Chem. Pharm. Bull.
30(9)3231—3238(1982)

Preparation and High-Performance Liquid Chromatographic Determination of Guaiacol Estrogen 17β-Conjugates: The Enzymatic *O*-Methylation Products of 2-Hydroxyestradiol 17β-Conjugates (Clinical Analysis on Steroids. XXII[1])

KAZUHIRO WATANABE and ITSUO YOSHIZAWA*

Hokkaido Institute of Pharmaceutical Sciences, 7-1, Katsuraoka-cho, Otaru, Hokkaido, 047-02, Japan

(Received March 6, 1982)

For the direct assay of the enzymatic *O*-methylation products of 2-hydroxyestradiol 17β-sulfate (**2**) and 17β-glucuronide (**3**), the corresponding guaiacol estrogens have been prepared as authentic specimens and their high-performance liquid chromatography (HPLC) was investigated.

The materials synthesized were: potassium 3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl sulfate (**7**), potassium 2-hydroxy-3-methoxyestra-1,3,5(10)-trien-17β-yl sulfate (**13**), potassium [3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid]uronate (**9**), and potassium [2-hydroxy-3-methoxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid]-uronate (**15**).

These sulfates and glucuronides were separated quantitatively by reversed-phase HPLC. The separation was performed with a mixture of acetate buffer (50 mM, pH 5.0) and methanol (50: 50) as the mobile phase on a column of ODS SIL. The eluates were monitored in terms of the absorbance at 280 nm. Calibration curves between the amounts of conjugated guaiacols and the peak heights on chromatograms were all linear.

The results obtained by proposed HPLC method for the quantification of *O*-methylated products obtained by the incubation of **2** and/or **3** with purified rat liver catechol *O*-methyltransferase in the presence of (^3H_3C)-*S*-adenosyl-L-methionine were in good agreement with the results obtained by a different procedure, the reverse isotope dilution method.

Keywords——catechol estrogen; guaiacol estrogen; steroidal conjugate; sulfate; glucuronide; high-performance liquid chromatography; *O*-methylation; catechol-*O*-methyltransferase (COMT)

The biological significance of catechol estrogen formed by hydroxylation at C-2 of the female hormone estrogen is now well-known and the accumulated data have been reviewed.[2] Recently, we have demonstrated 2-hydroxylation of estradiol 17β-sulfate by rat liver microsomes.[3]

Catechol estrogens in the body are metabolized by such conjugations as *O*-methylation, sulfation, glucuronidation, and glutathionation.[2c] Of these conjugations, *O*-methylations by the enzyme catechol *O*-methyltransferase (COMT) is important metabolically, because catechol estrogens act as strong competitive inhibitors for *O*-methylation of neurotransmitter substances, catecholamines.[4]

A previous report from our laboratory showed the directive effect of 17β-conjugate groups on enzymatic *O*-methylation of 17β-conjugates of 2-hydroxyestradiol (**1**), in that *O*-methylation of the C-2 phenolic hydroxyl group of potassium 2,3-dihydroxyestra-1,3,5(10)-trien-17β-yl sulfate (**2**) and potassium [2,3-dihydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid] uronate (**3**) exceeded those of C-3.[5] In these *O*-methylation studies, however, the reverse isotope dilution method employed was not satisfactory for the following reasons. Namely, the method: 1) is time-consuming, especially in the stage of hydrolysis, 2) requires large amounts of valuable carrier steroids, 3) uses expensive radioactive materials. Further, 4) the separation of guaiacols (**4** and **10**) is tedious and sometimes unsatisfactory as regards complete resolution.[6]

Therefore, it became necessary to establish direct assays for conjugated guaiacol estrogens without removal of the 17β-conjugate groups.

The present paper deals with the preparation of 17β-conjugated guaiacol estrogens as authentic materials, and also with the establishment of a quantitative assay method for these conjugates by high-performance liquid chromatography (HPLC). We have compared two different procedures, the reverse isotope dilution method and the present HPLC method, for the quantitative analyses of *O*-methylated products obtained from **2** and **3** with purified rat liver COMT in the presence of (^3H_3C)-*S*-adenosyl-L-methionine.

Preparation of Guaiacol Estrogen 17β-Conjugates

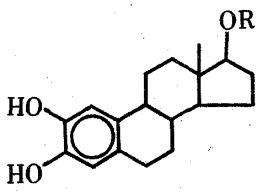
Schotten–Baumann reaction of 2-methoxyestra-1,3,5(10)-trien-3,17β-diol (2-methoxyestradiol), **4** with benzoyl chloride gave quantitatively 3-benzoyloxy-2-methoxyestra-1,3,5(10)-trien-17β-ol (**5**), which was treated with sulfur trioxide–pyridine complex in dry pyridine, followed by neutralization with potassium hydroxide to yield potassium 3-benzoyloxy-2-methoxyestra-1,3,5(10)-trien-17β-yl sulfate (**6**). Saponification of **6** with methanolic potassium hydroxide afforded a desired sulfate, potassium 3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl sulfate (**7**).

By means of the same reaction using benzoyl chloride, the isomeric monomethyl ether, 2-hydroxy-3-methoxyestra-1,3,5(10)-trien-2,17β-diol (**10**), was converted to the corresponding benzoate, 2-benzoyloxy-3-methoxyestra-1,3,5(10)-trien-17β-ol (**11**), which was derived to potassium 2-benzoyloxy-3-methoxyestra-1,3,5(10)-trien-17β-yl sulfate (**12**) by the same procedure as described above. Treatment of **12** in methanolic potassium hydroxide afforded the guaiacol sulfate, potassium 2-hydroxy-3-methoxyestra-1,3,5(10)-trien-17β-yl sulfate (**13**).

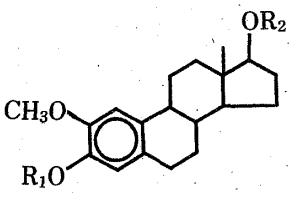
The structures of these sulfates were confirmed by instrumental analyses and also by mild solvolysis according to the method of Burstein and Liebermann,[7] by which the sulfates **7** and **13** gave only the corresponding intact steroids, **4** and **10**, respectively.

The isomeric glucuronides of the above guaiacols were prepared by the following procedures. Koenigs–Knorr reaction of **5** with methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate in the presence of silver carbonate in dry benzene gave methyl [3-benzoyloxy-2-methoxyestra-1,3,5(10)-trien-17β]-yl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (**8**). Saponification of **8** gave a desired glucuronide, potassium [3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid]uronate (**9**).

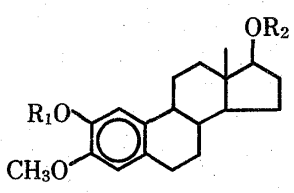
The same procedure was applied to the preparation of isomeric glucuronide. The benzoate



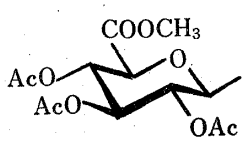
1: R=H
2: R=SO_3K
3: R=glucuronyl



4: R_1=H, R_2=H
5: R_1=PhCO, R_2=H
6: R_1=PhCO, R_2=SO_3K
7: R_1=H, R_2=SO_3K
8: R_1=PhCO, R_2=glucuronyl′
9: R_1=H, R_2=glucuronyl



10: R_1=H, R_2=H
11: R_1=PhCO, R_2=H
12: R_1=PhCO, R_2=SO_3K
13: R_1=H, R_2=SO_3K
14: R_1=PhCO, R_2=glucuronyl′
15: R_1=H, R_2=glucuronyl

glucuronyl′= 

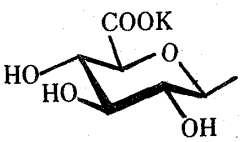
glucuronyl= 

Chart 1

(**11**) by Koenigs–Knorr reaction was converted to methyl [2-benzoyloxy-3-methoxyestra-1,3,5-(10)-trien-17β]-yl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (**14**), saponification of which gave potassium [2-hydroxy-3-methoxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid]uronate (**15**) as a final compound.

The structures of these glucuronides were confirmed by their instrumental analyses and the following enzymatic experiments. On hydrolysis by β-glucuronidase, the glucuronides **9** and **15** gave quantitatively their aglycones **4** and **10**, respectively. The mass spectral fragmentation patterns of these hydrolyzates were identical with those of the authentic specimens. This hydrolysis was strongly inhibited by addition of saccharo-1,4-lactone to the incubation media.

Separation and Quantification of Guaiacol Estrogen 17β-Conjugates by High-Performance Liquid Chromatography

The separation and quantification of two pairs of guaiacol estrogen 17β-conjugates were investigated by HPLC using a reversed-phase column. In our studies on the *O*-methylation of **2** and **3**, the separation of steroids may be sufficient for each group, namely for the substrate **2** and a pair of products **7** and **13**, and similarly for **3**, **9**, and **15**.

Initially, the separation of each group using several kinds of solvents as the mobile phase was investigated. When a mixture of acetate buffer (50 mM, pH 5.0) and methanol (50: 50) was employed, the conjugates in each group were separated satisfactorily as shown in Fig. 1. In this case, the column was maintained at 40°C, the best condition among those investigated, because the separation of each conjugate was considerably influenced by temperature.

Secondly, the quantification of these conjugates was investigated under the above conditions. The calibration curves between the amounts of guaiacols (**7**, **9**, **13**, and **15**) injected and their peak heights on the chromatograms were all linear as shown in Fig. 2.

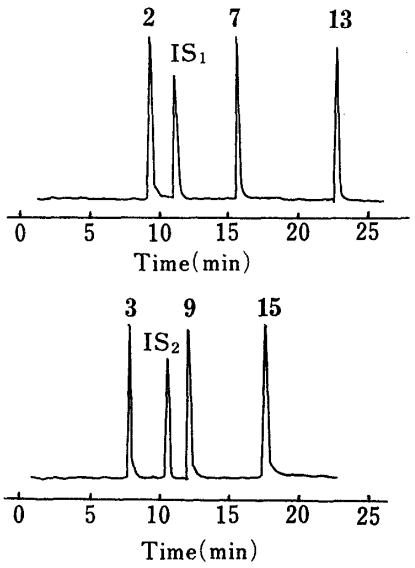


Fig. 1. Liquid Chromatographic Separation of 2-Hydroxyestradiol 17β-Sulfate (**2**) and 2-Hydroxyestradiol 17β-Glucuronide (**3**) and Their Mono *O*-Methylated Products (**7**, **13**, **9**, and **15**) with Estradiol 17β-Sulfate (IS_1) and Estradiol 17β-Glucuronide (IS_2) as Internal Standards

Conditions: TSK Gel LS 410 ODS SIL (5 μm) column at 40°C; mobile phase, acetate buffer (50 mM, pH 5.0) and methanol (50: 50), 110 kg/cm^2, 0.9 ml/min; detection, Uvidec UV detector (280 nm).

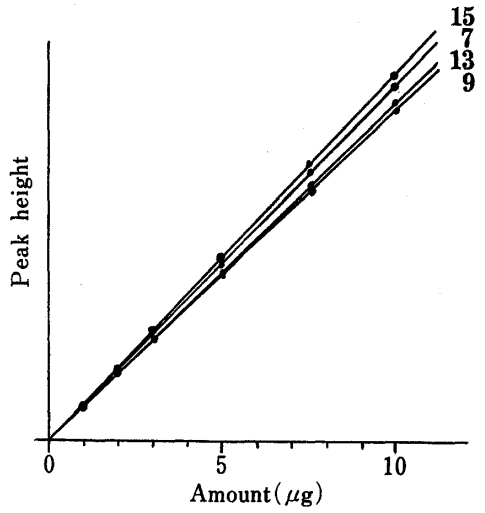


Fig. 2. Calibration Curves for Guaiacol Estrogen 17β-Sulfates (**7** and **13**) and 17β-Glucuronides (**9** and **15**)

Each plotted point is the mean of eight experiments. HPLC: the same conditions as described in the legend to Fig. 1.

High-Performance Liquid Chromatographic Determination of 17β-Conjugated Guaiacols formed from 2-Hydroxyestradiol 17β-Conjugates by Enzymatic *O*-Methylation

By using the above procedure, *O*-methylated products of **2** and **3** have been determined by HPLC.

First, we examined the recovery of each steroid from the incubation media. When the conjugates were extracted from the media by using Amberlite XAD-2 resin according to the method by Bradlow,[8] the recoveries were low (less than 50%). Therefore, the conjugates were extracted with *n*-butanol. Because 2- and 3-*O*-methyl products obtained by enzymatic *O*-methylation of conjugated catechols **2** and/or **3** are formed in different amounts, the recoveries of conjugates were tested at three different concentration levels. As shown in Table I, about 80% of guaiacol sulfates (**7** and **13**) were recovered when they were added to the incubation medium in amounts from 30 to 300 μg, while about 90% recovery was obtained in the case of glucuronides (**9** and **15**). Thus the mean recovery values of the conjugates were used for correction of the results obtained in the enzymatic *O*-methylation studies in the next experiments.

We next determined the yields of *O*-methylated products of **2** and **3** by the above method and compared the results with those obtained by the reverse isotope dilution method. The incubation of **2** and **3** with purified rat liver COMT in the presence of (^3H_3C)-*S*-adenosyl-L-

Table I. Recoveries of Guaiacol Estrogen 17β-Conjugates added to Incubation Mixtures containing Purified Rat Liver Catechol *O*-Methyltransferase

Conjugate	Added (μg)	Found[a] (μg)	Recovery (% ± S.D.)	Mean (% ± S.D.)
7	30.0	24.4±1.0	81.3±3.3	
	100	83.5±2.9	83.5±2.9	82.8±3.1
	300	250 ±9.0	83.3±3.0	
13	30.0	23.7±0.7	79.0±2.3	
	100	80.3±2.8	80.3±2.8	80.1±2.8
	300	243 ±9.6	80.9±3.2	
9	30.0	27.1±0.9	90.3±3.0	
	100	94.5±2.0	94.5±2.0	92.7±2.6
	300	279 ±8.1	93.0±2.7	
15	30.0	26.6±0.9	88.7±2.7	
	100	90.0±2.2	90.0±2.2	90.8±2.5
	300	281 ±7.8	93.7±2.6	

a) No detectable amounts of guaiacol estrogen 17β-conjugates were contained in steroid-free microsomes.

Table II. Comparison of the Yields and the Ratios of 2- and 3-*O*-Methyl Products of 2-Hydroxyestradiol 17β-Sulfate (**2**) and 2-Hydroxyestradiol 17β-Glucuronide (**3**) obtained by Two Different Procedures with Purified Male Rat Liver Catechol *O*-Methyltransferase[a]

Substrate	Method		Metabolites (%) 2-MeO-	Metabolites (%) 3-MeO-	Ratio (2-MeO-/3-MeO-)
2	RID	exp. 1	73.25 (644700 dpm)	20.05 (176500 dpm)	3.65
		2	78.35 (689400 dpm)	19.60 (172800 dpm)	3.99
	HPLC	exp. 1	70.2	17.6	4.0
		2	74.6	18.3	4.1
3	RID	exp. 1	50.20 (441200 dpm)	11.20 (98700 dpm)	4.50
		2	44.35 (390200 dpm)	9.25 (81300 dpm)	4.80
	HPLC	exp. 1	48.8	10.1	4.8
		2	42.2	9.3	4.5

a) RID, reverse isotope dilution method; HPLC, high-performance liquid chromatography.

methionine and Mg^{2+} yielded the corresponding *O*-methylated products, half of which was used for assay by HPLC, while the other half was analyzed by the reverse isotope dilution method as described previously.[5] The results obtained by the two different procedures were in good agreement, as shown in Table II.

Thus, in *O*-methylation studies of conjugated catechol estrogens, the reverse isotope dilution method can now be replaced by the present HPLC method, which offers greater speed and convenience, and similar accuracy.

Experimental

Melting points were determined on a micro-hot stage (Mitamura) and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a JNM-PMX-60 spectrometer (JEOL) at 60 MHz and chemical shifts are expressed relative to 1% tetramethylsilane as an internal standard. Abbreviations used are s=singlet, d=doublet, t=triplet, and m=multiplet. Infrared spectra (IR, ν_{max}) in KBr disks were recorded on a JASCO IR-2 (Nihon Bunko) and are given in cm^{-1}. Mass spectra (MS) were taken by the direct insertion method with a 9000 B (Shimadzu). Ultraviolet (UV) spectra were measured in ethanol with a model 200-20 spectrometer having an X-Y recorder (Hitachi) and are expressed as ν_{max} nm (ε), sh=shoulder. High-performance liquid chromatography was carried out on a model 803 chromatograph (Toyo Soda) with a column (30 cm × 0.4 cm, i.d.) packed with TSK-Gel LS 410 ODS SIL (5μm) (Toyo Soda). Uvidec UV detector (Nihon Bunko) was used for monitoring the absorbance at 280 nm. The temperature of the column was maintained at 40°C in a circulating water bath. Radioactivity countings were carried out on a Packard Tri-Carb 2650 liquid scintillation spectrometer. Toluene containing 6 g/l of 2,5-diphenyloxazole and 300 mg of 1,4-bis(5-phenyloxazol-2-yl)benzene was used as a scintillator. Aqueous samples were counted in Bray's system.

The steroidal materials, **1**, **4**, and **10** were prepared by the reported method.[9] The internal standards, potassium 3-hydroxyestra-1,3,5(10)-trien-17β-yl sulfate and potassium [3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid] uronate, were prepared in this laboratory from estradiol 3-benzoate by the method described in this paper. The conjugates, 2-hydroxyestradiol 17β-sulfate (**2**) and -glucuronide (**3**) were obtained in this laboratory by the reported method.[10] The enzyme β-glucuronidase (calf liver) was obtained commercially from Tokyo Zohki. For the determination of **4** and **10** in the enzymatic hydrolysis, a commercial kit (E_3-kit, Teikoku Hormone) composed of Amberlite XAD-2 resin and Kober reaction reagents was used. Silica gel used for column chromatography was Merck Kieselgel 60 (70—230 mesh, Art., 7734). Thin-layer chromatography was performed with Merck precoated Silica gel 60 F_{254} plates. Preparative thin-layer chromatographic plates were made from Wako gel B-5F (Wako Chemicals) in the usual way, with a thickness of 0.75 mm. Male rats (Wistar) weighing 200—250 g were used.

3-Benzoyloxy-2-methoxyestra-1,3,5(10)-trien-17β-ol (**5**)——To a stirred solution of **4** (1.85 g) in 10% NaOH (400 ml) was added dropwise 30 ml of 50% benzoyl chloride in ether, and the mixture was stirred at 20°C. After 4 h, the solution was neutralized by adding 3 N HCl and was adjusted to pH *ca.* 8 by adding $NaHCO_3$, followed by stirring overnight at room temperature. The solution was extracted with benzene and the combined extract was washed with 0.1 N KOH and water, dried over anhydrous Na_2SO_4, and concentrated. Crude benzoate was obtained (2.36 g), which was recrystallized from methanol to give **5** (1.20 g), mp 199.0—202.0°C. *Anal.* Calcd for $C_{26}H_{30}O_4$: C, 76.82; H, 7.44. Found: C, 76.51; H, 7.37. IR: 3355 (OH), 2835 (CH), 1718 (COO), 1603 (arom.). UV: 229.1 (20400), 281.2 (4380). NMR ($CDCl_3$) δ: 8.3—7.3 (5H, m, PhCOO), 6.94 (1H, s, C_1–H), 6.85 (1H, s, C_4–H), 3.78 (3H, s, OCH_3), 3.66 (1H, triplet-like, 17α-H), 0.79 (3H, s, 18-CH_3).

Potassium 3-Benzoyloxy-2-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (**6**)——A pyridine solution (150 ml) containing **5** (1.56 g) and sulfur trioxide-pyridine complex (6 g) was stirred for 3 d at room temperature. Pyridine was removed under reduced pressure at 50°C, and the resultant residue was dissolved in 0.5 N KOH (50 ml). The solution was extracted with benzene, followed by extraction with *n*-butanol. The butanol extract was washed with water and concentrated to give a powder (1.74 g), which was recrystallized from a mixture of ethanol and methanol to give **6** (0.88 g), mp 260.0—260.5°C. *Anal.* Calcd for $C_{26}H_{29}KO_7S$: C, 59.52; H, 5.57; S,6.11. Found: C, 58.84; H, 5.62; S, 6.05. IR: 2850 (CH), 1742 (COO), 1623 (arom.), 1220 (OSO_3). UV: 228.5 (18800), 282.5 (4130). NMR (DMSO-d_6) δ: 8.2—7.4 (5H, m, PhCOO), 7.01 (1H, s, C_1–H), 6.86 (1H, s, C_4–H), 4.15 (1H, t, J=6.0 Hz, 17α-H), 3.74 (3H, s, OCH_3), 0.76 (3H, s, 18-CH_3).

Potassium 3-Hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (**7**)——A methanolic solution of **6** (1.43 g in 150 ml) was treated with 150 ml of 0.2 N KOH, and the mixture was allowed to stand overnight at room temperature. After removal of methanol under reduced pressure at 50°C, the aqueous solution was extracted with *n*-butanol (30 ml × 5). The combined extract was washed with water and concentrated to give a crude product (610 mg), recrystallization of which from ethereal methanol afforded **7** as fine needles (390 mg), mp 209.5—212.0°C. *Anal.* Calcd for $C_{19}H_{25}KO_6S \cdot 1/2H_2O$: C, 53.12; H, 6.10; S, 7.47. Found:

C, 53.37; H, 6.36; S, 7.11. IR: 3211 (OH), 2839 (CH), 1623 (arom.), 1220 (OSO_3). UV: 215.2 (7250), 224.6 (6520, sh), 287.4 (4000). NMR (DMSO-d_6) δ: 6.77 (1H, s, C_1–H), 6.45 (1H, s, C_4–H), 4.12 (1H, t, J=6.0 Hz, 17α-H), 3.73 (3H, s, OCH_3), 0.71 (3H, s, 18-CH_3).

Methyl [3-Benzoyloxy-2-methoxyestra-1,3,5(10)-trien-17β-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (8)——Methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (2 g) and freshly prepared silver carbonate (5 g) were added to a solution of **5** (1.9 g) in 150 ml of benzene, and the mixture was stirred in the dark place for 5 d at room temperature. Additional reagent (1 g) was added to the reaction mixture every day. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give an oily product (6.1 g), which was subjected to silica gel column chromatography (2.5 cm, i.d.). From the eluate obtained with 50% chloroform in benzene, crystalline **8** (1.72 g) was obtained, and recrystallized from methanol to give **8** (1.10 g), mp 188.0—193.0°C. *Anal.* Calcd for $C_{39}H_{46}O_{13}$: C, 64.81; H, 6.41. Found: C, 64.95; H, 6.30. IR: 2930 (CH), 1760—1740 (COO), 1615 (arom.). UV: 228.6 (22500), 281.7 (4900). NMR ($CDCl_3$) δ: 8.3—7.3 (5H, m, PhCOO), 6.93 (1H, s, C_1–H), 6.86 (1H, s, C_4–H), 3.80 (6H, s, $COOCH_3$ and OCH_3), 2.08 and 2.04 (3H and 6H, respectively, both s, CH_3COO), 0.78 (3H, s, 18-CH_3). The multiplet peaks at 5.3—5.0 (3H), 4.7—4.5 (1H), 4.1—3.9 (1H), and 3.7—3.5 (1H) corresponding to the protons of the sugar moiety and 17α could not be assigned.

Potassium [3-Hydroxy-2-methoxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid]uronate (9)——A solution of **8** (1.3 g) in 150 ml of 0.2 N KOH in 90% methanol was allowed to stand overnight at room temperature. The mixture was concentrated under reduced pressure at 50°C to *ca.* 20 ml of aqueous solution, which was dissolved in 100 ml of *n*-butanol. The butanol solution was washed with water, and concentrated to give a residue (312 mg). Recrystallization of the material from aq. methanol afforded **9** as fine needles (119 mg), mp 275.0°C (dec.). *Anal.* Calcd for $C_{25}H_{33}KO_9 \cdot H_2O$: C, 56.17; H, 6.60. Found: C, 56.42; H, 6.60. IR: 3375 (OH), 2920 and 2840 (CH), 1598 (arom). UV: 214.2 (6850), 223.6 (6000, sh), 288.2 (3850). NMR (DMSO-d_6) δ: 6.75 (1H, s, C_1–H), 6.44 (1H, s, C_4–H), 3.51 (3H, s, OCH_3), 0.78 (3H, s, 18-CH_3). The multiplet peaks at 5.1—5.0, 4.4—4.2, 3.9—3.7, and 3.6—3.4 attributable to the protons of sugar moiety and 17α could not be assigned exactly.

2-Benzoyloxy-3-methoxyestra-1,3,5(10)-trien-17β-ol (11)——By the same procedure as described above, 2.84 g of the crude benzoate was obtained from 2.35 g of **10**, and recrystallization of the product from methanol gave **11** (1.90 g) as plates, mp 127.0—128.5°C and 167.0—168.5°C. *Anal.* Calcd for $C_{26}H_{30}O_4$: C, 76.82; H, 7.44. Found: C, 76.68; H, 7.52. IR: 3310 (OH), 2940 and 2860 (CH), 1737 (COO), 1605 (arom.). UV: 229.0 (23000), 281.0 (4800). NMR ($CDCl_3$) δ: 8.3—7.3 (5H, m, PhCOO), 7.03 (1H, s, C_1–H), 6.70 (1H, s, C_4–H), 3.77 (3H, s, OCH_3), 3.8—3.6 (1H, m, 17α-H), 0.77 (3H, s, 18-CH_3).

Potassium 2-Benzoyloxy-3-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (12)——By the same procedure as described above, was obtained 242 mg of the crude sulfate from 200 mg of **11** by using sulfur trioxide–pyridine complex (1 g) in pyridine (20 ml). The product was recrystallized from ethereal methanol to give **12** (130 mg), mp 210.0—215.0°C. *Anal.* Calcd for $C_{26}H_{29}KO_7S$: C, 59.52; H, 5.57; S, 6.11. Found: C, 59.76; H, 5.44; S, 5.98. IR: 3500—3400 (OH), 2920 (CH), 1730 (COO), 1610 (arom.), 1265—1245 (OSO_3). UV: 227.8 (11800), 282.6 (3960). NMR (DMSO-d_6) δ: 8.3—7.3 (5H, m, PhCOO), 7.10 (1H, s, C_1–H), 6.87 (1H, s, C_4–H), 4.13 (1H, t, J=6.0 Hz, 17α-H), 3.73 (3H, s, OCH_3), 0.73 (3H, s, 18-CH_3).

Potassium 2-Hydroxy-3-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (13)——The sulfate **12** (230 mg) was dissolved in 0.1 N KOH in 50% methanol (100 ml), and the mixture was allowed to stand overnight at room temperature. The mixture was concentrated under reduced pressure at 50°C to *ca.* 50 ml of aq. solution, which was extracted with *n*-butanol (20 ml×5). The combined extract was recrystallized from ethereal methanol to yield **13** (139 mg) as fine needles, mp 204.5°C (dec.). *Anal.* Calcd for $C_{19}H_{25}KO_6S$: C, 54.26; H, 5.99; S, 7.63. Found: C, 54.11; H, 5.83; S, 7.70. IR: 3550—3450 (OH), 2930 (CH), 1620 (arom.), 1230 (OSO_3). UV: 224.2 (5980, sh), 287.6 (3880). NMR (DMSO-d_6) δ: 6.68 (1H, s, C_1–H), 6.54 (1H, s, C_4–H), 4.04 (1H, t, J=6.0 Hz, 17α-H), 3.69 (3H, s, OCH_3), 0.69 (3H, s, 18-CH_3).

Methyl [2-Benzoyloxy-3-methoxyestra-1,3,5(10)-trien-17β-yl]-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (14)——By the same procedure as described for the preparation of **8**, an oily product (6.0 g) was obtained from 1.85 g of **11**, and subjected to column chromatography (2.5 cm, i.d.) on silica gel (150 g). A crude material (2.20 g) was obtained from the fractions eluted with 10—40% chloroform in benzene, and recrystallization from aq. methanol yielded **14** (1.51 g) as fine needles, mp 113.5—116.5°C. *Anal.* Calcd for $C_{39}H_{46}O_{13}$: C, 64.81; H, 6.41. Found: C, 64.92; H, 6.48. IR: 2920 and 2850 (CH), 1758 (COO), 1615 (arom.). UV: 228.6 (25200), 281.8 (5200). NMR ($CDCl_3$) δ: 8.3—7.3 (5H, m, PhCOO), 7.02 (1H, s, C_1–H), 6.75 (1H, s, C_4–H), 3.80 (6H, s, $COOCH_3$ and OCH_3), 2.02 (9H, s, CH_3COO), 0.75 (3H, s, 18-CH_3). The multiplet peaks at 5.3—5.0 (3H), 4.6—4.5 (1H), 4.1—3.9 (1H), and 3.7—3.5 (1H) attributable to the protons of the sugar moiety and 17α could not be assigned exactly.

Potassium [2-Hydroxy-3-methoxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid]uronate (15)——Saponification of **14** (1.20 g) in 0.5 N KOH in 90% methanol (120 ml) was carried out overnight at room temperature. The same treatment as described for **9** was applied to give the crude product (0.62 g), which was recrystallized from methanol to yield **15** (209 mg) as fine needles, mp 244.0°C (dec.). *Anal.* Calcd for $C_{25}H_{33}KO_9 \cdot H_2O$: C, 56.17; H, 6.60. Found: C, 56.48; H, 6.85. IR: 3410 (OH), 2830 (CH), 1610 (arom.). UV: 213.8 (6400), 223.8 (4980, sh), 288.2 (4060). NMR (DMSO-d_6) δ: 6.66 (1H, s, C_1–H), 6.55 (1H, s, C_4–H), 3.69 (3H, s, OCH_3),

0.78 (3H, s, 18-CH_3). Protons of the sugar moiety and at 17α could not be assigned.

Solvolysis of 7 and 13——About 5 mg each of **7** and **13** were dissolved in 10% H_2SO_4 (20 ml) and the solutions were extracted continuously with ether for 72 h. The extracts were washed with water, dried over anhydrous Na_2SO_4, and concentrated. Each oily product obtained showed a single spot on thin-layer chromatography when developed with the solvent systems, EtOAc–MeOH (4: 1) and benzene–acetone (10: 1). The MS of each product gave a molecular ion peak at *m/e* 302 and their fragmentation patterns were identical with those of the authentic steroids, **4** and **10**, respectively.

Enzymatic Hydrolysis of 9 and 15——β-Glucuronidase (final activity, 620 Fishman unit/ml) was added to acetate buffer solutions (10 ml, 100 mM, pH 4.9) containing an exact amount (*ca.* 2 mg) of **9** and **15**. The solutions were divided into two equal aliquots, to one of which was added 6 mg of saccharo-1,4-lactone as an inhibitor. These solutions were allowed to stand for 72 h at 37°C, followed by continuous extraction with ether for 48 h. The extracts were washed with water, dried, and concentrated. Aliquots of each product were assayed with the E_3-kit and the structural assignment was checked by MS.

In the absence of inhibitor, 91% of **9** was hydrolyzed to yield **4**. The MS of the hydrolyzate showed the parent ion *m/e* 302. The aqueous layer gave a positive naphthoresorcine reaction. In the presence of inhibitor, only 18% of **9** was hydrolyzed, and the MS of the aglycone still showed *m/e* 302 (M^+). The naphthoresorcine reaction of the aqueous layer was weakly positive.

Similarly, in the case of **15**, 21% and 88% of the aglycone **10** was liberated from the conjugate with and without inhibitor. The hydrolyzates from both experiments showed *m/e* 302, and both aqueous layers gave a positive naphthoresorcine reaction.

High-Performance Liquid Chromatography——In the separation work, standard solutions of each steroid (1000 μg) in methanol were prepared. From the standards, diluted solutions were prepared by dilution with methanol. Samples were injected into the chromatography with a microliter syringe (Hamilton). A mixture of acetate buffer (50 mM, pH 5.0) and methanol (50: 50) was used as the mobile phase at a flow rate of 0.9 ml/min, and a pressure of 110 kg/cm^2.

The amounts of steroids were determined by the peak-height method. The calibration curves between the amounts (μg) of conjugates (**7**, **9**, **13**, and **15**) and the peak heights (cm) on chromatograms were plotted (Fig. 2).

***In Vitro O*-Methylation of 2 and 3**——According to the previous paper,[5] 800 μg (2 μmol) of **2** and 900 μg (1.8 μmol) of **3** were incubated with purified male rat liver COMT (42 unit[11]) in the presence of (^3H_3C)-*S*-adenosyl-L-methionine (3.9×10^6 dpm/8.8 μmol) and Mg^{2+} in phosphate buffer (100 mM, pH 7.2) under a nitrogen stream for 3 h at 37°C. The reactions were stopped by heating the reaction vessels in boiling water for 1 min. After being diluted with 20 ml of water, the incubation mixtures were divided into two equal aliquots, one of which was extracted with *n*-butanol (5 ml $\times$ 5). The combined extracts were washed once with water and concentrated under reduced pressure below 50°C, followed by addition of estradiol 17β-sulfate or 17β-glucuronide, respectively, as an internal standard. The residues obtained were employed for HPLC assays as methanolic solutions.

The other halves of the incubation mixtures were used for quantifications by the reverse isotope dilution method. The incubation mixtures of **2** were converted to 10% H_2SO_4 solutions (*ca.* 50 ml), to which steroidal carriers **4** and **10** (*ca.* 30 mg each) were added, and the mixtures were extracted continuously with ether for 72 h.

After being diluted with the same steroidal carriers, the incubation mixtures of **3** were incubated with β-glucuronidase under the same conditions as described above, followed by continuous extraction with for 48 h.

Both extracts (from **2** and **3**) were washed with water, dried and concentrated. The oily products were subjected to preparative thin-layer chromatography using a mixture of benzene and acetone (98: 2) by continuous development. The identity of the separated materials was confirmed by NMR, and the products were acetylated in the usual way. Recrystallization of each acetate from petroleum ether to constant specific radioactivity was carried out.

Recovery Test——Exact amounts (30, 100, and 300 μg) of conjugates, **7**, **9**, **13** and **15**, were dissolved in the incubation medium (2 ml) as described. Each solution was then heated in boiling water for 1 min, and diluted with 10 ml of water. These solutions were extracted with *n*-butanol (5 ml $\times$ 5) and the combined extracts were washed once with water. Estradiol 17β-sulfate or 17β-glucuronide was added as an internal standard, and the mixture were concentrated under reduced pressure below 50°C to give residues, which were used for assays by HPLC as methanolic solutions. Six experiments were done on each compound.

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