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Studies on Steroid Conjugates. VIII. Synthesis of 16β-Hydroxyestrone 16-Glucuronide Acetate-Methyl Ester[1]

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As a part of our studies on the steroid conjugates we reported previously the synthesis of three possible 16-epiestriol monoglucuronides by the unequivocal routes.[3] The occurrence of 16-epiestriol 16-glucuronide in the late pregnancy urine could be confirmed by comparison with these synthetic samples.[4] A problem whether or not this conjugate is formed exclusively from 16-epiestriol appears to be of interest, since 16β-hydroxyestrone 16-glucuronide may possibly serve as a substrate leading to the conjugated 16-epiestriol with retention of the glucuronoside linkage. Moreover, the presence of 16β-hydroxyestrone 16-glucuronide in the pregnancy urine has not completely been clarified. In connection with these studies the titled compound has become requisite as a suitable reference for comparison with the naturally occurring product.

An initial attempt was made on the introduction of the sugar moiety into the 16β-hydroxy-17-oxosteroid. For this purpose the enol acetate (I) derived from estrone benzyl ether was chosen as a starting material. Treatment with lead tetraacetate in acetic acid gave the 16β-acetoxy-17-ketone (IIb) in reasonable yield. Subsequent hydrolysis with potassium bicarbonate under the mild conditions afforded the 16β-hydroxy-17-ketone (IIa), which could be backed to the acetate by reacetylation. When IIa 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,[5] condensation reaction proceeded to provide a small amount of the expected glucuronide (III) accompanied with 3-benzyloxy-17β-hydroxyestratrien-16-one formed by the ketol rearrangement.[6] Configurational retention of the C-16 substituent in III during Koenigs–Knorr reaction was confirmed by direct comparison with the known 16α-epimer.[7] Difficulties in both the availability of 16β-hydroxyestrone 3-benzyl ether and introduction of a glucuronyl residue necessitated the development of an alternative way to the desired compound.

Recently, Bernstein and his co-workers established a facile method to prepare estriol 16-glucuronide from estriol 3-benzyl ether by Koenigs–Knorr reaction where glucuronidation takes place exclusively at C-16.[8] This finding prompted us to synthesize 16-epiestriol 16-glucuronide by direct condensation with the 16β, 17β-glycol. When 16-epiestriol 3-benzyl ether (V) derivable from IIb was stirred with methyl acetobromoglucuronate in the presence of silver carbonate as the catalyst, two condensation products yielded in almost the equal amount. Separation of these positional isomers was efficiently achieved by the preparative

1) This paper constitutes Part LVI of the series entitled "Analytical Chemical Studies on Steroids"; Part LV: T. Nambara, M. Numazawa, and S. Ishioka, *Chem. Pharm. Bull.* (Tokyo), in press.
2) Location: *Aobayama, Sendai.*
3) *a*) T. Nambara, Y. Matsuki, and T. Chiba, *Chem. Pharm. Bull.* (Tokyo), **17**, 1636 (1969); *b*) T. Nambara, Y. Matsuki, and Y. Kawarada, *ibid.*, **19**, 844 (1971).
4) T. Nambara, Y. Matsuki, J. Igarashi, and Y. Kawarada, in preparation.
5) H.H. Wotiz, E. Smakula, N.N. Lichtin, and J.H. Leftin, *J. Am. Chem. Soc.*, **81**, 1704 (1959).
6) It is sufficiently substantiated that in the C/D-*trans* series the stability sequence of four isomeric 16, 17-ketols is as follows: 17β-OH, 16=O>17α-OH, 16=O>16α-OH, 17=O>16β-OH, 17=O (J. Fishman, *J. Am. Chem. Soc.*, **82**, 6143 (1960)).
7) T. Nambara and K. Imai, *Chem. Pharm. Bull.* (Tokyo), **15**, 1232 (1967).
8) J.P. Joseph, J.P. Dusza and S. Bernstein, *J. Am. Chem. Soc.*, **89**, 5078 (1967); J.P. Jeseph, J.P. Dusza, E.W. Cantrall and S. Bernstein, *Steroids*, **14**, 591 (1969).

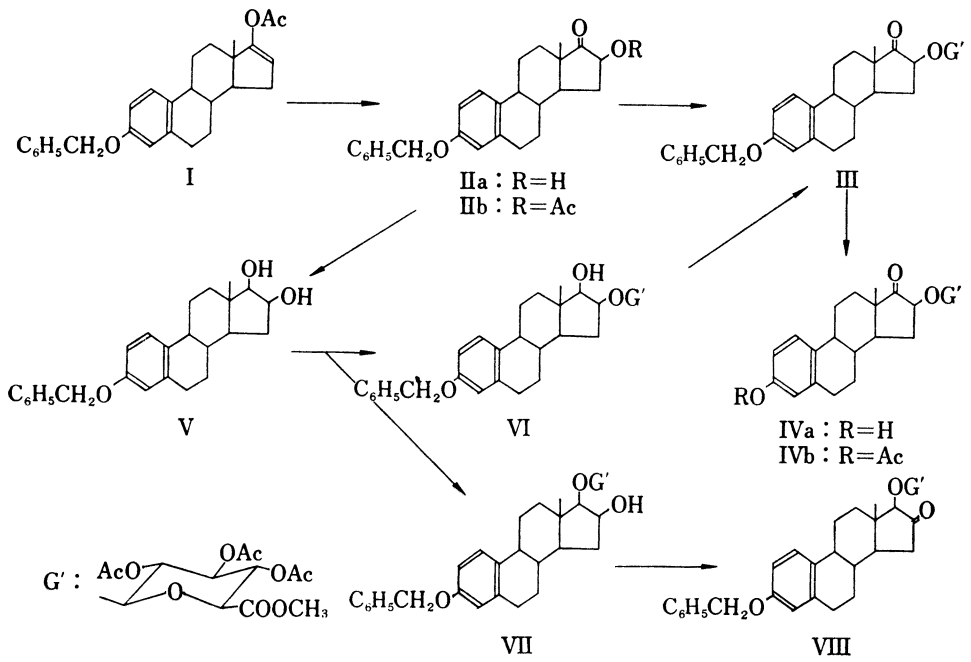

Chart 1

thin-layer chromatography (TLC) upon multiple development. The less polar isomer was elucidated to be the 16-glucuronide acetate-methyl ester (VI) by direct comparison with the authentic sample. Accordingly the remainder should be the 17-glucuronide acetate-methyl ester (VII), and the identity with the authentic specimen could be comfirmed by the usual criteria. It should be emphasized that the present method is of much more advantage for the synthesis of 16-epiestriol 16- and 17-monoglucuronides as compared with the previous one[3a] which needs the suitably protected steroid as a starting material.

This synthetic route seemed to be promising to obtain the desired compound with the more ease. The careful oxidation of VI with chromium trioxide-pyridine complex gave the corresponding 17-oxo compound (III), which proved to be identical with the substance obtained directly from the 16β-hydroxy-17-ketone. Debenzylation by hydrogenolysis over palladium-on-charcoal furnished the 3-hydroxylic compound (IVa), which on usual acetylation was led to the desired 3-acetate (IVb). Oxidation of VII with chromium trioxide gave similarly the known 16-oxo derivative (VIII) without disturbance of the 17β-substituent.

Experimental[9]

3-Benzyloxy-16β-hydroxyestra-1,3,5(10)-trien-17-one Acetate (IIb)——To a solution of 3-benzyloxyestra-1,3,5(10),16-tetraen-17-ol acetate (I) (4.5 g) in AcOH (90 ml)–Ac_2O (4.5 ml) was added $Pb(OAc)_4$ (12 g) and stirred at room temperature for 3 days. The resulting solution was concentrated *in vacuo* below 50° and extracted with ether. The organic layer was washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. After usual work-up the crude product obtained was chromatographed on Al_2O_3 (40 g). Rapid elution with hexane–benzene (8: 2 to 7: 3) and recrystallization of the eluate from MeOH gave IIb

9) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$. Infrared (IR) spectra were obtained by JASCO Model IR-S spectrometer. Nuclear magnetic resonance spectra were run on Hitachi Model R-20 spectrometer at 60 Mc in $CDCl_3$ using tetramethylsilane as an internal standard. Abbreviation used s=singlet and m=multiplet.

(1.73 g) as colorless needles. mp 131—132.5°. $[\alpha]_D^{20}$+130.4° (c=1.04). *Anal.* Calcd. for $C_{27}H_{30}O_4$: C, 77.48; H, 7.23. Found: C, 77.55; H, 7.30. NMR (5% solution in $CDCl_3$) δ: 1.00 (3H, s, 18-CH_3), 2.11 (3H, s, 16β-$OCOCH_3$), 5.01 (2H, s, -$CH_2C_6H_5$). 5.05 (1H, m, 16α-H).

3-Benzyloxy-16β-hydroxyestra-1,3,5(10)-trien-17-one (IIa)——To a solution of IIb (1.73 g) in acetone (100 ml) was added 1% $KHCO_3$ in MeOH–H_2O (5: 1) (200 ml) and stirred at room temperature for 2 hr. After neutralizing with 5% HCl the resulting solution was poured into ice-water, and the precipitate was filtered, washed with H_2O and dried. The crude product thus obtained was submitted to the preparative TLC using benzene–ether (3: 1) as developing solvent. The adsorbent corresponding to the spot (*Rf* 0.26) was eluted with $CHCl_3$ and the eluate was recrystallized from MeOH to give IIa (385 mg) as colorless needles. mp 169—170.5°. $[\alpha]_D^{20}$+81.0° (c=0.68). *Anal.* Calcd. for $C_{25}H_{28}O_3$: C, 79.75; H, 7.50. Found: C, 79.83; H, 7.75. NMR (5% solution in $CDCl_3$) δ: 0.99 (3H, s, 18-CH_3), 4.10 (1H, m, 16α-H), 5.04 (2H, s, -$CH_2C_6H_5$). Reacetylation of IIa (15 mg) with Ac_2O (0.1 ml) and pyridine (0.2 ml) in the usual manner gave IIb (7.2 mg). mp 134—134.5°. Mixed mp on admixture with the sample obtained from I showed no depression. The adsorbent corresponding to the spots (*Rf* 0.38 and 0.82) was eluted with $CHCl_3$ and the eluate was recrystallized from MeOH to give 3-benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16-one (24 mg) and unchanged IIb (1.06 g), respectively.

Methyl (3-Benzyloxy-17-oxoestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III)——i) To a solution of IIa (342 mg) in anhydrous benzene (55 ml) were added methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (530 mg) and freshly prepared Ag_2CO_3 (800 mg) and stirred for 24 hr in the dark place. After removal of the precipitate by filtration the filtrate was concentrated and submitted to the preparative TLC using benzene–ether (5: 1) as developing solvent. The adsorbent corresponding to the spot (*Rf* 0.46) was eluted with hot $CHCl_3$ and the eluate was recrystallized from MeOH to give III (58 mg) as colorless needles. mp 169.5—170°. $[\alpha]_D^{20}$+25.1° (c=1.19). *Anal.* Calcd. for $C_{38}H_{44}O_{12}$: C, 65.88; H, 6.40. Found: C, 65.39; H, 6.39. NMR (5% solution in $CDCl_3$) δ: 0.90 (3H, s, 18-CH_3), 2.03 (9H, s, pyranose-$COOCH_3$), 4.15 (2H, m, 16α-H, pyranose-C<u>H</u>-$OCOCH_3$), 5.03 (2H, s, -$CH_2C_6H_5$). The adsorbent corresponding to the spots (*Rf* 0.39 and 0.28) was eluted with hot $CHCl_3$ and the eluate was recrystallized from MeOH to give 3-benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16-one (81 mg) and unchanged IIa (53 mg), respectively.

ii) To a solution of VI (20 mg) in pyridine (0.5 ml) was added CrO_3–pyridine complex[10] (0.25 ml) and allowed to stand overnight at room temperature. The resulting solution was diluted with ether, washed with 10% AcOH, 5% $NaHCO_3$ and H_2O, successively and dried over anhydrous Na_2SO_4. After usual work-up an oily residue obtained was submitted to the preparative TLC using benzene–ether (3 : 1) as developing solvent. The adsorbent corresponding to the spot (*Rf* 0.58) was eluted with $CHCl_3$ and the eluate was recrystallized from MeOH to give III (3 mg) as colorless needles. mp 171—172°. Mixed mp on admixture with the sample obtained in i) showed no depression and IR spectra of two samples were entirely identical.

Methyl (3-Hydroxy-17-oxoestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IVa)——A solution of III (50 mg) in EtOH (20 ml) was shaken with 10% Pd/C (10 mg) in the stream of H_2 at room temperature for 2 days. After removal of the catalyst by filtration the filtrate was concentrated *in vacuo* to give an oily residue. The crude product was submitted to the preparative TLC using benzene–ether (1: 1) as developing solvent. The adsorbent corresponding to the spot (*Rf* 0.41) was eluted with $CHCl_3$ and the eluate was recrystallized from MeOH to give IVa (11 mg) as colorless needles. mp 170—172.5°. $[\alpha]_D^{16}$+22.7° (c=1.32). *Anal.* Calcd. for $C_{31}H_{38}O_{12}\cdot H_2O$: C, 59.99; H, 6.50. Found: C, 59.40; H, 6.85.

Methyl (3-Acetoxy-17-oxoestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IVb) Treatment of IVa (40 mg) with Ac_2O (0.2 ml) and pyridine (0.5 ml) in the usual manner and recrystallization of the crude product from MeOH gave IVb (20 mg) as colorless leaflets. mp 172—174°. $[\alpha]_D^{20}$+32.0° (c=0.94). *Anal.* Calcd. for $C_{33}H_{40}O_{13}$: C, 61.48; H, 6.26. Found: C, 60.91; H, 6.39.

3-Benzyloxyestra-1,3,5(10)-triene-16β,17β-diol (V)——To a solution of IIb (1.6 g) in anhydrous tetrahydrofuran (160 ml) was added $LiAlH_4$ (1 g) portionwise and stirred at room temperature for 15 hr. To the reaction mixture was added moist ether to decompose the excess of the reagent and acidified with 10% HCl. The organic layer was separated, washed with H_2O and dried over anhydrous Na_2SO_4. After usual work-up the crystalline product obtained was recrystallized from MeOH to give V (1.38 g) as colorless needles. mp 197.5—198.5°. Mixed mp on admixture with the authentic sample[3b] showed no depression.

Koenigs-Knorr Reaction with V——To a solution of V (282 mg) in anhydrous benzene (46 ml) were added methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (350 mg) and freshly prepared Ag_2CO_3 (350 mg) and stirred for 24 hr in the dark place. After removal of the precipitate by filtration the filtrate was concentrated and submitted to the preparative TLC using benzene–ether (3: 1) as developing solvent. After multiple development the adsorbent corresponding to the spot (^{3}*Rf* 0.35) was eluted with hot $CHCl_3$–acetone and the eluate was recrystallized from MeOH to give methyl (3-benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (VI) (77 mg) as colorless

10) G.I. Poos, G.E. Arth, R.E. Beyler, and L.H. Sarett, *J. Am. Chem. Soc.*, **75**, 422 (1953).

needles. mp 232—235°. Mixed mp on admixture with the authentic sample[3a] showed no depression and IR spectra of two samples were entirely identical. The adsorbent corresponding to the spot (^3Rf 0.29) was eluted with hot $CHCl_3$–acetone and the eluate was recrystallized from MeOH to give methyl (3-benzyloxy-16β-hydroxyestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (VII) (68 mg) as colorless needles. mp 256—257.5°. Mixed mp on admixture with the authentic sample[3a] showed no depression and IR spectra of two samples were entirely identical.

Methyl (3-Benzyloxy-16-oxoestra-1,3,5(10)-trien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (VIII)——To a solution of VII (9 mg) in acetone (0.3 ml) was added CrO_3 reagent (CrO_3 (1.33 g), H_2SO_4 (1.15 ml) diluted to 5 ml with H_2O) (0.01 ml) and stirred for 1.5 hr at 0—5°. The resulting solution was diluted with ether, washed with 5% $NaHCO_3$, H_2O and dried over anhydrous Na_2SO_4. After usual work-up the crystalline product obtained was recrystallized from MeOH to give VIII (3 mg) as colorless needles. mp 158—159°. Mixed mp on admixture with the authentic sample[3a] showed no depression and IR spectra of two samples were entirely identical.

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Studies on the Glucaric Acid Pathway in the Metabolism of D-Glucuronic Acid in Mammals. IV.[1] Fluorometric Method for the Determination of D-Glucaric Acid in Serum[2]

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In connection with the biochemical studies on D-glucaric acid, a normal constituent of human urine which is derived from D-glucuronolactone,[4] we developed a colorimetric method for the quantitative determination of this acid.[5] Thus, D-glucaric acid separated from mammalian urines using ion-exchange column chromatography was oxidized with periodic acid to give glyoxylic acid, which was further converted into the intensely colored 1,5-diphenylformazan[6] and determined colorimetrically. During the course of our studies on D-glucaric acid a much more highly sensitive method was required for the accurate estimation of normal serum level of D-glucaric acid in mammals.[1] The present paper deals with the fluorometric determination of D-glucaric acid which is based on the condensation of glyoxylic acid derived from D-glucaric acid with 4'-hydrazino-2-stilbazole to yield a highly fluorescent product using a modification of the procedure for the determination of α-oxo acids originally reported by Mizutani, *et al.* (Fig. 1).[7]

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3) Location: *Takada 3-Chome, Toshima-ku, Tokyo.*
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