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The Structure of Estriol Monoglucosiduronic Acid from Human Pregnancy Urine[1]

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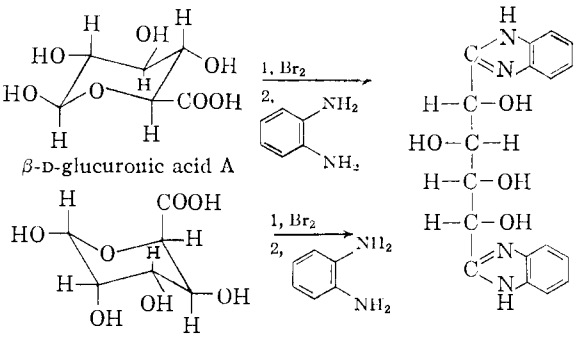
Crystalline "estriol monoglucosiduronic acid," isolated from late human pregnancy urine, was methylated partially with diazomethane, then exhaustively with acid-catalyzed diazomethane, and the totally methylated derivative was hydrolyzed with acid. The resulting estriol dimethyl ether was found to be identical with synthetic 3,17β-dimethoxyestra-1,3,5(10)-trien-16α-ol, and different from the 3,16α-dimethoxy isomer. The structure assigned to "estriol monoglucosiduronic acid," 3,17β-dihydroxyestra-1,3,5(10)-trien-16α-yl-β-D-glucopyranosiduronic acid, was confirmed by n.m.r. spectroscopy of methyl (3-methoxy-17β-acetoxyestra-1,3,5(10)-trien-16α-yl-2',3',4'-tri-O-acetyl-β-D-glucopyranosid)-uronate.

Steroid hormones of the ovaries, testes and adrenals have been encountered in many cases in conjugation with glucuronic acid, and in fewer cases with sulfuric acid.[3] Conceivably, as proposed by Fishman,[3,4] the metabolic conjugation of hormones serves to convert them to a "transport form" in their passage from the secreting gland to the target organ. Until the recent characterization of estrone sulfate in human plasma,[5] however, all previous evidence that circulating estrogen is present partly in conjugated form was only indirect. "The main difficulty is evident enough—lack of any precise knowledge concerning the molecular structure or state of conjugation of the estrogens under study."[6] Several conjugates of steroid hormones and of their metabolites have been isolated from human urine and have been characterized, such as "estriol glucuronide"[7] and the glucuronides of pregnanediol,[8] tetrahydrocortisone,[9] androsterone and etiocholanolone.[10] Nevertheless, the complete structures of only a few conjugates have been elucidated.[8c,8d,9,10]

Marrian, *et al.*, have demonstrated, in a classic series of investigations,[7] that their amorphous "estriol monoglucuronide" of m.p. 224–226° dec., $C_{24}H_{32}O_9$, had the following structural elements: first, a free phenolic group at the 3-position of the aglycone, as evidenced by the ultraviolet spectra of the glucuronide in neutral and alkaline media, and by the formation of estriol 3-methyl ether on methylation of the glucuronide with alkaline dimethyl sulfate, followed by acid hydrolysis; and secondly, a hexuronic acid moiety, combined with estriol in a 1:1 molar ratio, attached to the aglycone by a β-glycosidic linkage. This assignment was based on the following: a positive naphthoresorcinol test of the glucuronide; a negative Benedict test which became positive after treatment with acid; acid hydrolysis of the glucuronide, then a colorimetric determination of estriol; and hydrolysis with ox spleen β-glucuronidase, extractive removal of the aglycone, and finally bromine oxidation of the uronic acid moiety and isolation of the resulting saccharic acid as the bisbenzimidazole derivative, m.p. 242–243° dec., identical with that obtained from D-glucosaccharic acid, *which could have been derived from either* D-*glucuronic or* L-*guluronic acid.*



β-D-glucuronic acid A

β-L-glucuronic acid B

The objective of our present investigation was elucidation of the structural elements of our crystalline "estriol monoglucosiduronic acid" of m.p. 223–224° dec.[11,12] that remained undetermined by the previous investigators, namely, the exact identity and tautomeric form of the uronic acid moiety, and its position on the aglycone.[7e] The approach to this objective was, in principle, along a route of classical carbohydrate chemistry: exhaustive methylation of the glucuronide, fol-

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(2) Postdoctoral Fellow supported by Institutional Training Grant CRTY-5016 to Roswell Park Memorial Institute from the United States Public Health Service, on leave from Tokyo University, Tokyo, Japan.

(3) W. H. Fishman, Chemistry of Drug Metabolism," Charles C Thomas Co., Springfield, Ill., 1961, p. 121, lists glucuronides and/or sulfates of thirty steroid hormones described in the literature.

(4) W. H. Fishman and H. G. Sie, *J. Biol. Chem.*, **218**, 335 (1956).

(5) R. H. Purdy, L. L. Engel and J. L. Oncley, *ibid.*, **236**, 1043 (1961).

(6) L. L. Engel and C. B. Cameron, in "Hormones in Human Plasma," Little, Brown and Co., Boston, Mass., 1960, p. 406.

(7) (a) S. L. Cohen and G. F. Marrian, *Biochem. J.*, **30**, 57 (1936); (b) S. L. Cohen, G. F. Marrian and A. D. Odell, *ibid.*, **30**, 2250 (1936); (c) J. K. Grant and G. F. Marrian, *ibid.*, **43**, v (1948); (d) S. L. Cohen, *J. Biol. Chem.*, **184**, 417 (1950); (e) J. K. Grant and G. F. Marrian, *Biochem. J.*, **47**, 1 (1950).

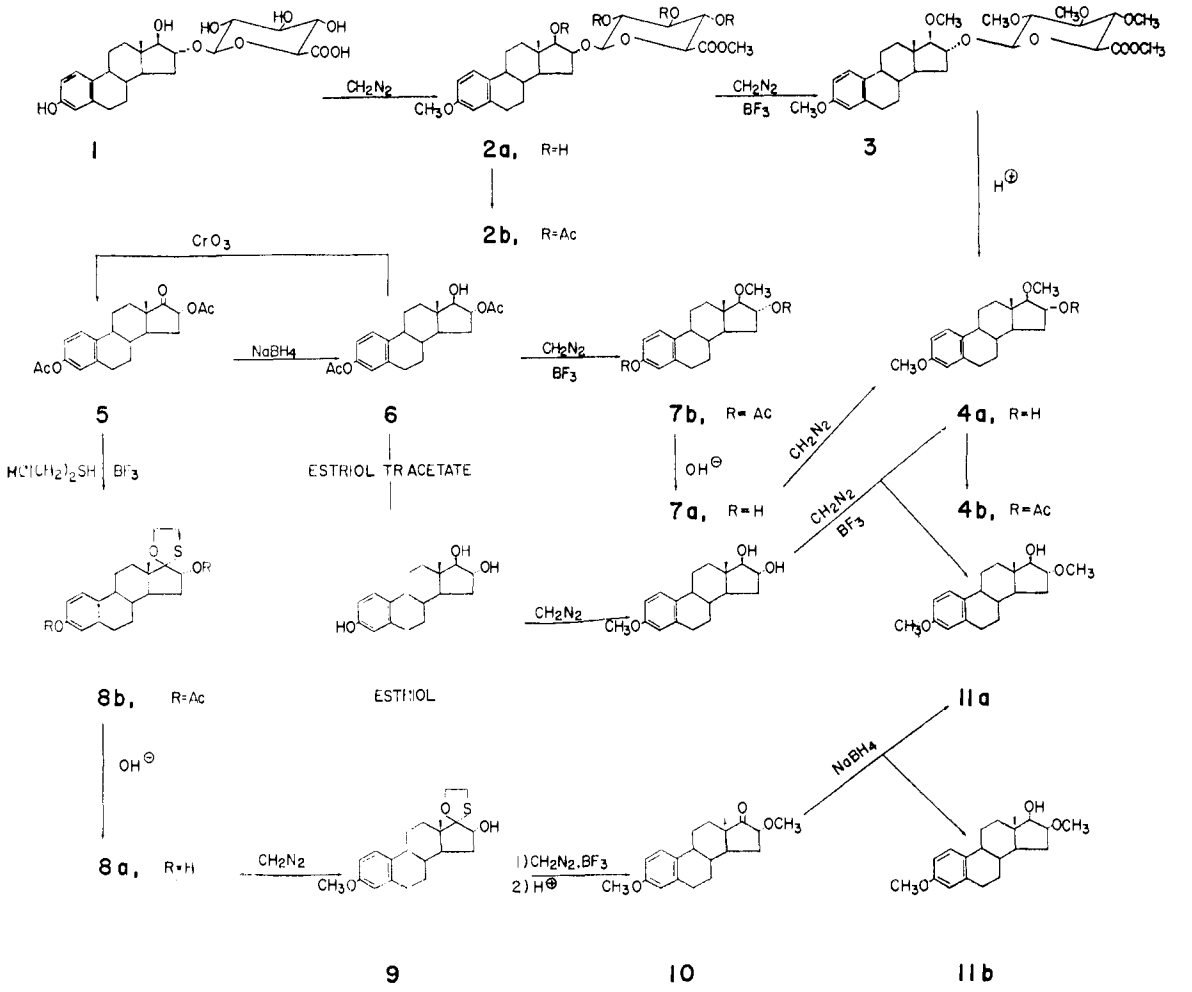
(8) A. D. Odell and G. F. Marrian, *ibid.*, **30**, 1533 (1936); (b) E. M. Venning and J. S. L. Browne, *Proc. Soc. Exp. Biol. Med.*, **34**, 792 (1936); (c) R. D. H. Heard, M. M. Hoffman and G. E. Mack, *J. Biol. Chem.*, **155**, 607 (1944); (d) C. F. Huebner, R. S. Overman and K. P. Link, *ibid.*, **155**, 615 (1944).

(9) J. J. Schneider, M. L. Lewbart, P. Levitan and S. Lieberman, *J. Am. Chem. Soc.*, **77**, 4184 (1955).

(10) H. E. Hadd and R. I. Dorfman, Abstracts, 137th National Meeting, Am. Chem. Soc., Cleveland, Ohio, 1960, p. 6-C.

(11) (a) M. Neeman and Y. Hashimoto, *Tetrahedron Letters*, No. **5**, 183 (1961); (b) Y. Hashimoto and M. Neeman, *J. Biol. Chem.*, in press (1962).

(12) (a) Our compound **1** of m.p. 223–224° dec. undoubtedly represents the first pure specimen of the major component of Cohen and Marrian's amorphous "estriol monoglucuronide" of m.p. 224–226° dec. (b) In retrospect it seems probable that the latter material may have been contaminated with glucuronides of 16-epiestriol, 16α-hydroxyestrone, etc., though there is no evidence that this was actually the case (private communication from Dr. G. F. Marrian).



lowed by acid hydrolysis of the glycosidic linkage and identification of the aglycone methylated on all but one of its hydroxyl groups. The methods used, however, were novel in that methylation of alcoholic hydroxyl groups in the uronic acid and aglycone portions was effected with acid-catalyzed diazomethane[13]; and the conformation, and hence the identity, of the uronic acid moiety was established mainly by nuclear magnetic resonance spectroscopy. Pure crystalline "estriol monoglucosiduronic acid" **1**, m.p. 224° dec.,[11] was converted with diazomethane in dichloromethane–methanol to the 3-methyl ether-methyl ester **2a**. This ester, without purification, was exhaustively methylated with diazomethane in dichloromethane in the presence of boron trifluoride catalyst to afford, after chromatography, in 49% overall yield from **1**, pure totally methylated glucuronide **3**, m.p. 144°, λ_{max} 278 mμ (ε 1800), 287 mμ (ε 1620). Complete methylation required the use of a large excess of methylating agent, a finding in line with previous observations.[13] Hydrolytic cleavage of the totally methylated glucuronide **3** did not occur under the conditions of perchloric acid-catalyzed hydrolysis in nearly anhydrous tetrahydrofuran.[14]

Boiling 2 *N* hydrochloric acid in 10% aqueous ethanol effected partial hydrolysis of the ester group, but little, if any, cleavage of the glycosidic linkage; whereas 4 *N* hydrochloric acid in 10% aqueous ethanol afforded the aglycone dimethyl ether **4a**, m.p. 169°, in 81% yield from **3**.

Two of the three estriol dimethyl ethers had to be considered as possible structures for the aglycone dimethyl ether **4a**, namely, the 3,16α- and 3,17β-dimethyl ethers. Both compounds, which were unknown, were synthesized by unequivocal routes, starting from 3,16α-diacetoxyestra-1,3,5-(10)-trien-17-one **5**, an intermediate previously used in the partial synthesis of estriol.[15] To obtain the 3,16α-dimethyl ether, the keto-diacetate **5** was converted to the thiohemiketal[16] diacetate **8b**, m.p. 178°; the acetoxyl groups were saponified, and the resulting thiohemiketal diol **8a**, m.p. 202°, was converted to the 3-methyl ether **9**, m.p. 167°, with diazomethane in dichloromethane–methanol. The 16α-alcoholic hydroxyl group was methylated with acid-catalyzed diazomethane,[13] and the 17-ketone was unmasked, to afford the 3,16α-dimethoxy ketone **10**, m.p. 97°. Sodium borohydride reduction of the 17-ketone was not highly stereoselective in this case; the

(13) M. Neeman, M. C. Caserio, J. D. Roberts and W. S. Johnson, *Tetrahedron*, **6**, 36 (1959).

(14) S. Burstein, G. M. Jacobsohn and S. Lieberman, *J. Am. Chem. Soc.*, **82**, 1226 (1960).

(15) N. S. Leeds, D. K. Fukushima and T. F. Gallagher, *ibid.*, **76**, 2943 (1954).

(16) L. F. Fieser, *ibid.*, **76**, 1945 (1954).

ratio of more abundant product **11a**, m.p. 140°, to the less abundant one **11b**, m.p. 117°, was only 11:9. The assignment of the configurations at C_{17} to the 140° and 117° compounds was therefore made on the basis of independent evidence, obtained by partial methylation of estriol 3-methyl ether with acid-catalyzed diazomethane, which afforded two dimethyl ethers, separable by chromatography on alumina, the more polar of which was identical with the more abundant reduction product **11a** of m.p. 140°, which therefore was assigned the 17β-ol configuration.

The isomeric 3,17β-dimethyl ether was synthesized from the 3,16α-diacetoxy-17-ketone **5** by sodium borohydride reduction of the latter at low temperature, which produced estriol 3,16-diacetate **6**, m.p. 156°, in this case with a high degree of stereoselectivity. The structure of the diacetate **6** was confirmed by its reoxidation to the diacetoxy ketone **5**, and by its conversion to estriol triacetate. The 3,16-diacetate **6** was methylated with acid-catalyzed diazomethane to the 17β-methoxy diacetate **7b**, m.p. 115°, the acetoxyl groups were saponified, and the resulting 17β-methoxy-diol **7a**, m.p. 192°, was treated with diazomethane in dichloromethane–methanol to give the 3,17β-dimethoxy-16α-ol **4a**, m.p. 169°, which was identical with the estriol dimethyl ether **4a** of unknown structure obtained by acid hydrolysis of the totally methylated "estriol monoglucosiduronic acid" **3** (Fig. 1). The synthetic 3,17β-dimethoxy-16α-ol **4a** of m.p. 169° was also identical with the less polar of the two estriol dimethyl ethers obtained by acid-catalyzed diazomethane methylation of estriol 3-methyl ether described above. Both isomeric estriol dimethyl ethers **4a** and **11a** were found to be completely stable on treatment with hydrochloric acid under the conditions employed in the hydrolysis of the totally methylated "estriol monoglucuronide" **3**. Thus, the position of the hexuronic acid moiety on estriol was proved to be at C_{16}.

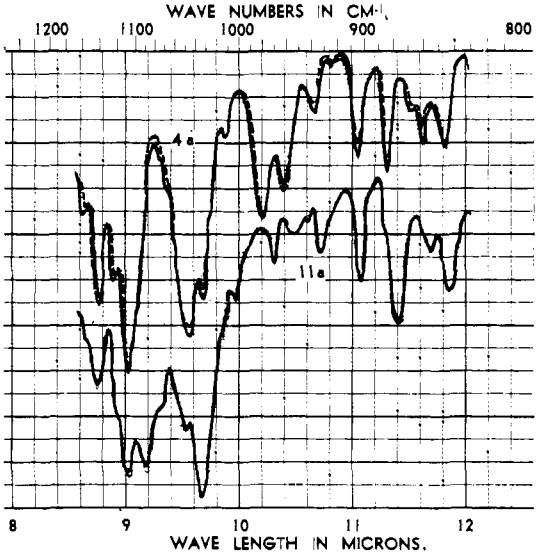


Fig. 1.—Infrared spectra of isomeric estriol dimethyl ethers: upper curve, 3,17β-dimethoxyestra-1,3,5(10)-trien-16α-ol (**4a**); ———, synthetic; – – – –, from estriol monoglucosiduronic acid (**1**); lower curve, 3,16α-dimethoxyestra-1,3,5(10)-trien-17β-ol (**11a**), synthetic.

Elucidation of the identity of the hexuronic acid moiety was possible by applying conformational analysis based on nuclear magnetic resonance spectroscopy[17,18] (Figs. 2 and 3). First, it was expected that derivatives of β-D-glucuronic acid **A** would have an all-equatorial chair conformation, whereas those of β-L-guluronic acid **B** would have one axial hydroxyl group.[19] An all-equatorial conformation would be expected to give rise to a single acetoxyl methyl signal at higher fields than the signal for axial acetoxyl.[18a,c,d] A second criterion, independent of the first, is provided by the Karplus correlation of spin–spin coupling constants as a function of the dihedral angles of vicinal protons,[17] as applied to the anomeric protons in carbohydrates.[18] Thus, the 1′-proton in β-D-glucuronic acid A is in a *trans*-diaxial relationship to the vicinal 2′-proton; whereas in β-L-guluronic acid **B**, the corresponding relationship is axial-equatorial.[19] Hence a larger spin–spin coupling constant, of about 7 c.p.s.,[18c] would be expected for structure **A** as compared with a coupling constant of about 3 c.p.s.[18c] for structure **B**. The n.m.r. spectrum at 60 mc. (Fig. 2) of the tetraacetoxy-methyl ether ester **2b** derived from estriol monoglucosiduronic acid **1**, which showed close resemblance to that of the analogous triacetoxy-methyl ester derived from borneol glucosiduronic acid, showed only *one* sharp signal (g), representing the protons of four equatorial acetoxyl methyl groups. Thus, the absence of signals assignable to axial acetoxyl of the uronic acid moiety, or to 16α-bisectional acetoxyl (Fig. 3, f), is in accord with the all-equatorial *glucuronide* structure **A**, and also confirms the assignment of the 16-position to the uronic acid moiety. The second, independent criterion, based on the spin–spin coupling constant of the anomeric hydrogen, was more difficult to apply, in view of some degree of uncertainty in the assignment to the 1′-proton of the doublet, representing one proton, found at 270 c.p.s. with $J = 8$ c.p.s. in the spectrum of the acetylated estriol monoglucosiduronic acid derivative **2b**. The same doublet appeared in the spectrum of acetylated borneol glucuronide, and similar doublets appeared at 256 c.p.s. with $J = 7$ c.p.s. in the spectrum of the totally methylated estriol monoglucosiduronic acid **3**, and at 258 c.p.s. with $J = 6$ c.p.s. in that of the corresponding trimethyl ether methyl ester of borneol

(17) (a) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959); (b) H. Conroy, in "Advances in Organic Chemistry: Methods and Results," Vol. II, Interscience Publishers, Inc., New York, N. Y., 1960, p. 311.

(18) (a) R. U. Lemieux, R. K. Kullnig, H. J. Bernstein and W. G. Schneider, *J. Am. Chem. Soc.*, **80**, 6098 (1958); (b) R. U. Lemieux, Abstracts, 18th International Congress of Pure and Applied Chemistry, Montreal, Canada, August, 1961, p. 268; (c) J. A. Pople, W. G. Schneider and H. J. Bernstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., New York, N. Y., 1959, p. 395–399. For discussions of the effects on J-values of variations in conformational rigidity of six-membered rings, see (d) J. I. Musher, *J. Chem. Phys.*, **34**, 594 (1961), and *J. Am. Chem. Soc.*, **83**, 1146 (1961); and (e) K. L. Williamson and W. S. Johnson, *ibid.*, **83**, 4623 (1961).

(19) The chair conformation of the β-L-guluronic acid derivative, alternative to B, in which the glycosidic linkage would be axial, would have two axial hydroxyl groups; the juxtaposition of the 1′- and 2′-protons, and that of the 4′- and 5′-protons, would be e-a, as compared with e-e in the conformation B (see footnote 21).

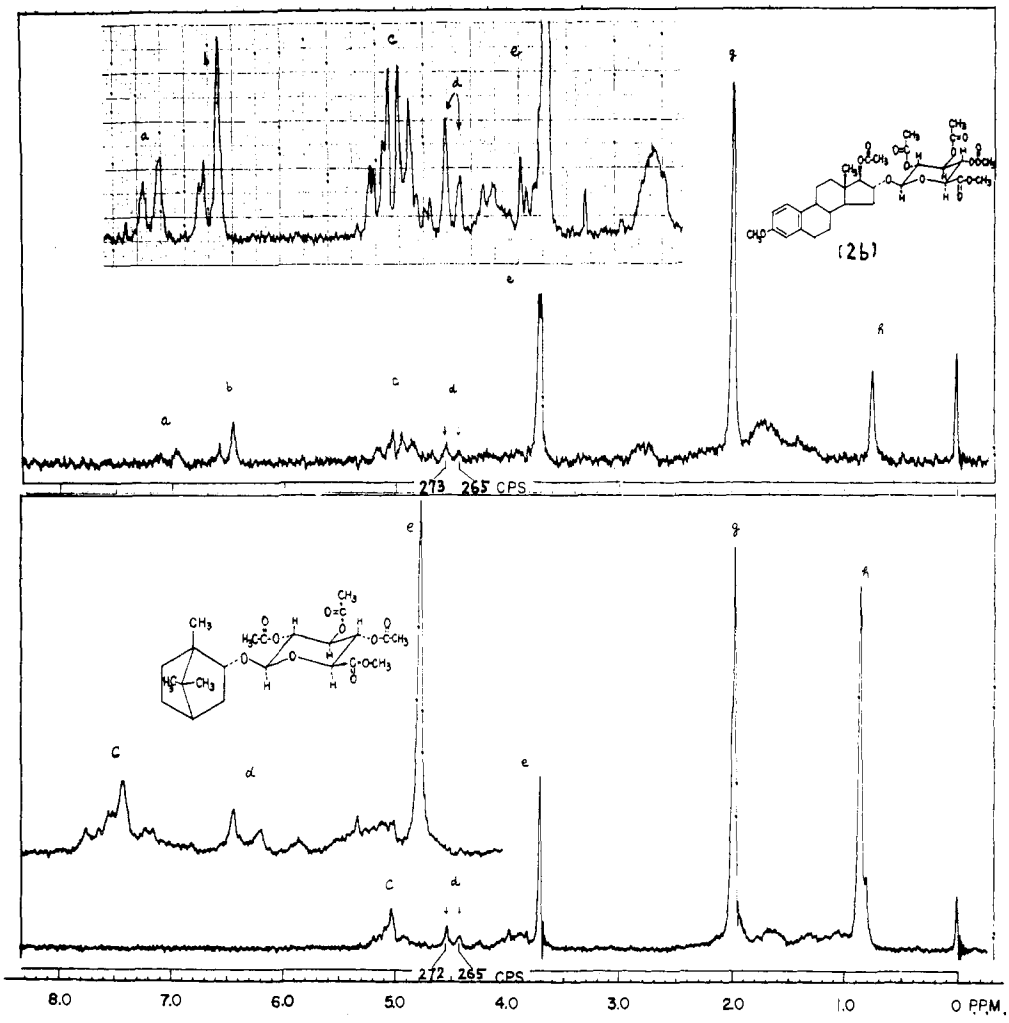


Fig. 2.—N.m.r. spectra in CCl_4 solution: upper curve, methyl (3,17β-dimethoxyestra-1,3,5(10)-trien-16α-yl-2',3',4'-tri-O-acetyl-β-D-glucopyranosid)-uronate (**2b**); lower curve, methyl (bornyl-2',3',4'-tri-O-acetyl-β-D-glucopyranosid)-uronate: a, b, aromatic protons; c, d, e, protons adjacent to oxygen; g, equatorial acetoxyl protons; h, angular, bridgehead or *gem*-methyl protons.

glucuronide.[20] Hence assignment of the doublet at 270 c.p.s. with $J = 8$ c.p.s. to the 1'-proton in a *trans*-diaxial relationship is reasonably certain,[21] and confirms the β-D-glucopyranosiduronic acid structure **A** for estriol monoglucosiduronic acid by exclusion of the only possible alternative structure derived from β-L-guluronic acid **B**.[7e] Hence urinary "estriol monoglucosiduronic acid" of m.p. 224° is 3,17β-dihydroxyestra-1,3,5(10)-trien-16α-yl-β-D-glucopyranosiduronic acid.

Experimental[22,23,24]

Methyl (3,17β-Dimethoxy-1,3,5(10)-estratrien-16α-yl-2',3',4'-tri-O-methyl-β-D-glucopyranosid)-uronate (3) by Exhaustive Methylation of Estriol Glucuronic Acid (1).—To a solution of 0.100 g. (0.22 mmole) of estriol glucuronic acid 1 in 10 ml. of methanol was added 8 mmoles of dry diazomethane (DM) in 33 ml. of methylene chloride. After 15 hours, excess DM was destroyed with a few drops of acetic acid, and the solvent was flash-evaporated under reduced pressure. The product, 0.145 g. of a colorless sirup, which showed λ_{max} 278 mμ, 287 mμ, was dissolved in 15 ml. of methylene chloride. The methyl ether-ester **2a** was exhaustively methylated by the acid-catalyzed diazomethane procedure,[13] a total of 19.2 mmoles of dry DM in 62 ml. of methylene chloride being employed. Seventeen 0.1-ml. aliquots, taken from a catalyst stock solution which contained 0.114 ml. of freshly distilled boron trifluoride etherate in 10 ml. of 1:1 anhydrous diethyl ether–methylene chloride, were added to the reaction mixture at intervals in the course of the methylation. Excess of DM was destroyed by addition of acetic acid, the solution was filtered, the filtrate was washed with aqueous sodium bicarbonate and with

(20) Compound described by J. Pryde and R. T. Williams, *Biochem. J.*, **27**, 1197 (1933).

(21) An alternative assignment of the 270 c.p.s. doublet to the 5'-proton seems unlikely. However, a *trans*-diaxial juxtaposition of the 5'- and 4'-protons would lead to the same structural conclusions as the preferred assignment to the 1'-proton.

(22) Melting points of analytical specimens were determined with a Hershberg m.p. apparatus and are corrected for stem exposure. Melting points reported in our preliminary communication were determined on a Kofler Audiohm Thermistor microscope hot-stage of A. H. Thomas Co. (ref. 11a, footnote 5, p. 184).

(23) Ultraviolet absorption spectra were determined on a Cary recording spectrophotometer model 14 PM, 95% ethanol being employed as the solvent. Infrared spectra were determined on a Baird double-beam infrared recording spectrophotometer model AB 2, methylene chloride being used as the solvent. Optical rotations were determined with a Rudolph photoelectric spectropolarimeter, model 200 AS/800/650.

(24) Alumina used for chromatographic columns was reagent grade acid-washed aluminum oxide, Merck. Petroleum ether had a b.p. 40–50°.

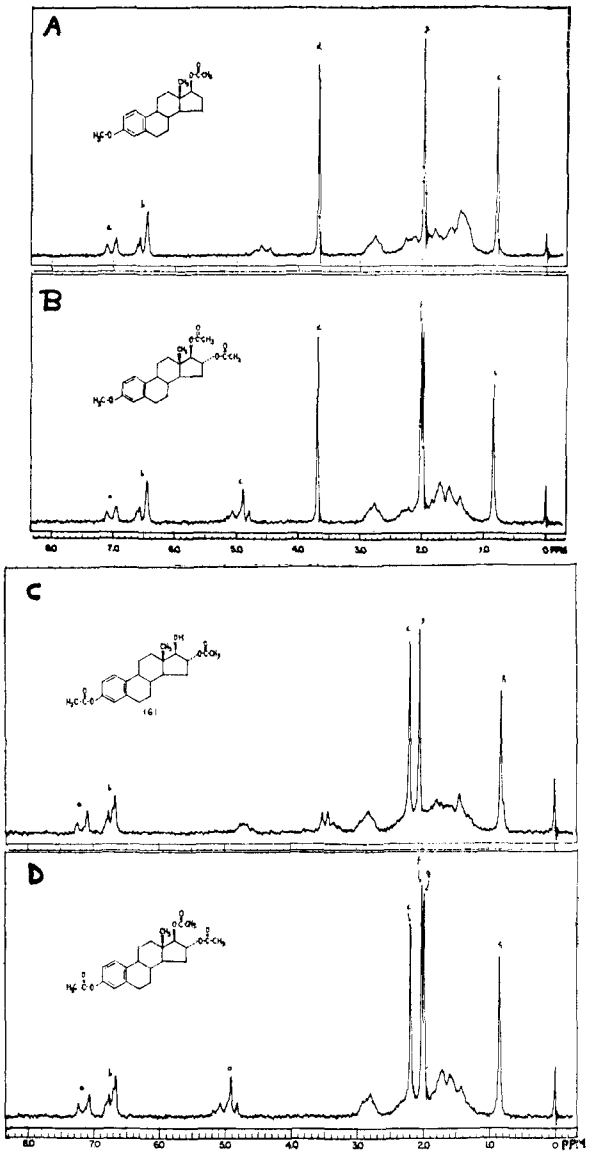


Fig. 3.—N.m.r. spectra of derivatives of estradiol and of estriol in CCl_4 solution: A, estradiol 3-methyl ether 17-acetate; B, estriol 3-methyl ether 16,17-diacetate; C, estriol 3,16-diacetate **6**; D, estriol triacetate; a, b, aromatic protons; c, d, protons adjacent to oxygen; e, 3-acetoxyl protons; f, 16α-acetoxyl (bisectional) protons; g, 17β-acetoxyl (quasi-equatorial) protons; h, angular methyl (C_{18}) protons.

water, dried over anhydrous sodium sulfate, and flash-evaporated under reduced pressure. The residue, 0.194 g. of a yellow sirup which showed no O–H stretching band in the infrared, was chromatographed on 6.0 g. of Florisil. Fractions eluted with 1:200 to 1:32 acetone–benzene amounted to 0.099 g. (A), and crystallized on addition of a small amount of methanol. A further 0.016 g. of a sirupy product (B) was eluted with 1:32 to 1:20 acetone–benzene. The crystalline product (A) was recrystallized from methanol, affording colorless thin plates of the totally methylated ether-ester **3**, 0.048 g. (first crop), m.p. 143–144°, 0.010 g. (second crop), m.p. 137–142°. The over-all yield of the crystalline methyl ether-ester **3** was thus 49% from the urinary estriol glucuronic acid **1**. The mother liquor afforded 0.040 g. of a sirupy residue (C). Two further recrystallizations of a specimen of the first crop material gave plates of **3**, m.p. 143–144°, λ_{max} 278 mμ (ε 1800), 287 mμ (ε 1620); λ_{max} 5.72; 8.77 μ; $\alpha^{24}D$ −2.1° (*c* 1 in $CHCl_3$).

Anal. Calcd. for $C_{30}H_{44}O_8$: C, 65.67; H, 8.08; CH_3O, 33.9. Found: C, 65.78; H, 8.13; CH_3O, 32.8.

Acid Hydrolysis of 3 to 3,17β-Dimethoxyestra-1,3,5(10)-trien-16α-ol (4a).—A solution of the methyl ether-ester **3**, 0.040 g., in 4 ml. of ethanol containing 36 ml. of 2 *N* hydrochloric acid, was refluxed for 2 hr. After cooling, the reaction mixture was extracted with methylene chloride, and the extract was washed with 10% aqueous sodium hydroxide and with water. The sodium hydroxide washing was combined with the aqueous layer remaining after extraction of the reaction mixture. The methylene chloride extract gave 0.030 g. of a sirupy material which showed a strong band in the ester carbonyl region, whereas the combined aqueous layers showed the phenol methyl ether chromophore at 278 and 287 mμ. These findings indicated incomplete hydrolysis. The sirupy extracted material was again refluxed for 2 hr. with 4 ml. of ethanol and 36 ml. of 4 *N* hydrochloric acid. The combined aqueous layers were also rehydrolyzed in the same manner after having been adjusted to 4 *N* with concentrated hydrochloric acid. Both hydrolysis mixtures were combined and worked up as before, providing 0.029 g. of slightly yellow crystals, m.p. 134–157°. This product was purified by gradient elution chromatography on a column of 15.0 g. of alumina, using 700 ml. of 1:10^4 ethanol–benzene as the recipient solvent and 100 ml. of 1:50 ethanol-benzene as the donor solvent. Fractions between 480 ml. and 610 ml. eluent volume were crystalline; their m.p. ranged from 160 to 170°. They showed essentially identical infrared spectra, and amounted to 0.019 g. (81% yield). This material was recrystallized from methanol to give colorless plates of the dimethyl ether **4a**, m.p. 168–169.5°; λ_{max} 278 mμ (ε 1990), 287 mμ (ε 1810); $\alpha^{24}D$ + 41° (*c* 0.6 in $CHCl_3$). The m.p. was undepressed on admixture with synthetic 3,17β-dimethoxyestra-1,3,5-(10)-trien-16α-ol **4a** of m.p. 168–169.5° obtained from the diol **7a** described below, and the infrared spectra of the two specimens were identical. On the other hand, the melting point of the dimethyl ether **4a** obtained by acid hydrolysis, was strongly depressed on admixture of synthetic 3,16α-dimethoxyestra-1,3,5(10)-trien-17β-ol (**11a**) of m.p. 139–140.5°, described below, and the infrared spectra of these two compounds were clearly different (Fig. 1). Acetic anhydride–pyridine acetylation of the dimethyl ether **4a** obtained by acid hydrolysis afforded the dimethoxy-acetate **4b**, m.p. 121.5–124°, which was undepressed on admixture with synthetic 3,17β-dimethoxy-16α-acetoxyestra-1,3,5-(10)-trien (**4b**) of m.p. 123.5–124°; the infrared spectra of both specimens were identical.

After purification of two 0.2-mmole batches of **3**, the sirupy fractions (B) and (C) remaining were combined (0.08 g.) and were hydrolyzed by refluxing for 3 hr. with 4 ml. of a 4 *N* solution of hydrochloric acid in 10% ethanol, and were worked up as before. The brown sirupy product thus obtained was chromatographed on 15.0 g. of alumina. The fractions eluted with 1:500 ethanol–benzene were crystalline (0.006 g.) and identical with the 3,17β-dimethyl ether **4a**. Thus, no evidence was found for the presence of the isomeric 3,16α-dimethyl ether **11a** in the hydrolyzate of either the crystalline portion or the sirupy residues of the methylation product **3**.

Acid Treatment of Dimethyl Ethers 4a and 11a.—Solutions of 0.105 mmole of each isomeric dimethyl ether **4a** and **11a** in 20 ml. of 4 *N* hydrochloric acid in 10% ethanol were refluxed for 3 hours. Each reaction mixture was extracted with methylene chloride, and the extract was washed with 10% aqueous sodium hydroxide and with water, and was dried. Recovery of unchanged starting materials was quantitative in both cases.

Methyl-(3-Methoxy-17β-acetoxyestra-1,3,5(10)-trien-16α-yl-2',3',4'-tri-O-acetyl-β-D-glucopyranosid)-uronate (2b).—A 0.093-mmole batch of estriol glucuronic acid **1** was converted to the methyl ester-ether **2a** with DM in methanol as described under **3**. The crude ester-ether, 0.074 g. of a colorless sirup, λ_{max} 278 and 287 mμ, was acetylated, without purification, with acetic anhydride–pyridine at room temperature overnight, and for a further 5 min. at 70°. The acetylation mixture was evaporated under reduced pressure to a gel, which solidified on addition of a small amount of water. The solid product was dried under reduced pressure, was dissolved in 1:1 petroleum ether–benzene, and was absorbed on a column of 4.0 g. of Florisil. Fractions of the product, which were eluted with 1:50 to 1:20 acetone–benzene, crystallized on removal of the eluent.

They were combined and recrystallized from methanol to afford 0.024 g. (first crop) of silky needles of **2b**, which sintered at 166–167°, resolidified, and remelted at 185.5–186°; λ_{max} 278 mμ (ε 1950), 287 mμ (ε 1700); λ_{max} 5.65, 8.20 μ (broad); and 0.019 g. of needles (second crop) which sintered at 165° and melted at 185–186° (67% total yield).

Anal. Calcd. for $C_{34}H_{44}O_{13}$: C, 61.79; H, 6.71. Found: C, 61.81; H, 6.90.

3,16α-Diacetoxyestra-1,3,5(10)-trien-17β-ol (6).—After dissolving 2.25 g. of 3,16α-diacetoxyestra-1,3,5(10)-trien-17-one **5** in 200 ml. of methanol by slight warming, this solution was cooled to 0° in an ice–salt-bath, and a solution of 0.350 g. of sodium borohydride in 50 ml. of cold methanol was added with stirring. After 1 hr., 10 ml. of 10% acetic acid was added. Flash-evaporation of the solvent gave a white solid, which afforded white needles on washing with water. These were recrystallized from 60 ml. of 50% ethanol to give silky needles, which lost solvent of crystallization at about 100° and melted at 155–157°. Drying the crystals under reduced pressure over phosphorus pentoxide for 6 hr. at 60° and 3 hr. at 110° provided the solvent-free diacetate **6**, 1.68 g. (74% yield), m.p. 155–157° (reported[25] 158–160°); λ_{max} 268 mμ (ε 710), 275 mμ (ε 650); λ_{max} 2.75, 2.80 (broad), 5.69, 5.79, 8.25 (broad) μ. Acetylation of **6** afforded estriol triacetate, m.p. 128–129°.

16α-Acetoxy-1,3,5(10)-triene-3,17β-diol.—Isolation of the diacetate **6**, from another batch, by alumina chromatography, yielded in addition to the diacetate **6** a minor crystalline fraction, which was recrystallized from 50% ethanol and was dried under reduced pressure over phosphorus pentoxide at 60° for 10 hr. providing needles of 16α-acetoxyestra-1,3,5(10)-trien-3,17β-diol, m.p. 198–200°, λ_{max} 281 mμ (ε 2060).

Anal. Calcd. for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.78; H, 7.97.

Oxidation of Diacetate 6 to 5.—To a solution of 0.019 g. of **6** in 2 ml. of pyridine was added a solution of 0.30 g. of chromic anhydride in 3 ml. of pyridine. After standing at 24° overnight, the reaction mixture was poured into 100 ml. of water, and the product was extracted with 1:1 diethyl ether–benzene. The extract was washed with dilute hydrochloric acid, with aqueous sodium bicarbonate, and with water, and was dried over anhydrous sodium sulfate. After removal of the solvent, the residue was crystallized from ethanol, affording 0.068 g. of colorless plates (first crop), m.p. 173.5–174°, undepressed on admixture with ketone **5**. The infrared spectra of both specimens of **5** were identical. A second crop of 0.041 g. of crystals had m.p. 172–173.5° (63% total yield). Complete evaporation of the mother liquor gave 0.041 g. of less pure crystals, m.p. 141–150°.

3,16α-Diacetoxy-17β-methoxyestra-1,3,5(10)-triene (7b).—A solution of 0.376 g. of the diacetate **6** in 10 ml. of methylene chloride was methylated by the acid-catalyzed diazomethane procedure, as described under **3**, using 7.1 mmoles of DM and 0.15 ml. of boron trifluoride solution. The pale yellow sirupy product was chromatographed on 7.0 g. of alumina. The fractions eluted with 1:4 benzene–petroleum ether amounted to 0.243 g. and crystallized on trituration with methanol. The product was recrystallized from methanol, giving 0.134 g. (34% yield) of colorless needles, m.p. 114.5–115.5°; λ_{max} 268 mμ (ε 810), 275 mμ (ε 780); λ_{max} 5.68, 5.78, 8.28 (broad) μ.

Anal. Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82; CH_3O, 8.03. Found: C, 71.30; H, 8.08; CH_3O, 7.85.

17β-Methoxyestra-1,3,5(10)-trien-3,16α-diol (7a).—Saponification of 0.096 g. of the diacetate **7b** was effected by refluxing for 4.5 hr. in 8 ml. of 10% aqueous sodium hydroxide. Neutralization of the solution with dilute hydrochloric acid resulted in the deposition of white crystals, which were collected by filtration, washed with water, dried, and recrystallized from benzene, giving 0.048 g. (first crop) of silky needles of **7a**, m.p. 192–192.5°, λ_{max} 281 mμ (ε 2010); and 0.015 g. (second crop) of needles, m.p. 190–191° (83% yield). The analytical sample melted at 192–192.5°.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67; CH_3O, 10.26. Found: C, 75.27; H, 8.69; CH_3O, 10.59.

3,17β-Dimethoxyestra-1,3,5(10)-trien-16α-ol (4a) from Diol 7a.—To a solution of 0.048 g. (0.16 mmole) of diol **7a** in 5 ml. of methanol was added 3.7 mmoles of DM in 12 ml. of methylene chloride, and the reaction mixture was kept at room temperature overnight. A small amount of acetic acid was added to decompose excess DM. Crystallization from methanol afforded colorless plates of **4a**, 0.013 g. (first crop), m.p. 168–169.5°; 0.008 g. (second crop), m.p. 167–169°; and 0.030 g. (residue), m.p. 161–165°. The first and second crops were combined and were recrystallized from methanol, giving colorless plates which melted at 168–169.5°, λ_{max} 278 mμ (ε 1990), 287 mμ (ε 1810); λ_{max} 2.75, 2.85 (broad) μ; $\alpha^{24}D$ +41° (*c* 1.5 in $CHCl_3$).

Anal. Calcd. for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92; CH_3O, 19.62. Found: C, 76.03; H, 8.90; CH_3O, 19.70.

Acetic anhydride–pyridine acetylation of **4a** obtained from the diol **7a** afforded **3,17β-dimethoxy-16α-acetoxyestra-1,3,5(10)-triene (4b)**, which was recrystallized from *n*-hexane to give colorless needles, m.p. 123.5–124°; λ_{max} 5.80, 8.12 μ.

Anal. Calcd. for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 74.07; H, 8.37.

3,16α-Diacetoxyestra-1,3,5(10)-trien-17-one Ethylenehemithioketal (8b).—To a solution of 1.238 g. of the keto diacetate **5** in 60 ml. of acetic acid was added 3.0 ml. of 2-mercaptoethanol and 5.5 ml. of boron trifluoride etherate.[16] After 3 hr. at 24°, 120 ml. of water was added, and the mixture was extracted with chloroform. The extract was washed with water, aqueous sodium bicarbonate, and water, and dried over anhydrous sodium sulfate. Evaporation of the solvent left a colorless sirup, which crystallized on the addition of ethanol. The crystals collected by filtration amounted to 0.535 g. (35% yield), m.p. 166–176°. Recrystallization from ethanol afforded plates of the hemithioketal **8b**, m.p. 177.5–179°; λ_{max} 268 mμ (ε 700), 275 mμ (ε 640); λ_{max} 5.65–5.71 (broad), 8.1–8.3 (broad) μ.

Anal. Calcd. for $C_{24}H_{30}O_5S$: C, 66.96; H, 7.02. Found: C, 66.76; H, 7.19.

3,16α-Dihydroxyestra-1,3,5(10)-trien-17-one Ethylenehemithioketal (8a).—A solution of 0.200 g. of the diacetate **8b** in 50 ml. of 10% sodium hydroxide in 80% ethanol was refluxed for 1.5 hr. under nitrogen. The solution was cooled and was neutralized with 4 *N* hydrochloric acid. Evaporation of the solvent gave a white deposit, which was washed with water and dried, providing 0.160 g. of crystals (99% yield), m.p. 192–196°. Recrystallization from methanol afforded colorless plates of the diol **8a**, m.p. 201–202°, λ_{max} 281 mμ (ε 2100).

Anal. Calcd. for $C_{20}H_{26}O_3S$: C, 69.34; H, 7.57; S, 9.24. Found: C, 68.93; H, 7.46; S, 8.96.

3-Methoxy-16α-hydroxyestra-1,3,5(10)-trien-17-one Ethylenehemithioketal (9).—A solution of 0.320 g. (0.93 mmole) of the diol **8a** in 30 ml. of methanol was treated with 14 mmoles of DM in 38 ml. of methylene chloride. Concentration of the solution gave 0.219 g. (66% yield) of plates, m.p. 163–166°, which were collected by filtration and washed with methanol. Further concentration of the filtrate afforded 0.062 g. (second crop) of plates, m.p. 156–162°, and 0.030 g. (third crop) of plates, m.p. 142–151°. The first crop material was crystallized from absolute ethanol, giving 0.100 g. of colorless plates of 3-methyl ether **9**, m.p. 166–167°, λ_{max} 278 mμ (ε 2050), 287 mμ (ε 1800); λ_{max} 2.85 (broad) μ.

Anal. Calcd. for $C_{21}H_{28}O_3S$: C, 69.97; H, 7.83; S, 8.88. Found: C, 70.13; H, 8.02; S, 8.94.

3,16α-Dimethoxyestra-1,3,5(10)-trien-17-one (10).—A solution of 0.149 g. (0.41 mmole) of the 3-methyl ether **9** in 5 ml. of methylene chloride was treated with 20 mmoles of DM in 70 ml. of methylene chloride and 2 ml. of boron trifluoride solution, as described under **3**. The reaction mixture was allowed to stand at room temperature overnight. The sirupy product, isolated in the usual manner, was chromatographed on 7.0 g. of alumina. Fractions eluted with 1:4 to 1:1 benzene–petroleum ether afforded 0.064 g. of a sirup (A), which showed no infrared absorption in the O–H stretching region.

The product (B) from the middle peak, eluted with 1:1 petroleum ether–benzene, showed λ_{max} 5.73 μ and amounted to 0.013 g. (10% yield). Starting material **9** was recovered from the final eluate with 1:99 ethanol–benzene, and amounted to 0.016 g. The sirupy fraction (A) was hydrolyzed, without further purification, by refluxing for 3 hr. in a mixture of 20 ml. of ethanol, 1 ml. of water and 0.8 ml. of concentrated hydrochloric acid. The reaction mixture was

(25) J. Fishman, *J. Am. Chem. Soc.*, **82**, 6143 (1960).

neutralized with aqueous sodium bicarbonate, the solvent was removed under reduced pressure, and the residue was extracted with methylene chloride. The extract was washed with water and was dried over anhydrous sodium sulfate. The yellow sirupy residue was chromatographed on 7.0 g. of alumina, first by gradient elution, using 100 ml. of benzene as the donor and 350 ml. of 1:9 benzene–petroleum ether as the recipient solvent, and subsequently by 100 ml. of benzene. The fractions from 310 ml. to 360 ml. of eluent contained a material which showed neither O–H stretching nor C=O stretching bands in the infrared spectrum, possibly unchanged 3,16α-dimethoxyestra-1,3,5(10)-trien-17-one ethylenehemithioketal. The peak from 420 ml. to 550 ml. gave 0.030 g. (23% yield from **9**) of a keto-compound identical with the product (B). Recrystallization from methanol afforded prisms of 3,16α-dimethoxyestra-1,3,5(10)-trien-17-one (**10**), m.p. 96–97.5°, λ_{max} 5.73 μ.

Anal. Calcd. for $C_{20}H_{26}O_3$: C, 76.40; H, 8.34. Found: C, 75.98; H, 8.14.

3,16α-Dimethoxyestra-1,3,5(10)-trien-17β-ol (11a) and 17α-ol (11b).—A solution of 0.030 g. of ketone **10** in methanol was treated with 0.010 g. of sodium borohydride at ice–salt bath temperature for 30 min., and at 24° for another 30 min. After addition of a small amount of acetic acid, the solvent was flash-evaporated. The residue was mixed with water and extracted with methylene chloride. The usual work-up provided 0.027 g. of a sirup, which was chromatographed on 7.0 g. of alumina. The first peak, eluted with 1:10⁴ ethanol–benzene, gave 0.008 g. (27% yield) of crystals, which were recrystallized from 80% aqueous methanol, affording prisms of the 17α-ol **11b**, m.p. 116–117.5°, λ_{max} 2.80 μ (somewhat broad).

Anal. Calcd. for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92. Found: C, 75.42; H, 8.89.

The second peak, eluted with 1:200 ethanol–benzene, gave 0.010 g. (33%) yield of crystals, which were recrystallized from 80% aqueous methanol, giving small plates of the 17β-ol **11a**, m.p. 139–140.5°; λ_{max} 278 mμ (ε 2030), 287 mμ (ε 1790); λ_{max} 2.75, 2.85 (broad) μ.

Anal. Calcd. for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92. Found: C, 75.82; H, 8.76.

Acetylation of **11a** with acetic anhydride–pyridine afforded prisms of **3,16α-dimethoxy-17β-acetoxyestra-1,3,5(10)-triene**, m.p. 119–120°.

Anal. Calcd. for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 73.83; H, 8.17.

Methylation of Estriol 3-Methyl Ether to 3,16α-Dimethoxyestra-1,3,5(10)-triene-17β-ol (11a) and 3,17β-Dimethoxyestra-1,3,5(10)-triene-16α-ol (4a).—A suspension of 0.050 g. (0.15 mmole) of estriol 3-methyl ether, m.p. 160–161°, in 10 ml. of methylene chloride was methylated with 2.4 mmoles of DM in 8 ml. of methylene chloride and 0.18 ml. of boron trifluoride catalyst solution, as described under **3**. The sirupy product (0.063 g.) was chromatographed on 15.0 g. of alumina. Fractions eluted with benzene afforded 0.020 g. of a crystalline product which showed no O–H stretching band in the infrared region, probably estriol trimethyl ether. Fractions eluted with 1:200 ethanol–benzene gave 0.025 g. of a mixture of isomeric estriol dimethyl ethers, which was rechromatographed on 15.0 g. of alumina. The middle portions of the first peak, eluted with 1:500 ethanol–benzene, contained 3,17β-dimethoxyestra-1,3,5(10)-trien-16α-ol (**4a**); and the second peak, eluted with 1:500 ethanol–benzene, afforded the 3,16α-isomer **11a**. The identity of both isomers was confirmed by direct comparison with the specimens of unequivocal structures **4a** obtained from diol **7a**, and **11a** obtained from ketone **10**. The ratio of the amount of **4a** to **11a** was approximately 1:2. The final washing of the first chromatography column with 50 ml. of 1:1 ethanol–benzene contained 0.005 g. of recovered estriol 3-methyl ether.

Methyl (Bornyl-2',3',4'-tri-O-acetyl-β-D-glucopyranosid)-uronate.—Methylation of 0.70 g. of bornyl β-D-glucopyranosiduronic acid[26] with 40 mmoles of DM in dichloromethane–methanol gave 0.77 g. of a sirupy methyl ester which was acetylated with acetic anhydride–pyridine without further purification. Evaporative removal of the solvents from the reaction mixture gave a sirupy product which solidified on addition of water. Drying of the solid and recrystallization from *n*-hexane afforded 0.60 g. of colorless prisms of the methyl ester triacetate, m.p. 138–142° (71% yield). The analytical sample melted at 139–142°, $\alpha^{19}D$ −41.5° (*c* 2.0 in $CHCl_3$).

Anal. Calcd. for $C_{23}H_{34}O_{10}$: C, 58.71; H, 7.28. Found: C, 58.96; H, 7.23.

Nuclear magnetic resonance (n.m.r.) spectra were determined with an A-60 analytical n.m.r. spectrometer of Varian Associates, except for the insert, Fig. 2, upper curve, which was determined with an HR-60 n.m.r. spectrometer of Varian Associates. We wish to thank Dr. James N. Shoolery for his kind coöperation in determining the spectra, and for his helpful discussion.

(26) Commercial borneol glucuronic acid of Sigma Chemical Co., St. Louis, Mo., m.p. 156–158.5°, $\alpha^{20}D$ −59.5° (*c* 2.0 in H_2O).

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The Structure of Serratamolide[1–3]

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Serratamolide, $C_{26}H_{46}O_5N_2$, has been isolated from cultures of *Serratia* and has been shown to have structure II. Mild hydrolysis leads to serratamic acid (I). Formation of the following derivatives is described: diacetylserratamolide, the ditetrahydropyranyl ether, ditosylserratamolide and the dityril ether. Reduction of the free hydroxyl groups by the sequence –OH → –O–Mesyl → –Br → –H followed by hydrolysis with HCl yields alanine as well as 3-hydroxydecanoic acid.

During studies[4a,b] on the metabolic products from *Serratia marcescens*, strain HY-3[5a] and its mutant strains SM 9-3-3[5b] and P-1,[5c] we have isolated a neutral colorless compound, melting at 159–160°, which we have named serratamolide. This material was found on working up the mother liquors in the purification of prodigiosin and its C_{10}-precursor, and was first obtained from methylene chloride extracts of SM 9-3-3 liquid cultures. Subsequently it was isolated in greatly improved yield from HY-3, SM 9-3-3 or P-1, grown on glycerol–peptone agar medium.

(1) For a preliminary account of this work, see H. H. Wasserman, J. J. Keggi and J. E. McKeon, *J. Am. Chem. Soc.*, **83**, 4107 (1961).

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(3) Taken from the doctoral theses of J. J. Keggi (1962) and J. E. McKeon (1961), Yale University.

(4) (a) H. H. Wasserman, J. E. McKeon, L. Smith and P. Forgione, *J. Am. Chem. Soc.*, **82**, 506 (1960); (b) H. H. Wasserman, J. E. McKeon and U. V. Santer, *Biochem. and Biophys. Res. Comm.*, **3**, 146 (1960).

(5) (a) Strain HY-3 was provided by Dr. Mary I. Bunting; see E. L. Labrums and M. I. Bunting, *J. Bacteriol.*, **65**, 394 (1953); (b) U. V. Santer, Ph.D. Dissertation, Yale University, 1958; (c) M. T. M. Rizki, *Proc. Nat. Acad. Sci.*, **40**, 1057 (1954).