[Chem. Pharm. Bull. 17(8)1636—1640(1969)]

UDC 547.92.07

Studies on Steroid Conjugates. II. Synthesis of 16-Epiestriol Monoglucuronides[1]

Toshio Nambara, Yasuhiko Matsuki and Tomomi Chiba

Pharmaceutical Institute, Tohoku University[2]

(Received February 4, 1969)

16-Epiestriol 16- and 17-monoglucuronides (XI, V) were prepared from 3-benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16-one (I) employing Koenigs-Knorr reaction as shown in Chart 1. These synthetic glucuronides readily underwent hydrolysis with beef-liver β-glucuronidase preparation to furnish 16-epiestriol and free glucuronic acid.

It is well known that a large amount of estrogens is excreted in human pregnancy urine and a variation in their quantity can serve as an index of the functional state of feto-placental unit. For this purpose numerous methods for determination of the urinary estrogens with more accuracy and precision have been proposed. Almost all methods require the hydrolysis of the conjugates as an initial step, but the naturally occurring conjugates have not yet fully been characterized. In connection with these problems the authors have previously reported the synthesis of estriol monoglucuronides.[3] As for 16-epiestriol the presence of the glucuronides in pregnancy urine has also been suggested,[4] but the complete structure still remains unknown because of unavailability of the authentic specimen. In addition the multiplicity of the UDP glucuronyl transferase which involves the formation of the steroid glucuronide,[5] and the physiological significance of conjugation appeared to be quite attractive subjects. In this paper the authors wish to report the synthesis of 16-epiestriol 16- and 17-monoglucuronides in respect of these biochemical problems.[6]

The first proiect was directed to the preparation of the 17-glucuronide starting from the suitably protected estrone derivative, that is, 3-benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16-one (I).[3,6,7] The introduction of the glucuronyl moiety was accomplished by the use of Koenigs-Knorr reaction in the usual manner.[8] When the 17β-hydroxy-16-ketone and methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate were stirred in dry benzene with freshly prepared silver carbonate, condensation reaction took place to give methyl (3-benzyloxy-16-oxoestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (II) in 11% yield. The stereospecific reduction of the 16-oxo function was achieved without disturbance of the ester groups by treatment with sodium borohydride at $-10°$. It seemed very likely that the existence of the bulky substituent at 17β would favor the preferential attack of the

1) This paper constitutes Part XXIX of the series entitled "Analytical Chemical Studies on Steroids"; Part XXVIII: T. Nambara, M. Usui, and H. Hosoda, *Chem. Pharm. Bull.* (Tokyo), **17**, 1611 (1969).
2) Location: *Aobayama, Sendai.*
3) T. Nambara and K. Imai, *Chem. Pharm. Bull.* (Tokyo), **15**, 1232 (1967).
4) M.G.B.A. Rahman and R. Hähnel, *Clin. Chim. Acta*, **17**, 59 (1967); E.R. Smith and A.E. Kellie, *Biochem. J.*, **104**, 83 (1967).
5) K.J. Isselbacher, "Recent Progress in Hormone Research," Vol. 12, ed. by G. Pincus, Academic Press, New York, N.Y., 1956, pp. 134—147.
6) During the course of this work Elce, *et al.* reported the attempt to prepare 16-epiestriol 17-glucuronide. However, the structural assignment could not definitely be established, since the equivocal synthetic route was employed (J.S. Elce, J.G.D. Carpenter, and A.E. Kellie, *J. Chem. Soc.* (*C*), **1967**, 542).
7) It is sufficiently substantiated that the 17β-hydroxy-16-ketone is the most stable 16,17-ketol among the four possible isomers in C/D-*trans* series (J. Fishman, *J. Am. Chem. Soc.*, **82**, 6143 (1960)).
8) H.H. Wotiz, E. Smakula, N.N. Lichtin, and J.H. Leftin, *J. Am. Chem. Soc.*, **81**, 1704 (1959).

reagent from the α-side of the molecule yielding the 16β-hydroxy compound (IIIa) as a single product. In order to facilitate the subsequent elaboration, the reduction product was then led to the 16-acetate (IIIb) with acetic anhydride and pyridine as usual. The catalytic hydrogenation over palladium-on-charcoal gave methyl (3-hydroxy-16β-acetoxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IV) with loss of the benzyl group at C-3. Then, simultaneous removal of the protecting groups in both steroid and sugar moiety proceeded with success leading to desired sodium 17-glucosiduronate (V), when treated with methanolic sodium hydroxide under mild conditions.[9]

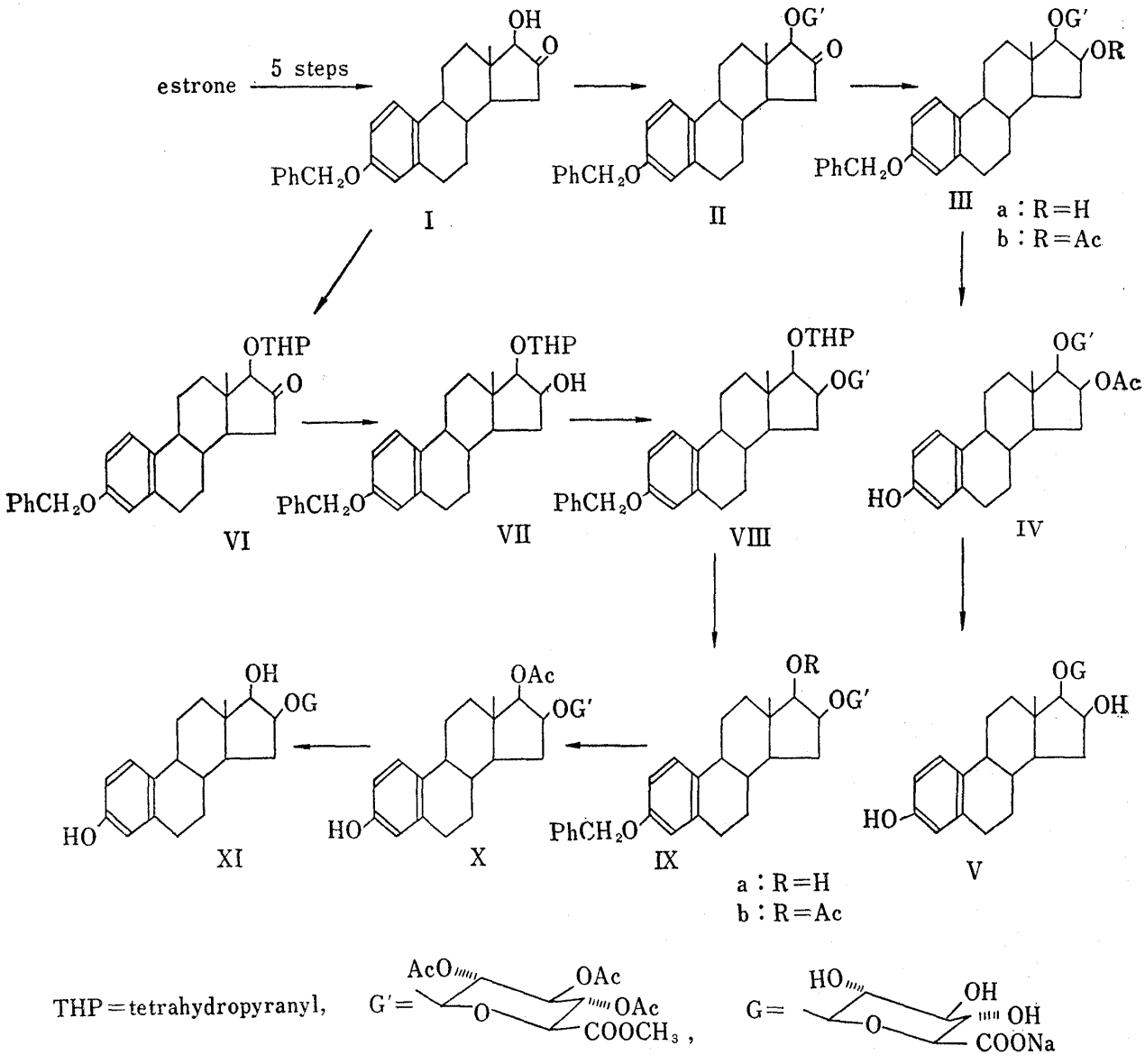

Chart 1

The second project was focused on the synthesis of the isomeric 16-glucuronide, which required the somewhat more involved reaction sequence. An initial attempt to prepare 16,17-*cis*-β-glycol 17-monoacetate from the corresponding α-ketol acetate by borohydride reduction resulted in failure, since acetyl migration did take place in part along with reduction of the 16-ketone yielding a mixture of the isomeric 16- and 17-acetates in a ratio of *ca.* 3 to 1.[10]

9) R. Emilliozzi, *Bull. Soc. Chim. France*, **1968**, 738.
10) T. Nambara, Y. Matsuki, and T. Kudo, to be published.

Therefore introduction of the tetrahydropyranyl group, which is stable in basic media,[11] into the 17-hydroxyl function was undertaken. When I and 3,4-dihydro-2H-pyran were brought into reaction under catalysis by anhydrous *p*-toluenesulfonic acid, the tetrahydropyranyl ether (VI) was formed in satisfactory yield. The borohydride reduction of the 16-oxo group again proceeded stereoselectively from the rear side to furnish solely the 16β-hydroxy compound (VII), probably due to the presence of the bulky group at 17β. Condensation of methyl acetobromoglucuronate with the 16β-hydroxy derivative could be attained under the similar conditions as in the case of the isomeric 17-glucuronide. Subsequent elimination of the tetrahydropyranyl residue was accomplished with ease on brief exposure to the mineral acid. Usual acetylation with acetic anhydride and pyridine gave the 17-acetate (IXb) as the more favorable material to deal with in the subsequent steps. Upon hydrogenolysis over palladiumon-charcoal the 17-acetate underwent debenzylation to provide methyl (3-hydroxy-17β-acetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (X) almost quantitatively. Then, hydrolytic cleavage with methanolic sodium hydroxide resulted in formation of sodium 16-glucosiduronate (XI) with complete removal of the protecting groups.

The nuclear magnetic resonance spectra of the acetylated glucuronides were indicative of the formation of the β-glucoside linkage by the present synthetic way. The anomeric proton of the sugar moiety appeared at 4.5—4.6 ppm as doublet (J=7 cps) indicating a *trans*-diaxial relationship to the vicinal 2′-proton. Further evidence for the β-glucuronoside structure present in 16-epiestriol monoglucuronides thus prepared was definitely demonstrated by characterizing 16-epiestriol and free D-glucuronic acid after incubation with beef-liver β-glucuronidase by means of coloration test and thin-layer chromatography.

It is hoped that these synthetic samples will serve as the standard to characterize the naturally occurring 16-epiestriol conjugates. Furthermore, availability of these specimens together with the estriol glucuronides as models for the natural conjugates may provide the valuable information on the hydrolysis prior to analysis of the urinary estrogens.

Experimental[12]

Methyl (3-Benzyloxy-16-oxoestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (II)——To a solution of 3-benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16-one (I)[6] (1.0 g) in anhydrous benzene (150 ml) were added methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (1.5 g) and freshly prepared Ag_2CO_3 (1.2 g), and the mixture was stirred for 24 hr in the dark place. After removal of the precipitate by filtration the filtrate was concentrated, and the residue obtained was chromatographed on silica gel (30 g)–celite (10 g). Elution with hexane–benzene–ether (5:5:2) and recrystallization of the eluate from EtOH gave II (260 mg) as colorless prisms. mp 162—163°. $[\alpha]_D^{17}$ +73.8° (c=0.16). *Anal.* Calcd. for $C_{38}H_{44}O_{12}$: C, 65.88; H, 6.40. Found: C, 65.62; H, 6.49.

Methyl (3-Benzyloxy-16β-hydroxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IIIa)——To a solution of II (230 mg) in DMF (2 ml)–MeOH (2 ml) was added a solution of KBH_4 (80 mg) in aq. MeOH (1.5 ml), and the resulting solution was allowed to stand at −10——13° for 6 hr. The reaction mixture was acidified with AcOH and then poured into ice–water. The precipitate was filtered, washed with H_2O and then dried. Recrystallization from EtOH gave IIIa (225 mg) as colorless needles. mp 259—260°. $[\alpha]_D^{12}$ −24.2° (c=0.21). *Anal.* Calcd. for $C_{38}H_{46}O_{12}$: C, 65.69; H, 6.67. Found: C, 65.69; H, 6.67.

Methyl (3-Benzyloxy-16β-acetoxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IIIb)——A solution of IIIa (210 mg) in pyridine (2.5 ml) and Ac_2O (1.2 ml) was heated at 70—80° for 6 hr. The resulting solution was diluted with $CHCl_3$, washed with 5% HCl, 5% $NaHCO_3$ and H_2O, successively and dried over anhydrous Na_2SO_4. After usual work-up recrystallization of the crude product

11) J.F.W. McOmie, "Advances in Organic Chemistry: Methods and Results," Vol. 3, ed. by R.A. Raphael, E.C. Taylor, and H. Wynberg, Interscience Publishers, Inc., New York, N.Y., 1963, pp. 191—294.

12) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$ unless otherwise stated. Nuclear magnetic resonance spectra were obtained on Hitachi Model H-60 spectrometer at 60 Mc in $CDCl_3$ using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet and m=multiplet. Infrared spectra measurements were run on Hitachi Model EPI-2 spectrophotometer.

from EtOH gave IIIb (185 mg) as colorless needles. mp 180—182°. $[\alpha]_D^{12}$ +3.71° (c=0.13). *Anal.* Calcd. for $C_{40}H_{48}O_{13}$: C, 65.20; H, 6.57. Found: C, 64.89; H, 6.60.

Methyl (3-Hydroxy-16β-acetoxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IV)——A solution of IIIb (200 mg) dissolved in AcOEt (5 ml)–EtOH (50 ml) was shaken with 5% Pd/C (150 mg) under a current of H_2 for 40 hr at room temperature. After removal of catalyst by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from EtOH gave IV (110 mg) as colorless prisms. mp 223—225°. $[\alpha]_D^{24}$ +19.0° (c=0.16). *Anal.* Calcd. for $C_{33}H_{42}O_{13} \cdot \frac{1}{2}H_2O$: C, 60.45; H, 6.61. Found: C, 60.23; H, 6.69. NMR (4% solution in $CDCl_3$) δ: 0.91 (3H, s, 18–CH_3), 2.04 (9H, s, pyranose-$OCOCH_3$), 2.08 (3H, s, 16β-$OCOCH_3$), 3.53 (1H, d, J=6 cps, 17α-H), 3.76 (3H, s, –$COOCH_3$), 4.63 (1H, d, J=7 cps, pyranose-C_1-H), 5.25 (4H, m, 16α-H, pyranose-C<u>H</u>–$OCOCH_3$). IR cm^{-1}(KBr): 790 (pyranose breathing), 890 (C_1-H).

Sodium (3,16β-Dihydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosid)uronate (V)——To a solution of IV (80 mg) in MeOH (3 ml) was added 1N NaOH (0.7 ml), and the resulting solution was allowed to stand at room temperature for 48 hr. The precipitated crystalline product was filtered, washed with MeOH and dried. Recrystallization from MeOH gave V (54 mg) as colorless prisms. mp>310°. $[\alpha]_D^{26}$ −6.2° (c=0.16, H_2O). *Anal.* Calcd. for $C_{24}H_{31}O_9Na \cdot 2H_2O$: C, 55.16; H, 6.75. Found: C, 55.49; H, 7.03.

3-Benzyloxy-17β-(2-tetrahydropyranyloxy)estra-1,3,5(10)-trien-16-one (VI)——To a solution of I (650 mg) in benzene (10 ml) were added 3,4-dihydro-2H-pyran (7.5 ml) and anhydrous p-TsOH (15 mg), and the resulting solution was stirred at room temperature for 3 hr. The reaction mixture was diluted with benzene, washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. After usual work-up recrystallization of the crude product from MeOH gave VI (685 mg) as colorless needles. mp 164—167°. $[\alpha]_D^{26}$ −85.6° (c=0.11). *Anal.* Calcd. for $C_{30}H_{36}O_4$: C, 78.23; H, 7.88. Found: C, 79.01; H, 7.73.

3-Benzyloxy-17β-(2-te trahydropyranyloxy)estra-1,3,5(10)-trien-16β-ol (VII)——To a solution of VI (685 mg) in benzene (3 ml)-MeOH(81 ml) was added a solution of KBH_4 (100 mg) in aq. MeOH (3 ml), and the resulting solution was stirred at room temperature for 2 hr. The reaction mixture was diluted with benzene, washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. After usual work-up recrystallization of the crude product from MeOH gave VII (600 mg) as colorless needles. mp 142—146°. $[\alpha]_D^{26}$ +85.2° (c= 0.12). *Anal.* Calcd. for $C_{30}H_{38}O_4 \cdot \frac{1}{2}H_2O$: C, 76.39; H, 8.33. Found: C, 76.49; H, 7.96.

Methyl (3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)-uronate (IXa)——To a solution of VII (400 mg) in anhydrous benzene (40 ml) were added methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (600 mg) and freshly prepared Ag_2CO_3 (800 mg), and the mixture was stirred for 20 hr in the dark place. After removal of the precipitate by filtration the filtrate was concentrated to give an oily residue. To a solution of this crude product in acetone (40 ml) was added 5% HCl (3 ml), and the solution was heated at 70° for 5 min. The resulting solution was neutralized with 5% $NaHCO_3$ and concentrated. The residue obtained was dried and chromatographed on silica gel (9 g)–celite (3 g). Elution with hexane–benzene–AcOEt (4:1:1 to 2:2:1) and recrystallization of the eluate from MeOH gave IXa (145 mg) as colorless needles. mp 232—234°. $[\alpha]_D^{24}$ +26.2° (c=0.13). *Anal.* Calcd. for $C_{38}H_{46}O_{12}$: C, 65.69; H, 6.67 Found: C, 65.66; H, 6.51. NMR (4% solution in $CDCl_3$) δ: 0.84 (3H, s, 18–CH_3), 2.00 (6H, s, pyranose-$OCOCH_3$), 2.05 (3H, s, pyranose-$OCOCH_3$), 3.43 (1H, d, J=7 cps, 17α-H), 3.75 (3H, s, –$COOCH_3$), 4.06 (1H, m, 16α-H), 4.67 (1H, d, J=6 cps, pyranose-C_1-H), 5.01 (2H, s, -$CH_2C_6H_5$), 5.11 (3H, m, pyranose-C<u>H</u>-$OCOCH_3$).

Methyl (3-Benzyloxy-17β-acetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IXb)——A solution of IXa (260 mg) in pyridine (2 ml) and Ac_2O (1 ml) was heated at 70—80° for 5 hr. The resulting solution was diluted with ether, washed with 5% HCl, 5% $NaHCO_3$ and H_2O, successively and dried over anhydrous Na_2SO_4. After usual work-up recrystallization of the crude product from EtOH gave IXb (180 mg) as colorless needles. mp 196—198°. $[\alpha]_D^{23}$ +43.0° (c=0.14). *Anal.* Calcd. for $C_{40}H_{48}O_{13}$: C, 65.20; H, 6.57. Found: C, 65.00; H, 6.33. NMR (5% solution in $CDCl_3$) δ: 0.88 (3H, s, 18-CH_3), 1.93 (6H, s, pyranose-$OCOCH_3$), 1.99 (3H, s, pyranose-$OCOCH_3$), 2.02 (3H, s, 17β-$OCOCH_3$), 3.70 (3H, s,–$COOCH_3$), 4.23 (1H, m, 16α-H), 4.47 (1H, d, J=7 cps, pyranose-C_1-H), 4.95 (2H, s, -$CH_2C_6H_5$), 4.98 (4H, m, 17α-H, pyranose-C<u>H</u>-$OCOCH_3$).

Methyl (3-Hydroxy-17β-acetoxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid) uronate (X)——A solution of IXb (100 mg) dissolved in AcOEt (5 ml)–EtOH (40 ml) was shaken with 5% Pd/C (80 mg) under a current of H_2 for 24 hr at room temperature. After removal of catalyst by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from MeOH gave X (75 mg) as colorless needles. mp 173—174°. $[\alpha]_D^{24}$ +69.6° (c=0.14). *Anal.* Calcd. for $C_{33}H_{42}O_{13} \cdot \frac{1}{2}H_2O$: C, 60.45; H, 6.61. Found: C, 60.72; H, 6.71. NMR (4% solution in $CDCl_3$) δ: 0.89 (3H, s, 18-CH_3), 2.01 (6H, s, pyranose-$OCOCH_3$), 2.08 (3H, s, pyranose-$OCOCH_3$), 2.14 (3H, s, 17β-$OCOCH_3$), 3.78 (3H, s, –$COOCH_3$), 4.00 (1H, m, 16α-H), 4.47 (1H, d, J=8 cps, pyranose-C_1-H), 5.15 (4H, m, 17α-H, pyranose-C<u>H</u>-$OCOCH_3$). IR cm^{-1}(KBr): 785 (pyranose breathing), 888 (C_1-H).

Sodium (3,17β-Dihydroxyestra-1,3,5(10)-trien-16β-yl-β-D-glucopyranosid)uronate (XI)——To a solution of X (80 mg) in MeOH (3 ml) was added 1N NaOH (0.7 ml), and the resulting solution was allowed to stand at room temperature for 48 hr. The precipitated crystalline product was filtered, washed with MeOH

and dried. Recrystallization from MeOH gave XI (50 mg) as colorless needles. mp 278—281° (decomp.). $[\alpha]_D^{15}$ −36.0° (c=0.11, H_2O). *Anal.* Calcd. for $C_{24}H_{31}O_9Na \cdot 2H_2O$: C, 55.16; H, 6.75. Found: C, 54.82; H, 6.92.

Hydrolysis of 16-Epiestriol Monoglucuronides with β-Glucuronidase——To an aq. solution (2 ml) of 16-epiestriol monoglucuronide (*ca.* 0.6 mg) were added 0.1M acetate buffer (pH 4.7, 20 ml) and beef-liver β-glucuronidase (Tokyo Zōki Kagaku Co., Ltd.) (13000 Fishman Unit/ml, 2 ml), and the mixed solution was incubated at 37° for 24 hr. The incubated fluid was saturated with NaCl and extracted with ether (30 ml ×2, 10 ml×1). The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. A portion of the residue thus obtained was submitted to TLC employing silica gel HF (E. Merck AG) as adsorbent and ether–AcOEt (2:1) as developing solvent. The test sample exhibited a spot at *Rf* 0.58, which proved to be identical with that of the authentic sample (16-epiestriol). Another portion of the hydrolyzate was colorimetrically determined by the Nocke's method.[13] The test sample gave the characteristic Kober coloration (λ_{max}517 mμ) and almost quantitative recovery of 16-epiestriol. Free glucuronic acid liberated was characterized by the method of Fishman, *et al.* with use of naphthoresorcinol as coloring reagent.[14]

Acknowledgement The authors are indebted to all the staffs of the central analytical laboratory of this Institute for elemental analyses, IR and NMR spectral measurements. Thanks are also due to Teikoku Hormone Manufacturing Co., Ltd. for generous supply of estrone. This work was supported in part by a Grant-in-Aid from the Ministry of Education, which is gratefully acknowledged.

13) W. Nocke, *Biochem. J.*, **78**, 593 (1961).
14) W.H. Fishman and S. Green, *J. Biol. Chem.*, **215**, 527 (1955).