.3$ Hz, H-21), 1.18, 1.21 (3H each, s, H-26, 27), 2.01 (6H, s, 2×OAc), 2.02 (3H, s, OAc), 3.65 (1H, m, H-3α), 3.74 (3H, s, $COOCH_3$), 4.00 (1H, d, $J=9.6$ Hz, H-5′), 4.70 (1H, d, $J=7.9$ Hz, H-1′), 4.93—5.01, 5.17—5.30 (total 3H, m, 3×CHOAc), 5.39, 5.58 (1H each, m, H-7, 6). EI-MS *m/z*: 716 (M^+).

Compound **5** [TLC: CH_2Cl_2–AcOEt (6:1), *Rf* 0.63; 10.1 mg, 1.66%] was also obtained from the above fraction as a colorless amorphous substance (from Et_2O). mp 142—145 °C. $[\alpha]_D^{19}$ −68.3° ($c=0.10$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.61 (3H, s, H-18), 0.92 (3H, s, H-19), 0.96 (3H, d, $J=6.3$ Hz, H-21), 1.18, 1.21 (3H each, s, H-26, 27), 2.02 (12H, s, 4×OAc), 2.05 (6H, s, 2×OAc), 3.65 (1H, m, H-3α), 3.74, 3.75 (3H each, s, 2×$COOCH_3$), 4.00, 4.02 (1H each, d, $J=9.6$ Hz, 2×H-5′), 4.67, 4.70

(1H each, d, J=7.6 Hz, 2×H-1′), 4.93—5.01, 5.17—5.29 (total 6H, m, 6×CHOAc), 5.38, 5.57 (1H each, m, H-7, 6). FAB-MS m/z: 1031 $(M-H)^-$.

Methyl(3β-acetoxycholesta-5,7-dien-25-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (4b) Compound **4a** (5.2 mg) was dissolved in pyridine–Ac_2O (2:1; 0.75 ml) and stirred at room temperature for 2 h. After the additon of H_2O, the mixture was extracted with AcOEt. The organic layer was washed (H_2O, 5% HCl, 5% $NaHCO_3$ and brine), dried and evaporated. The obtained residue was purified by prep. TLC [CH_2Cl_2–AcOEt (50:1)]. The zone corresponding to *Rf ca.* 0.4 was extracted with AcOEt to yield **4b** (5.1 mg, 92.6%) as a colorless semi-solid. ^{1}H-NMR ($CDCl_3$) δ: 0.61 (3H, s, H-18), 0.93 (3H, d, J=6.6 Hz, H-21), 0.95 (3H, s, H-19), 1.18, 1.21 (3H each, s, H-26, 27), 2.01 (6H, s, 2×OAc), 2.02 (3H, s, OAc), 2.04 (3H, s, OAc), 3.74 (3H, s, $COOCH_3$), 4.00 (1H, d, J=9.6 Hz, H-5′), 4.70 (1H, m, H-3α), 4.70 (1H, d, J=7.6 Hz, H-1′), 4.94—5.00, 5.16—5.30 (total 3H, m, 3×CHOAc), 5.38, 5.56 (1H each, m, H-7, 6). EI-MS m/z: 758 (M^+).

Methyl[(5*Z*,7*E*)-(3*S*)-25-hydroxy-9,10-secocholesta-5,7,10(19)-trien-3-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (6a) A solution of **3** (75 mg) in Et_2O–CH_2Cl_2 (400:1, 401 ml) was irradiated intermittently (for 10, 5, 5 and 3 s) with a 400 W high pressure mercury lamp through a Vycor filter at 0 °C with argon bubbling through the solution. After removal of the solvent under reduced pressure, the residue was dissolved in hexane–tetrahydrofuran (THF) (4:1, 25 ml) and stored in the dark under argon at room temperature for 7 d. The solvent was evaporated off and the crude product was purified by prep. TLC [hexane–AcOEt (8:5)]. The zone corresponding to ^{2}*Rf ca.* 0.5 was extracted with $CHCl_3$–MeOH (5:1) to yield **6a** (15.6 mg, 20.8%) as a colorless amorphous substance (from CH_2Cl_2–hexane). mp 126—128 °C. $[\alpha]_D^{19}$ +2.3° (c=0.10, $CHCl_3$). UV λ_{max} nm: 264, λ_{min} nm: 227. ^{1}H-NMR ($CDCl_3$) δ: 0.54 (3H, s, H-18), 0.94 (3H, d, J=5.9 Hz, H-21), 1.22 (6H, s, H-26, 27), 1.96, 2.01, 2.02 (3H each, s, 3×OAc), 3.75 (3H, s, $COOCH_3$), 3.96 (1H, m, H-3α), 4.03 (1H, d, J=9.6 Hz, H-5′), 4.68 (1H, d, J=7.6 Hz, H-1′), 4.80, 5.03 (1H each, br s, H-19), 4.95—5.01, 5.17—5.31 (total 3H, m, 3×CHOAc), 6.00, 6.18 (total 2H, ABq, J=11.2 Hz, H-7, 6). EI-MS m/z: 716 (M^+).

[(5*Z*,7*E*)-(3*S*)-25-Hydroxy-9,10-secocholesta-5,7,10(19)-trien-3-yl]-β-D-glucopyranosiduronic Acid (6b) A solution of 0.1 M NaOH–MeOH (1.5 ml) was added to a solution of **6a** (17 mg) in MeOH–CH_2Cl_2 (4:1, 2.5 ml) and the mixture was stirred at room temperature for 17 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 dried and evaporated and the obtained residue was submitted to prep. TLC [$CHCl_3$–MeOH–H_2O (70:30:4)]. The zone corresponding to *Rf ca.* 0.3 was extracted with $CHCl_3$–MeOH–H_2O (70:30:4) to yield **6b** (11.5 mg, 84.6%) as a colorless amorphous substance (from MeOH). mp 165—170 °C (dec.). $[\alpha]_D^{19}$ +6.0° [c=0.10, $CHCl_3$–MeOH–H_2O (70:30:4)]. UV λ_{max} nm: 264, λ_{min} nm: 227. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 0.54 (3H, s, H-18), 0.94 (3H, d, J=5.6 Hz, H-21), 4.82, 5.05 (1H each, br s, H-19), 6.02, 6.24 (total 2H, ABq, J=11.2 Hz, H-7, 6). FAB-MS m/z: 575 $(M-H)^-$. Its purity was confirmed by HPLC [J'sphere ODS-M80, MeCN–0.5% $AcONH_4$ (pH 5.0) (2:3), t_R 10.4 min].

Methyl[(5*Z*,7*E*)-(3*S*)-3-hydroxy-9,10-secocholesta-5,7,10(19)-trien-25-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (7a) A solution of **4a** (26 mg) in Et_2O–CH_2Cl_2 (400:1, 401 ml) was irradiated intermittently (for 6 and 4 s) with a 400 W high pressure mercury lamp as described in **6a**. After removal of the solvent under reduced pressure, the residue was dissolved in hexane–THF (4:1, 25 ml) and stored in the dark under argon at room temperature for 3 d. The solvent was evaporated off and the crude product thus obtained was purified by prep. TLC [hexane–AcOEt (5:3)]. The zone corresponding to ^{2}*Rf ca.* 0.3 was extracted with $CHCl_3$–MeOH (5:1) to yield **7a** (2.5 mg, 9.6%) as a colorless oily substance. UV λ_{max} nm: 264, λ_{min} nm: 227. ^{1}H-NMR ($CDCl_3$) δ: 0.54 (3H, s, H-18), 0.93 (3H, d, J=7.3 Hz, H-21), 1.17, 1.20 (3H each, s, H-26, 27), 2.01 (6H, s, 2×OAc), 2.02 (3H, s, OAc), 3.74 (3H, s, $COOCH_3$), 3.97 (1H, m, H-3α), 4.00 (1H, d, J=9.6 Hz, H-5′), 4.70 (1H, d, J=7.6 Hz, H-1′), 4.82, 5.02 (1H each, br s, H-19), 4.94—4.97, 5.16—5.30 (total 3H, m, 3×CHOAc), 6.03, 6.24 (total 2H, ABq, J=11.2 Hz, H-7, 6). EI-MS m/z: 716 (M^+).

[(5*Z*,7*E*)-(3*S*)-3-Hydroxy-9,10-secocholesta-5,7,10(19)-trien-25-yl]-β-D-glucopyranosiduronic Acid (7b) A solution of 0.1 M KOH–MeOH (1 ml) was added to a solution of **7a** (9.2 mg) in MeOH–CH_2Cl_2 (4:1; 1.25 ml) and the mixture was treated as described in **6b**. The residue obtained was submitted to prep. TLC [$CHCl_3$–MeOH–H_2O (70:30:4)] and the zone corresponding to *Rf ca.* 0.3 was extracted with $CHCl_3$–MeOH–H_2O (70:30:4) to yield **7b** (4.8 mg, 69%) as a colorless semi-solid. UV λ_{max} nm: 264, λ_{min} nm: 227. FAB-MS m/z: 575 $(M-H)^-$. Its purity was confirmed by HPLC [J'sphere ODS-M80, MeCN–0.5% $AcONH_4$ (pH 5.0) (2:3), t_R 7.6 min]. The compound dissolved in $CDCl_3$–CD_3OD decomposed spontaneously, the reason for which is unknown, so ^{1}H-NMR spectrum was not obtained.

Methyl[(22*E*)-ergosta-5,7,22-trien-25-ol-3β-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (11b), Methyl[(22*E*)-ergosta-5,7,22-trien-3β,25-diyl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (13) Freshly prepared Ag_2CO_3 (167.8 mg, 0.61 mmol) and Br-sugar (96.7 mg, 0.24 mmol) were added to a solution of **10a**[8] (49.6 mg, 0.12 mmol) in anhydrous $CHCl_3$ (7.0 ml), and the reaction mixture was stirred at room temperature. After 3 and 17 h, additional amounts of Ag_2CO_3 (86.0 mg, 0.31 mmol) and Br-sugar (56.0 mg, 0.14 mmol) were added to the mixture, respectively. Ag_2CO_3 (88.5 mg, 0.32 mmol) and Br-sugar (95.5 mg, 0.24 mmol) were added after 25 h. After 47.5 h, the reaction mixture was filtered, the filtrate was evaporated *in vacuo* and the crude product was purified by flash chromatography [32×1.0 cm i.d., hexane–AcOEt (2:1)] to yield **11b** [TLC: hexane–AcOEt (3:2), *Rf* 0.45; 25.8 mg, 29.4%] as a colorless amorphous substance (from Et_2O–hexane). mp 133.5—135.0 °C. $[\alpha]_D^{18}$ −33.8° (c=0.26, $CHCl_3$). *Anal.* Calcd for $C_{41}H_{60}O_{11}\cdot 1/2H_2O$: C, 66.73; H, 8.33. Found: C, 66.54; H, 8.42. ^{1}H-NMR ($CDCl_3$) δ: 0.63 (3H, s, H-18), 0.92 (3H, s, H-19), 1.02 (3H, d, J=6.9 Hz, H-24[1]), 1.06 (3H, d, J=6.6 H, H-21), 1.13, 1.17 (3H each, s, H-26, 27), 2.02 (6H, s, 2×OAc), 2.05 (3H, s, OAc), 3.62 (1H, m, H-3α), 3.75 (3H, s, $COOCH_3$), 4.03 (1H, d, J=9.2 Hz, H-5′), 4.67 (1H, d, J=7.9 Hz, H-1′), 4.95—5.02 (1H, m, CHOAc), 5.21—5.36 (5H, m, H-7, 22, 23, 2×CHOAc), 5.56 (1H, m, H-6). FAB-MS m/z: 751 $(M+Na)^+$, 729 $(M+H)^+$.

The eluate from the hexane–AcOEt (1:1) from the above flash chromatography was evaporated *in vacuo* to yield **13** [TLC: hexane–AcOEt (3:2), *Rf* 0.27; 31.7 mg, 25.2%] as a colorless amorphous substance (from acetone–hexane). mp 158.0—160.0 °C. $[\alpha]_D^{18}$ −33.3° (c=0.29, $CHCl_3$). *Anal.* Calcd for $C_{54}H_{76}O_{20}\cdot 1/2H_2O$: C, 61.52; H, 7.36. Found: C, 61.46; H, 7.50. ^{1}H-NMR ($CDCl_3$) δ: 0.61 (3H, s, H-18), 0.91 (3H, s, H-19), 0.93 (3H, d, J=9.2 Hz, H-24[1]), 1.02 (3H, d, J=5.9 Hz, H-21), 1.07, 1.19 (3H each, s, H-26, 27), 2.01 (12H, s, 4×OAc), 2.05 (6H, s, 2×OAc), 3.62 (1H, m, H-3α), 3.75 (6H, s, 2×$COOCH_3$), 4.00, 4.02 (1H each, d, J=9.6 Hz, 2×H-5′), 4.67, 4.72 (1H each, d, J=7.9 Hz, 2×H-1′), 4.95—5.03 (2H, m, 2×CHOAc), 5.16—5.30 (6H, m, H-22, 23, 4×CHOAc), 5.37 (1H, m, H-7), 5.55 (1H, m, H-6). FAB-MS m/z: 1067 $(M+Na)^+$, 1045 $(M+H)^+$, 1043 $(M-H)^-$.

25-Tetrahydropyranyloxy-3β-hydroxy-4′-phenyl-3′,5′-dihydro-5,8-[1,2]epi[1,2,4]triazolo-5α,8α-(22*E*)-ergosta-6,22-diene-3′,5′-dione (9) Compound **9** (a colorless semi-solid; 556.2 mg, 58.8%) was prepared from compound **8** (768.2 mg)[8] in two steps according to the procedure described by Tsuji *et al.*[9] ^{1}H-NMR ($CDCl_3$) δ: 3.46 (1H, m, THPH-6), 3.95 (1H, m, THPH-6), 4.45 (1H, m, H-3α), 4.77 (1H, m, THPH-2), 5.16—5.39 (2H, m, H-22,23), 6.24, 6.40 (total 2H, ABq, J=8.3 Hz, H-7, 6), 7.26—7.43 (5H, m, ArH). Its structure was confirmed by conversion to the known compound **10a**.[8]

(22*E*)-25-Tetrahydropyranyloxyergosta-5,7,22-trien-3β-ol (10b) Compound **9** (56.6 mg) was dissolved in 1,1,3,3-tetramethylguanidine (1.5 ml) and refluxed for 1.5 h. The reaction mixture was diluted with AcOEt, which was washed with brine, dried and evaporated. The crude product was purified by column chromatography [8.5×1.3 cm i.d., hexane–AcOEt (4:1)] to yield **10b** (28.4 mg, 67.9%) as a colorless solid. ^{1}H-NMR ($CDCl_3$) δ: 0.63 (3H, s, H-18), 0.95 (3H, s, H-19), 3.45 (1H, m, THPH-6), 3.64 (1H, m, H-3α), 3.96 (1H, m, THPH-6), 4.78 (1H, m, THPH-2), 5.35 (3H, m, H-7, 22, 23), 5.57 (1H, m, H-6).

Methyl[(22*E*)-25-tetrahydropyranyloxyergosta-5,7,22-trien-3β-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (11a) Freshly prepared Ag_2CO_3 (264.3 mg, 0.96 mmol) and Br-sugar (187.7 mg, 0.47 mmol) were added to a solution of **10b** (157.3 mg, 0.32 mmol) in anhydrous $CHCl_3$ (15 ml), and the reaction mixture was stirred at room temperature for 9 h. After further addition of Ag_2CO_3 (93.6 mg, 0.34 mmol) and Br-sugar (130.1 mg, 0.328 mmol), the mixture was stirred for 17 h. After filtration, the filtrate was evaporated *in vacuo* and the crude product was purified by flash chromatography [38×1.0 cm i.d., hexane–AcOEt (3:1)] to yield **11a** (165.0 mg, 64.1%) as a colorless solid. ^{1}H-NMR ($CDCl_3$) δ: 0.63 (3H, s, H-18), 0.92 (3H, s, H-19), 2.02 (6H, s, 2×OAc), 2.05 (3H, s, OAc), 3.45 (1H, m, THPH-6), 3.63 (1H, m, H-3α), 3.75 (3H, s, $COOCH_3$), 3.96 (1H, m, THPH-6), 4.03 (1H, d, J=9.2 Hz, H-5′), 4.68 (1H, d,

J=7.6 Hz, H-1′), 4.78 (1H, br s, THPH-2), 4.99 (1H, m, CHOAc), 5.18—5.39 (5H, m, H-7, 22, 23, 2×CHOAc), 5.56 (1H, m, H-6).

Methyl[(22*E*)-ergosta-5,7,22-trien-25-ol-3β-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (11b) PPTS (2.6 mg, 10.4 μmol) was added to a solution of **11a** (165.1 mg, 0.20 mmol) in 95% EtOH–CH_2Cl_2 (3:1, 2.13 ml), and the mixture was stirred at 54—65 °C for 5.5 h. Brine was added to the mixture and then extracted with AcOEt, which was washed with brine. The organic layer was dried and evaporated, and the residue was purified by column chromatography [8.5×2 cm i.d., hexane–AcOEt (2:1)] to yield **11b** (109.1 mg, 73.7%) as a colorless amorphous substance. Its structure was confirmed by ^{1}H-NMR spectrum and chromatographic behavior using normal [hexane–isopropanol (95:5), 2.0 ml/min, t_R 8.3 min]- and reversed [Develosil ODS-5, MeOH–H_2O (9:1), t_R 6.0 min]-phase HPLC using **11b** as an authentic sample, which was prepared by direct glucuronidation.

Methyl[(5*Z*,7*E*,22*E*)-(3*S*)-25-hydroxy-9,10-secoergosta-5,7,10(19),22-tetraen-3-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (14a) A solution of **11b** (84.3 mg) in Et_2O–$CHCl_3$ (400:1, 401 ml) was irradiated intermittently (for 20 and 8 s) with a 400 W high pressure mercury lamp as described in **6a**. After removal of the solvent under reduced pressure, the residue was dissolved in hexane–THF (4:1, 25 ml) and stored in the dark under argon at room temperature for 6 d. The solvent was evaporated and the crude product was purified by flash chromatography [36×1.0 cm i.d., hexane–AcOEt (2:1)]. The obtained product was further purified by prep. HPLC [MeOH–H_2O (7:1), t_R 8.3—9.4 min] to yield **14a** (11.4 mg, 13.5%) as a colorless semi-solid. UV λ_{max} nm: 265, λ_{min} nm: 229. ^{1}H-NMR ($CDCl_3$) δ: 0.55 (3H, s, H-18), 0.99 (3H, d, J=6.9 Hz, H-24^1), 1.03 (3H, d, J=6.3 Hz, H-21), 1.13, 1.17 (3H each, s, H-26, 27), 1.95, 2.01, 2.02 (3H each, s, 3×OAc), 3.75 (3H, s, $COOCH_3$), 3.96 (1H, m, H-3α), 4.03 (1H, d, J=9.2 Hz, H-5′), 4.68 (1H, d, J=7.6 Hz, H-1′), 4.79 (1H, d, J=2.6 Hz, H-19), 4.94—5.02 (2H, m, H-19, CHOAc), 5.17—5.42 (4H, m, H-22, 23, 2×CHOAc), 6.00, 6.17 (total 2H, ABq, J=11.2 Hz, H-7, 6). EI-MS m/z: 728 (M^+).

[(5*Z*,7*E*,22*E*)-(3*S*)-25-Hydroxy-9,10-secoergosta-5,7,10(19),22-tetraen-3-yl]-β-D-glucopyranosiduronic Acid (14b) A solution of 0.1 M NaOH–MeOH (0.5 ml) was added to a solution of **14a** (7.4 mg) in MeOH–CH_2Cl_2 (10:3, 1.3 ml) and the mixture was treated as described in **6b**. The residue obtained was submitted to prep. TLC [$CHCl_3$–MeOH–H_2O (70:30:4)]. The zone corresponding to *Rf ca.* 0.4 was extracted with $CHCl_3$–MeOH–H_2O (70:30:4) to yield **14b** (4.7 mg, 78.6%) as a colorless semi-solid. UV λ_{max} nm: 263.5, λ_{min} nm: 227.5. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 0.56 (3H, s, H-18), 1.00 (3H, d, J=6.9 Hz, H-24^1), 1.04 (3H, d, J=6.6 Hz, H-21), 1.12, 1.15 (3H each, s, H-26, 27), 3.99 (1H, m, H-3α), 4.23 (1H, dd, J=1.8, 5.8 Hz, H-5′), 4.44 (1H, d, J=7.6 Hz, H-1′), 4.79 (1H, br s, H-19), 5.03 (1H, br s, H-19), 5.27—5.34 (2H, m, H-22, 23), 6.02, 6.24 (total 2H, ABq, J=10.7 Hz, H-7, 6). FAB-MS m/z: 633 ($M+2Na-H)^+$, 611 ($M+Na)^+$, 587 ($M-H)^-$. Its purity was confirmed by HPLC [Develosil ODS-5, MeCN–2% $NaClO_4$ (pH 3.0) (3:2), t_R 8.1 min, MeOH–2% $NaClO_4$ (pH 3.0) (4:1), t_R 13.2 min].

(22*E*)-3β-Acetoxyergosta-5,7,22-trien-25-ol (10c) Compound **10a** (338.4 mg) was dissolved in pyridine–Ac_2O (2:1, 9.0 ml) and stirred at room temperature for 11.5 h. After the additon of H_2O, the mixture was extracted with AcOEt. The organic layer was washed (5% HCl, 5% $NaHCO_3$ and brine), dried and evaporated. The residue obtained was purified by column chromatography [13.5×3.5 cm i.d., $CHCl_3$–AcOEt (100:1)] to yield **10c** (233.4 mg) as colorless leaflets (from MeOH–CH_2Cl_2). mp 174.5—176.0 °C. $[\alpha]_D^{17}$ −94.4° (c=0.25, $CHCl_3$). *Anal.* Calcd for $C_{30}H_{46}O_3$: C, 79.24; H, 10.20. Found: C, 78.76; H, 10.14. ^{1}H-NMR ($CDCl_3$) δ: 0.63 (3H, s, H-18), 0.95 (3H, s, H-19), 1.00 (3H, d, J=6.9 Hz, H-24^1), 1.06 (3H, d, J=6.6 Hz, H-21), 1.13, 1.16 (3H each, s, H-26, 27), 4.70 (1H, m, H-3α), 5.34 (3H, m, H-7, 22, 23), 5.56 (1H, m, H-6). EI-MS m/z: 436 (M^+-H_2O), 394 (M^+–AcOH), 376 (M^+-H_2O–AcOH).

Methyl[(22*E*)-3β-acetoxyergosta-5,7,22-trien-25-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (12) Freshly prepared Ag_2CO_3 (95.4 mg, 0.35 mmol) and Br-sugar (182.1 mg, 0.46 mmol) were added to a solution of **10c** (51.8 mg, 0.114 mmol) in anhydrous $CHCl_3$ (2.0 ml) and the mixture was stirred at room temperature for 11 h. After filtration, the filtrate obtained was purified by flash chromatography [34×1.0 cm i.d., hexane–AcOEt (3:1)] to yield **12** (27.9 mg, 31.8%) as colorless leaflets (from Et_2O). mp 199.5—201.0 °C. $[\alpha]_D^{20}$ −58.0° (c=0.26, $CHCl_3$). *Anal.* Calcd for $C_{43}H_{62}O_{12}$: C, 66.99; H, 8.11. Found: C, 66.81; H, 8.08. ^{1}H-NMR ($CDCl_3$) δ: 0.62 (3H, s, H-18), 0.94 (3H, d, J=5.9 Hz, H-24^1), 0.95 (3H, s, H-19), 1.02 (3H, d, J=6.9 Hz, H-21), 1.08, 1.20 (3H each, s, H-26, 27), 2.02—2.04 (12H, m, 4×OAc), 3.75 (3H, s, $COOCH_3$), 4.00 (1H, d, J=9.6 Hz, H-5′), 4.70 (1H, m, H-3α), 4.72 (1H, d, J=7.9 Hz, H-1′), 5.00 (1H, m, CHOAc), 5.21—5.30 (4H, m, H-22, 23, 2×CHOAc), 5.39, 5.56 (1H each, m, H-7, 6). FAB-MS m/z: 793 ($M+Na)^+$.

Methyl[(5*Z*,7*E*,22*E*)-(3*S*)-3-acetoxy-9,10-secoergosta-5,7,10(19),22-tetraen-25-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid]uronate (15a) A solution of **12** (41.1 mg) in Et_2O–$CHCl_3$ (400:1, 401 ml) was irradiated intermittently (6, 2, 2 and 2 s) with a 400 W high pressure mercury lamp as described in **6a**. After removal of the solvent under reduced pressure, the residue was dissolved in EtOH (20 ml), stored in the dark under argon at room temperature for 5 d and then kept at 48—55 °C for 2.5 h. After the solvent was again removed, the crude product was purified by flash chromatography [24.5×1.0 cm i.d., $CHCl_3$–AcOEt (50:1)]. The product obtained was further purified by prep. HPLC [MeOH–H_2O (93:7), t_R 8.0—8.7 min] to yield **15a** (5.6 mg, 13.6%) as a colorless semi-solid. UV λ_{max} nm: 264, λ_{min} nm: 227. ^{1}H-NMR ($CDCl_3$) δ: 0.54 (3H, s, H-18), 0.94 (3H, d, J=6.9 Hz, H-24^1), 1.00 (3H, d, J=6.6 Hz, H-21), 1.07, 1.19 (3H each, s, H-26, 27), 2.01—2.05 (12H, m, 4×OAc), 3.75 (3H, s, $COOCH_3$), 4.00 (1H, d, J=9.6 Hz, H-5′), 4.72 (1H, d, J=7.9 Hz, H-1′), 4.83 (1H, d, J=2.3 Hz, H-19), 4.94—5.00 (3H, m, H-3, 19, CHOAc), 5.17—5.30 (4H, m, H-22, 23, 2×CHOAc), 6.02, 6.21 (total 2H, ABq, J=11.4 Hz, H-7, 6). EI-MS m/z: 770 (M^+).

[(5*Z*,7*E*,22*E*)-(3*S*)-3-Hydroxy-9,10-secoergosta-5,7,10(19),22-tetraen-25-yl]-β-D-glucopyranosiduronic Acid (15b) A solution of 0.1 M KOH–MeOH (0.35 ml) was added to a solution of **15a** (4.9 mg) in MeOH–CH_2Cl_2 (7:2, 0.9 ml), the mixture was treated as described in **14b**, and the crude product was purified by prep. TLC [$CHCl_3$–MeOH–H_2O (70:30:4)]. The zone corresponding to *Rf ca.* 0.3 was extracted with $CHCl_3$–MeOH–H_2O (70:30:4) to yield **15b** (2.9 mg, 77.5%) as a colorless semi-solid. UV λ_{max} nm: 263.5, λ_{min} nm: 227.5. FAB-MS m/z: 633 ($M+2Na-H)^+$, 611 ($M+Na)^+$, 589 ($M+H)^+$. ESI-MS m/z: 587 ($M-H)^-$. Its purity was confirmed by HPLC [Develosil ODS-5, MeCN–2% $NaClO_4$ (pH 3.0) (3:2), t_R 6.6 min, MeOH–2% $NaClO_4$ (pH 3.0) (5:1), t_R 8.8 min]. The compound dissolved in $CDCl_3$–CD_3OD decomposed spontaneously, the reason for which is unknown, so ^{1}H-NMR spectrum was not obtained.

Enzymatic Hydrolysis of Monoglucuronide The monoglucuronide [**6b**, **7b**, **14b** or **15b**: each 0.25 nmol in EtOH (20 μl)] obtained above and β-glucuronidase (from bovine liver: 500 Fishman units), each of which was dissolved in acetate buffer (0.88 ml), were separately pre-incubated at 37 °C for 15 min. The two solutions were mixed and incubated at 37 °C for 2 h. The reaction mixture was extracted with AcOEt and the organic layer was evaporated *in vacuo*. The residue was submitted to the following HPLC and 25(OH)D_3 or 25(OH)D_2 was identified using an authentic sample. HPLC: 25(OH)D_3 [J'sphere ODS-H80, MeOH–H_2O (21:4), t_R 19.9 min], 25(OH)D_2 [J'sphere ODS-H80, MeCN–H_2O (7:3), t_R 22.4 min].

Determination of Substrate Specificity of β-Glucuronidase Two monoglucuronides [pair of **6b** and **7b** or **14b** and **15b**: each 0.125 nmol in EtOH (10 μl)] dissolved in acetate buffer (0.88 ml) and β-glucuronidase (500 Fishman units) in acetate buffer (0.1 ml) were separately pre-incubated at 37 °C for 15 min. The two solutions were mixed and incubated at 37 °C for 2 h. The reaction was stopped by the addition of AcOEt (1 ml). After the addition of IS, the reaction mixture was extracted with AcOEt, the organic layer was evaporated *in vacuo* and the residue obtained was submitted to the following HPLC to determine the liberated genin and remaining substrate. IS: for 25(OH)D_3; 25-hydroxy-7-dehydrocholesterol MBOTAD adduct [0.4 nmol in EtOH (20 μl)], for 25(OH)D_3G; 25-hydroxy-7-dehydrocholesterol 3G MBOTAD adduct [0.4 nmol in EtOH (20 μl)], for 25(OH)D_2; 25(OH)D_3 [0.25 nmol in EtOH (20 μl)], for 25(OH)D_2G; 25(OH)$D_3$3G [0.125 nmol in EtOH (20 μl)]. HPLC: for 25(OH)D_3, J'sphere ODS-H80, MeOH–H_2O (21:4): for 25(OH)D_2, J'sphere ODS-H80, MeOH–H_2O (6:1): for 25(OH)DG, J'sphere ODS-M80, MeCN–0.5% $AcONH_4$ (pH 5.0) (2:3). The determination of liberated genin and the remaining substrate was done by addition of the authentic sample to the incubation medium or heat-denatured incubation medium, respectively, whose recovery was taken as 100%. The absolute recoveries of genin and the substrate at two levels were more than 88.1% (0.03 and 0.25 nmol/tube; mean, n=2). The calibration curves (0.03—0.25 nmol) were constructed by the peak height ratio method and the obtained linear relationships were as follows: 25(OH)D_3, y=4.452x−0.004 (r=0.998); 25(OH)D_2, y=2.962x−0.004 (r=0.997); 25(OH)$D_3$3G, y=4.168x−0.004 (r=0.997); 25(OH)$D_3$25G,

$y=5.893x-006$ ($r=0.999$); 25(OH)$D_2$3G, $y=5.118x+0.008$ ($r=0.989$); 25(OH)$D_2$25G, $y=7.243x+0.008$ ($r=0.990$). Details of the assay method will be reported elsewhere.

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