[Chem. Pharm. Bull. 34( 5 )2231—2235(1986)]

# Synthesis and High-Performance Liquid Chromatography of 3,4-Guaiacol Estrogen 17-Sulfates[1)]

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(Received October 11, 1985)

For studies on the enzymatic *O*-methylation of 3,4-dihydroxyestra-1,3,5(10)-trien-17$\beta$-ol 17-sulfate (Ie), its two monomethyl ethers, 4-hydroxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-ol 17-sulfate (IId) and 3-hydroxy-4-methoxyestra-1,3,5(10)-trien-17$\beta$-ol 17-sulfate (IIId), were prepared as authentic specimens.

An assay procedure for these guaiacols including the isomeric sulfates, 3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17$\beta$-ol 17-sulfate (Ic) and 2-hydroxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-ol 17-sulfate (Id), was established by using reversed-phase high-performance liquid chromatography. The devised technique involves elution with a mixture of 0.5% $NH_4H_2PO_4$ (pH 3.0) and methanol (55 : 45, v/v) on a TSK gel ODS-120A column and monitoring with an electrochemical detector.

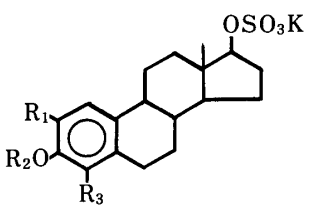
**Keywords**——*O*-methylation of catechol estrogen; guaiacol estrogen; estrogen sulfate; HPLC; electrochemical detection

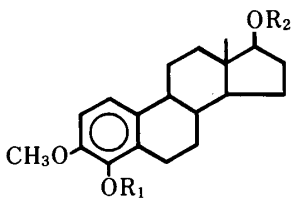
The main route of metabolism of the female hormone estrogen in humans and in laboratory animals is oxidation at the *ortho* positions of the C-3 phenolic hydroxyl group to produce 2- and 4-hydroxylated metabolites, which are members of the group of catechol estrogens. Catechol estrogens are formed not only in the liver, which is the most active site for their production, but also in the brain, kidney and other organs.[2)]

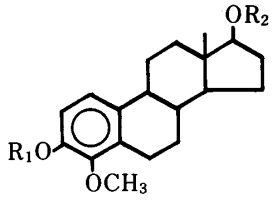
Endogenous catechol estrogens are in part *O*-methylated in the body by the enzyme catechol *O*-methyltransferase (COMT) to give the corresponding guaiacol estrogens. For example, 2-hydroxyestradiol, a representative catechol estrogen, as well as 2-hydroxyestrone, is transformed by *O*-methylation to the 2,3-guaiacols, 3-hydroxy-2-methoxyestra-1,3,5(10)-trien-17$\beta$-ol and 2-hydroxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-ol. This is also the case for another type of catechol estrogen, 3,4-dihydroxyestra-1,3,5(10)-trien-17$\beta$-ol, which is converted to the 3,4-guaiacols, 4-hydroxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-ol (IIa) and 3-hydroxy-4-methoxyestra-1,3,5(10)-trien-17$\beta$-ol (IIIa).[3)]

Previously, we have demonstrated that estradiol 17-sulfate (Ia) is metabolized by microsomal enzymes in various rat organs, including the liver, to produce 2-hydroxyestradiol 17-sulfate (Ib).[4)]Further, we have shown a directive effect of the 17-sulfate group upon the *O*-methylation of Ib by COMT, in that *O*-methylation of the C-2 phenolic hydroxyl group exceeded that of the C-3 group.[5)] Recently, we have observed the 4-hydroxylation of Ia by rat hepatic microsomes to produce the 3,4-catechol estrogen, 4-hydroxyestradiol 17-sulfate (Ie).[6)]

To elucidate the entire metabolic pattern of Ia, it became necessary to investigate the enzymatic *O*-methylation of Ie. The present paper describes our synthetic method for the preparation of two isomeric 3,4-guaiacol estrogen 17-sulfates (IId and IIId) as authentic compounds, and also describes a method for assay of these sulfates including their isomers (Ic and Id) by high-performance liquid chromatography (HPLC) using an electrochemical detector (ECD).




| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Ia: | $R_1=H$ | $R_2=H$ | $R_3=H$ | IIa: | $R_1=H$ | $R_2=H$ | IIIa: | $R_1=H$ | $R_2=H$ |
| Ib: | $R_1=OH$ | $R_2=H$ | $R_3=H$ | IIb: | $R_1=Ph\cdot CH_2$ | $R_2=H$ | IIIb: | $R_1=Ph\cdot CH_2$ | $R_2=H$ |
| Ic: | $R_1=OCH_3$ | $R_2=H$ | $R_3=H$ | IIc: | $R_1=Ph\cdot CH_2$ | $R_2=SO_3K$ | IIIc: | $R_1=Ph\cdot CH_2$ | $R_2=SO_3K$ |
| Id: | $R_1=OH$ | $R_2=CH_3$ | $R_3=H$ | IId: | $R_1=H$ | $R_2=SO_3K$ | IIId: | $R_1=H$ | $R_2=SO_3K$ |
| Ie: | $R_1=H$ | $R_2=H$ | $R_3=OH$ | | | | | | |

Chart 1

## 1) Synthesis of 3,4-Guaiacol Estrogen 17-Sulfates

Benzylation of 4-hydroxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-ol (IIa) using benzyl chloride and anhydrous potassium carbonate in refluxing ethanol gave 4-benzyloxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-ol (IIb) in 50% yield. Treatment of IIb with sulfur trioxide–pyridine complex in dry pyridine, followed by treatment with ion exchange resin, gave potassium 4-benzyloxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-yl sulfate (IIc) quantitatively. Hydrogenolysis of IIc on palladium-on-charcoal gave the desired compound, potassium 4-hydroxy-3-methoxyestra-1,3,5(10)-trien-17$\beta$-yl sulfate (IId). The overall yield from the starting material (IIa) was about 27%.

By means of the same reaction as described for IIb, the isomeric monomethyl ether (IIIa)[7] was converted to the corresponding derivative, 3-benzyloxy-4-methoxyestra-1,3,5(10)-trien-17$\beta$-ol (IIIb). Similarly, this compound was derived to the 17-sulfate, potassium 3-benzyloxy-4-methoxyestra-1,3,5(10)-trien-17$\beta$-yl sulfate (IIIc). Hydrogenolysis of IIIc gave potassium 3-hydroxy-4-methoxyestra-1,3,5(10)-trien-17$\beta$-yl sulfate (IIId), the overall yield of which from IIIa was about 30%.

The structures of these synthetic compounds were confirmed by elemental analyses and examination of the ultraviolet (UV), infrared (IR), $^1$H-nuclear magnetic resonance (NMR), and mass spectra. The homogeneity of the sulfates was confirmed by the finding that IId and IIId on mild solvolysis[8] gave IIa and IIIa, respectively, as the sole products.

## 2) Separation and Quantification of Guaiacol Estrogen 17-Sulfates by HPLC

The separation and quantification of guaiacol estrogen 17-sulfates were investigated by HPLC using a reversed-phase column. For the quantification of the *O*-methylated products of Ib and Ie, complete separation of these catechols and their guaiacols is necessary.

Initially, the separation of these sulfates by using several kinds of solvents as the mobile phase was investigated. When a mixture of 0.5% $NH_4H_2PO_4$ (pH 3.0)–methanol (55 : 45, v/v) was employed, all the conjugates were separated satisfactorily as shown in Fig. 1

The development of a method for the quantification of guaiacols was then undertaken. The calibration curves for IId and IIId were constructed by plotting the peak height ratios of guaiacols with respect to the internal standard (estradiol 17-sulfate, Ia) against the amount of the former, and satisfactory linearities were obtained in the appropriate ranges. The detection limits of IId and IIId (injected amount) were 1 and 0.5 ng, respectively (S/N: about 3). Similar results were obtained for other guaiacols (Ic and Id).

In order to confirm the validity of the present method for determination of the guaiacols, recovery tests were carried out using authentic samples under the same conditions as used for *in vitro O*-methylation studies.[5] Known amounts of guaiacols were added to the incubation medium, and the steroids recovered through the whole clean-up procedure were determined

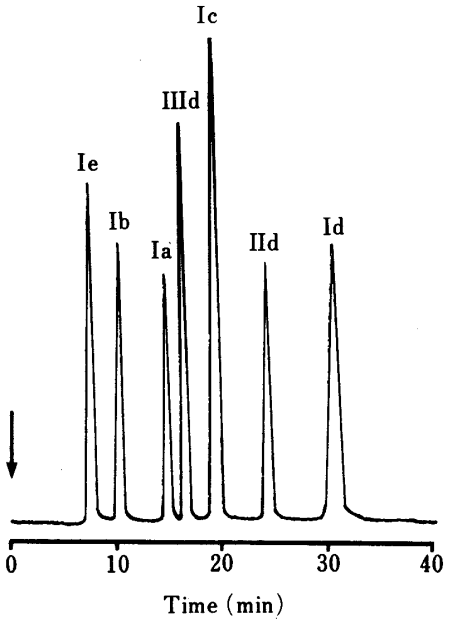



Fig. 1. HPLC Showing the Separation of Catechol Estrogen 17-Sulfates and Their Monomethylated Products

Ia: estradiol 17-sulfate as an internal standard.

TABLE I. Recoveries of Guaiacol Estrogen 17-Sulfates Added to the Incubation Medium Containing Rat Liver Catechol *O*-Methyltransferase

| Added ($\mu$g) | Recovery (%)[a] | | | |
|---|---|---|---|---|
| | Ic | Id | IId | IIId |
| 50 | 97.2±5.3 | 92.8±4.9 | 93.2±3.3 | 95.9±1.3 |
| 5 | 99.8±3.7 | 98.6±4.8 | 95.7±5.0 | 97.6±1.3 |
| 0.5 | 96.6±3.9 | 95.0±4.8 | 98.9±2.0 | 97.9±4.1 |

*a*) Mean±S.D. ($n$=6).

by HPLC. It is evident from the data in Table I that the conjugated guaiacols were recovered to a satisfactory extent.

Thus, a simple and highly sensitive analytical method for the determination of guaiacol estrogen 17-sulfates by using ECD/HPLC was established. This method should be extremely useful for enzymatic studies, especially kinetic work, on the *O*-methylation of conjugated estrogen catechols.

## Experimental

Melting points were determined on a micro-hot stage (Mitamura, Tokyo) and are uncorrected. UV spectra were measured in ethanol with a model 200-20 spectrometer equipped with X-Y type recorder (Hitachi, Tokyo) and data are given as $\lambda_{max}$ nm ($\varepsilon$). IR spectra ($\nu_{max}$) in KBr disks were recorded on a model IR-A-102 (Nihon Bunko, Tokyo) and are given in $cm^{-1}$. NMR spectra were recorded on a JNM-FX-100 spectrometer (JEOL,Tokyo) at 100 MHz and chemical shifts are expressed relative to 1% tetramethylsilane as an internal standard. Abbreviations used are s=singlet, d=doublet, t=triplet, m=multiplet, and br=broad. Mass spectra (MS) were taken by the direct insertion method with a 9000B machine (Shimadzu, Kyoto). HPLC was carried out on a model 803 chromatograph equipped with an EC-8 electrochemical detector at 0.9 V *vs.* the Ag/AgCl reference electrode (Toyo Soda, Tokyo). A stainless steel column (25 cm×4.6 mm, i.d.) packed with TSK gel ODS-120A (5 $\mu$m, Toyo Soda) was used, and maintained at 40 °C in a circulating water bath. The following solutions were used as mobile phases: system A, 0.5% $NH_4H_2PO_4$ (pH 3.0)–methanol (55:45, v/v); system B, 0.5% $NH_4H_2PO_4$ (pH 3.0)–acetonitrile (60:40, v/v). Systems A and B were used for the analyses of sulfates and free steroids, respectively.

**Steroids and Chemicals**——Steroidal compounds, Ia,[9] Ib,[10] Ic,[11] Id,[11] Ie,[6] IIa,[12] and IIIa,[7] were prepared in this laboratory according to the known methods. Other chemicals were reagent grade products obtained com-

mercially.

**4-Benzyloxy-3-methoxyestra-1,3,5(10)-trien-17β-ol (IIb)**——An ethanolic solution (20 ml) containing IIa (106 mg), benzyl chloride (0.23 ml) and anhydrous $K_2CO_3$ (250 mg) was refluxed on an oil bath for 4 h. After cooling, the reaction mixture was concentrated under reduced pressure to give a residue, to which was added 50 ml of water. The solution was extracted with benzene (3 × 30 ml), and the combined organic layer was washed with water (2 × 20 ml), dried over anhydrous $Na_2SO_4$, and concentrated. The crude material (126 mg) obtained was recrystallized from methanol to give colorless fine needles (68 mg), mp 78—80 °C. *Anal.* Calcd for $C_{26}H_{32}O_3$: C, 79.55; H, 8.22. Found: C, 79.95; H, 8.23. IR: 3350 (OH), 2950, 2850 (CH), 1600, 1490 (aromatic ring). NMR (chloroform-*d*) δ: 0.78 (3H, s, $C_{18}$-H), 3.70 (1H, br, $C_{17}$-H), 3.86 (3H, s, $OCH_3$), 4.97 (2H, s, Ph·$\underline{CH_2}$), 6.98 (1H, d, $J$=9 Hz, $C_2$-H), 7.04 (1H, d, $J$=9 Hz, $C_1$-H), 7.23—7.57 (5H, m, $\underline{Ph}$·$CH_2$). MS *m/z*: 392 ($M^+$), 301 ($M^+ - Ph\cdot CH_2$). UV: 209 (34500), 275 (1700), 283 (1600).

**Potassium 4-Benzyloxy-3-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (IIc)**——Freshly prepared sulfur trioxide–pyridine complex (30 mg) was added to a pyridine solution (4 ml) of IIb (67 mg), and the mixture was heated at 50 °C for 2 h, followed by stirring for 20 h at room temperature. Pyridine was removed under reduced pressure to give an oily product, which was dissolved in 5 ml of water. The solution was neutralized by addition of 0.1 N KOH, applied to a column (20 × 1 cm, i.d.) packed with Dowex 50W (× 8, 200—400 mesh, $K^+$ form), and eluted with water. The eluate was concentrated under reduced pressure below 50 °C to give a colorless powder (97 mg). Recrystallization from methanol afforded colorless fine needles (81 mg), mp 229—231 °C. *Anal.* Calcd for $C_{26}H_{31}KO_6S\cdot H_2O$: C, 59.06; H, 6.29, S, 6.06. Found: C, 58.85; H, 5.92; S, 5.80. IR: 3450 (OH), 2950, 2850 (CH), 1640—1600, 1490 (aromatic ring), 1240—1220 ($OSO_3K$). NMR (pyridine-$d_5$) δ: 0.96 (3H, s, $C_{18}$-H), 3.79 (3H, s, $OCH_3$), 4.15 (1H, t, $J$=8.5 Hz, $C_{17}$-H), 5.13 (2H, s, Ph·$\underline{CH_2}$), 6.91 (1H, d, $J$=9 Hz, $C_2$-H), 7.07 (1H, d, $J$=9 Hz, $C_1$-H), 7.20—7.50 (5H, m, $\underline{Ph}$·$CH_2$). UV: 210 (29000), 230 (8000, shoulder), 276 (1750), 283 (1650).

**Potassium 4-Hydroxy-3-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (IId)**——Absolute ethanol (20 ml) containing 65 mg of 10% Pd/C was stirred in hydrogen gas at atmospheric pressure for 1 h at room temperature. Then IIc (64 mg) was added, and the mixture was stirred under the same conditions for 6 h. Filtration of the catalyst, followed by the evaporation of the filtrate gave 41 mg of crude product, which was recrystallized from methanol to afford colorless fine needles (32 mg), mp 225 °C (dec.). *Anal.* Calcd for $C_{19}H_{25}KO_6S\cdot H_2O$: C, 52.03; H, 6.21; S; 7.31. Found: C, 51.84; H, 6.10; S, 7.48. IR: 3450 (OH), 2950, 2850 (CH), 1620, 1490 (aromatic ring), 1240 ($OSO_3K$). NMR (methanol-$d_4$) δ: 0.84 (3H, s, $C_{18}$-H), 3.81 (3H, s, $OCH_3$), 4.30 (1H, t, $J$=8.5 Hz, $C_{17}$-H), 6.72 (2H, s, $C_1$- and $C_2$-H). UV: 210 (30000), 222 (7700, shoulder), 278 (1780), 282 (1650).

**3-Benzyloxy-4-methoxyestra-1,3,5(10)-trien-17β-ol (IIIb)**——This compound was prepared from IIIa (352 mg) by using benzyl chloride (0.71 ml) and anhydrous $K_2CO_3$ (820 mg) in the same manner as described for IIb. The crude product (418 mg) obtained was recrystallized from methanol to give colorless fine needles (296 mg), mp 65—67 and 126—127 °C. *Anal.* Cald for $C_{26}H_{32}O_3$: C, 79.55; H, 8.22. Found: C, 79.76;H, 8.19. IR: 3450 (OH), 2940, 2860 (CH), 1650—1605, 1490 (aromatic ring). NMR (chloroform-*d*) δ: 0.78 (3H, s, $C_{18}$-H), 3.60 (1H, br, $C_{17}$-H), 3.85 (3H, s, $OCH_3$), 5.10 (2H, s, Ph·$\underline{CH_2}$). 6.78 (1H, d, $J$=8.5 Hz, $C_2$-H), 6.98 (1H, d, $J$=8.5 Hz, $C_1$-H), 7.25—7.50 (5H, m, $\underline{Ph}$·$CH_2$). MS *m/z*: 392 ($M^+$), 301 ($M^+ - Ph\cdot CH_2$). UV:206 (31000), 230 (8700, shoulder), 275 (1650), 283 (1400).

**Potassium 3-Benzyloxy-4-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (IIIc)**——The title compound was prepared from IIIb (195 mg) by using sulfur trioxide–pyridine complex (90 mg) in dry pyridine (12 ml) in the same manner as described for IIc. The crude product (239 mg) was recrystallized from methanol to give colorless fine needles (221 mg), mp 262—264 °C. *Anal.* Calcd for $C_{26}H_{31}KO_6\cdot H_2O$: C, 59.06; H, 6.29; S, 6.06. Found: C, 58.87; H, 6.35; S, 5.77. IR: 3450 (OH), 2900, 2850 (CH), 1630—1600, 1490 (aromatic ring), 1250—1220 ($OSO_3K$). NMR (methanol-$d_4$) δ: 0.84 (3H, s, $C_{18}$-H), 3.78 (3H, s, $OCH_3$), 4.31 (1H, t, $J$=8 Hz, $C_{17}$-H), 5.07 (2H, s, Ph·$\underline{CH_2}$), 6.82 (1H, d, $J$=9 Hz, $C_2$-H), 6.98 (1H, d, $J$=9 Hz, $C_1$-H), 7.20—7.50 (5H, m, $\underline{Ph}$·$CH_2$). UV: 212 (26000), 227 (10000, shoulder), 274 (1600), 282 (1400).

**Potassium 3-Hydroxy-4-methoxyestra-1,3,5(10)-trien-17β-yl Sulfate (IIId)**——This compound was prepared from IIIc (166 mg) by using 10% Pd/C (170 mg) in absolute ethanol (50 ml) in the same manner as described for IId. The crude product (127 mg) obtained was recrystallized from methanol to give colorless fine needles (75 mg), mp 232—234 °C. *Anal.* Calcd for $C_{19}H_{25}KO_6S\cdot 3/2H_2O$: C, 50.98; H, 6.30; S, 7.17. Found: C, 50.89; H, 6.26; S, 7.22. IR: 3450 (OH), 2950, 2850 (CH), 1630, 1490 (aromatic ring), 1250—1210 ($OSO_3K$). NMR (methanol-$d_4$) δ: 0.84 (3H, s, $C_{18}$-H), 3.73 (3H, s, $OCH_3$), 4.30 (1H, d, $J$=8 Hz, $C_{17}$-H), 6.62 (1H, d, $J$=9 Hz, $C_2$-H), 6.88 (1H, d, $J$=9 Hz, $C_1$-H). UV: 208 (17500), 222 (7500, shoulder), 279 (1600), 283 (1480).

**Solvolysis of IId and IIId**——About 5 mg of a sulfate (IId or IIId) was dissolved in 10 ml of 10% $H_2SO_4$ containing 1 mg of Ia (as an internal standard), and the solution was extracted continuously with ether for 72 h. The organic layer was washed with water, dried (anhydrous $Na_2SO_4$), and concentrated. The oily product obtained was dissolved in methanol (1 ml), and the solution was passed through a Column guard (Millipore Co., Mass, U.S.A.). The filtrates were subjected to HPLC using system B as a mobile phase at a flow rate of 1.1 ml/min.

**HPLC of Guaiacol Estrogen 17-Sulfates**——For the separation study, standard solutions of each steroid (1000 μg) in methanol (10 ml) were prepared. From the standards, diluted solutions were prepared by dilution with methanol. Mixtures of these solutions (10 ml, each sulfate 10—100 μg) were subjected to HPLC using system A at a

flow rate of 1.0 ml/min, and a pressure of 110 kg/cm$^2$. Estradiol 17-sulfate (Ia) was used as an internal standard.

The calibration curves for the guaiacol estrogens (Ic, Id, IId, and IIId) were prepared by the internal standard method (internal standard: Ia). Amounts of the conjugates were determined by the peak-height method.

**Recovery Test**——A known amount of each guaiacol estrogen 17-sulfate was added to the incubation medium (1.5 ml) containing rat liver homogenate, *S*-adenosyl-L-methionine, and $MgSO_4$ in 0.1 M phosphate buffer.[5]Each solution was heated in boiling water for 1 min, diluted with 10 ml of water and supplemented with an exact amount of Ia. The mixtures were passed through Sep-Pak $C_{18}$ cartridges (Waters Assoc., Milford, Mass, U.S.A.). After washing of each cartridge with 2.0 ml of water, the conjugate fractions were obtained by elution with methanol (4.0 ml). Each eluate was passed through a column guard and evaporated under a nitrogen stream to give the residue, which was dissolved in 100 $\mu$l of methanol. These solutions were subjected to HPLC using system A as a mobile phase under the same conditions as described above. Six experiments were done on each sulfate.

**Acknowledgement** The authors are grateful to the staff of the Analytical Center of Hokkaido University (Sapporo, Hokkaido) for elemental analyses. This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

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