Ba(OH)₂ solution. Although the emission of CO₂ subsided within 30 min in most cases, N-formylation of the reduction product was imperfect under this condition (see Table II). Succeedingly the mixture was heated for additional two hour at 120—125°. After this, the reaction solution was evaporated under reduced pressure to remove TMAF. The residual liquid was distilled under high reduced pressure to give the product, N-alkyl-N-(α -alkylbenzyl)formamide. In all the runs yields of the product are about 90% as shown in Table II. Physical and analytical data of the products and the corresponding free N-alkyl- α -alkylbenzylamines are recorded in Table V and VI.

Acknowledgement We are grateful to the members of Analytical Center of this college for the elemental analyses.

[Chem. Pharm. Bull.
19(8)1727—1730(1971)]

UDC 547.92.04 : 615.32.076.9

C-2 Hydroxylation of 2-Deuterioestrogen in the Rat. Lack of "NIH Shift"¹⁾

TOSHIO NAMBARA, SETSUKO AKIYAMA, and SEIJIRO HONMA

Pharmaceutical Institute, Tohoku University²⁾

(Received February 1, 1971)

In a previous paper the occurrence of "NIH shift" during aromatic ring hydroxylation with 3-deoxyestrone has been reported.³⁾ Further interest in hydroxylation mechanism prompted us to examine whether or not *in vivo* formation of the catechol estrogen would similarly be accompanied by a migration of the heavy isotope labeled at C-2.

For this purpose an initial project was focused to the preparation of 2-deuterioestrogen as a substrate. First, 2-hydroxyestrone 3-benzyl ether (I) derivable from estrone in several steps⁴⁾ was taken as a starting compound. Condensation with 1-phenyl-5-chlorotetrazole in the presence of potassium carbonate⁵⁾ provided phenyltetrazolyl ether (II) in satisfactory yield. Hydrogenolysis over palladium-on-barium carbonate with use of deuterium gas proceeded readily to give 2-deuterioestrone (IIIa).

This synthetic route, however, appeared to be of disadvantage in the availability of the starting material. Accordingly, an attempt was then made on the utilization of 2-haloestrogen for the preparation of the specifically deuterated substrate. Treatment of estradiol with bromine in acetic acid resulted in formation of a mixture of 2- and 4-bromo derivatives in a ratio of *ca.* 1 to 1.⁶⁾ The isolation of the desired 2-bromo isomer (IV) could be attained by preparative thin-layer chromatography (TLC) upon multiple development. However, separation of these two positional isomers was a tedious work because of close similarity in their chromatographic behaviors. Therefore, the more suitable method for preparation of 2-halo derivative was explored. When estrone was treated with iodine in the presence of mercuric acetate as catalyst,⁷⁾ halogenation occurred selectively at C-2 yielding

1) This paper constitutes Part XLVII of the series entitled, "Analytical Chemical Studies on Steroids"; Part XLVI: T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 990 (1971). When this work was almost completed we learned a paper dealing with the preparation of C-2 isotope labeled estradiol from 2-bromo derivative and the loss of tritium during *in vivo* hydroxylation at C-2 in man (J. Fishman, H. Guzik, and L. Hellman, *Biochemistry*, **9**, 1593 (1970)).

2) Location: Aobayama, Sendai.

3) T. Nambara, M. Numazawa, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **19**, 153 (1971).

4) T. Nambara, S. Honma, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 474 (1970).

5) W.J. Musliner and J.W. Gates, Jr., *J. Am. Chem. Soc.*, **88**, 4271 (1966).

6) T. Utne, R.B. Jobson, and F.W. Landgraf, *J. Org. Chem.*, **33**, 1654 (1968).

7) A. Hillmann-Elies, G. Hillmann, and U. Schiedt, *Z. Naturforschung (B)*, **8**, 436 (1953).

brane permeability upon conjugation with sulfuric acid. Recently the biochemical significance of conjugation, in particular sulfate formation, has become an attractive problem associated with the metabolism of physiologically active substances.^{12,13} It is hoped that further work in progress in this laboratory will provide the more precise knowledge on this problem.

Experimental¹⁴

Synthesis of Substrates

3-Benzoyloxy-2-(1-phenyl-5-tetrazolyloxy)estra-1,3,5(10)-trien-17-one (II)—To a stirred solution of 2-hydroxyestrone 3-benzyl ether (I)⁴ (213 mg) and 1-phenyl-5-chlorotetrazole (242 mg) in AcOEt (50 ml) was added anhydrous K₂CO₃ (400 mg) and the suspended solution was refluxed for 5.5 hr. After removal of the precipitate by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from aq. MeOH gave II (271 mg) as colorless needles. mp 153—154.5°. $[\alpha]_D^{25} + 94.8^\circ$ ($c = 0.11$, CHCl₃). *Anal.* Calcd. for C₃₂H₃₂O₃N₄: C, 73.82; H, 6.20; N, 10.76. Found: C, 73.63; H, 6.34; N, 10.76.

2-Bromoestra-1,3,5(10)-triene-3,17 β -diol (IV), 4-Bromoestra-1,3,5(10)-triene-3,17 β -diol—To a solution of estradiol (96 mg) in glacial AcOH (5 ml) was added dropwise a solution of Br₂ (60 mg) in AcOH (4 ml) at 17° and then stirred for 24 hr. On usual work-up the crude product obtained was submitted to preparative TLC using benzene-ether (5:1) as developing solvent. After development was repeated three times, the adsorbent corresponding to the spot (*R_f* 0.45) was eluted with AcOEt. Recrystallization of the eluate from ether and then from EtOH gave IV (8 mg) as colorless needles. mp 196.5—197.5°. $[\alpha]_D^{25} + 106.4^\circ$ ($c = 0.08$, CHCl₃). *Anal.* Calcd. for C₁₈H₂₂O₂Br: C, 61.54; H, 6.60. Found: C, 61.64; H, 6.53. NMR (3% solution in acetone) δ : 0.76 (3H, s, 18-CH₃), 6.67 (1H, s, 4-H), 7.30 (1H, s, 1-H). (Reported⁹): mp 197—198°. $[\alpha]_D^{25} + 104^\circ$ ($c = 0.1$, CHCl₃). Elution of the adsorbent corresponding to the spot (*R_f* 0.38) with AcOEt and recrystallization of the eluate from ether and then from EtOH gave 4-bromoestradiol (20 mg) as colorless needles. mp 181.5—183°. $[\alpha]_D^{25} + 99.0^\circ$ ($c = 0.11$, CHCl₃). *Anal.* Calcd. for C₁₈H₂₂O₂Br: C, 61.54; H, 6.60. Found: C, 61.33; H, 6.66. NMR (4% solution in acetone) δ : 0.76 (3H, s, 18-CH₃), 6.71 (1H, d, *J* = 9 cps, 2-H), 7.08 (1H, d, *J* = 9 cps, 1-H). (Reported⁹): mp 213.5—215°. $[\alpha]_D^{25} + 43^\circ$ ($c = 0.1$, CHCl₃).

2-Iodoestra-1,3,5(10)-trien-17-one (Va)—To a solution of estrone (540 mg) in glacial AcOH (10 ml) was added dropwise a solution of Hg(OAc)₂ (165 mg) in AcOH (20 ml) saturated with I₂ (520 mg) over a period of 5 min and stirred at 45—55° for 1 hr. After removal of the precipitate by filtration the filtrate was poured into Na₂S₂O₃ solution. The precipitate was collected by filtration, washed with H₂O and dried. The crude product was dissolved in benzene and chromatographed on Al₂O₃ (30 g). Elution with benzene and recrystallization of the eluate from AcOH and then from MeOH gave Va (409 mg) as colorless needles. mp 200—205°. $[\alpha]_D^{25} + 154.6^\circ$ ($c = 0.11$, CHCl₃). *Anal.* Calcd. for C₁₈H₂₁O₂I: C, 54.56; H, 5.34. Found: C, 54.78; H, 5.44. NMR (2.5% solution in acetone) δ : 0.90 (3H, s, 18-CH₃), 6.66 (1H, s, 4-H), 7.54 (1H, s, 1-H).

2-Iodoestra-1,3,5(10)-trien-17 β -ol (Vb)—i) To a solution of estradiol (404 mg) in glacial AcOH (20 ml) was added dropwise a solution of Hg(OAc)₂ (123 mg) in AcOH (15 ml) saturated with I₂ (440 mg) over a period of 5 min and stirred at 45—55° for 17 hr. After removal of the precipitate by filtration the filtrate was poured into Na₂S₂O₃ solution. The precipitate was collected by filtration, washed with H₂O and dried. The crude product was submitted to preparative TLC using hexane-AcOEt (4:1) as developing solvent. After development was repeated five times, the adsorbent corresponding to the spot (*R_f* 0.32) was eluted with AcOEt. Recrystallization of the eluate from benzene and then from acetone gave Vb (56 mg) as colorless needles. mp 161—162°. $[\alpha]_D^{25} + 82.0^\circ$ ($c = 0.12$, CHCl₃). *Anal.* Calcd. for C₁₈H₂₃O₂I: C, 54.28; H, 5.82. Found: C, 54.26; H, 6.19. NMR (5% solution in acetone) δ : 0.78 (3H, s, 18-CH₃), 6.65 (1H, s, 4-H), 7.50 (1H, s, 1-H).

ii) To a solution of Va (15 mg) in MeOH (5 ml) was added portionwise NaBH₄ (5 mg) under ice-cooling and the resulting solution was allowed to stand at room temperature for 2 hr. After evaporation of solvent the residue was diluted with AcOEt, washed with H₂O and dried over anhydrous Na₂SO₄. On usual work-up a crystalline product was obtained. Recrystallization from acetone gave Vb (10 mg) as colorless prisms. mp 162—163.5°. Mixed melting point on admixture with the sample obtained in i) showed no depression.

12) H.E. Hadd and R.T. Blickenstaff, "Conjugates of Steroid Hormons," Academic Press, 1969, p.293.

13) D.S. Layne, "Metabolic Conjugation and Metabolic Hydrolysis," Vol. 1, ed. by W.H. Fishman, Academic Press, 1970, p. 43.

14) All melting points were taken on a micro hot-stage apparatus and are uncorrected. NMR spectra were recorded on Hitachi Model R-20 spectrometer using tetramethylsilane as an internal standard (TMS = 0.0 ppm). Mass spectral measurements were run on Hitachi Model RMU-6D spectrometer. For preparative TLC Silica Gel H (E. Merck AG) was used as an adsorbent.

2-Deuterio-3-hydroxyestra-1,3,5 (10)-trien-17-one (2-Deuterioestrone) (IIIa)—A solution of II (20 mg) in AcOEt (6 ml) was shaken with 5% Pd/BaCO₃ (40 mg) under a stream of D₂ gas for 15 hr. After removal of the catalyst by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from acetone gave IIIa (6 mg) as colorless needles. mp 252–254°. Mixed melting point on admixture with the authentic sample showed no depression. By mass spectrometry the deuterio steroid thus obtained was calculated to consist of 49.8% monodeuterio species along with 24.6% dideuterio and 25.6% normal estrone.

2-Deuterioestra-1,3,5 (10)-triene-3,17 β -diol (2-Deuterioestradiol) (IIIb)—A solution of Vb (or IV) (600 mg) in AcOEt (25 ml) was shaken with 5% Pd/BaCO₃ (1.4 g) under a stream of D₂ gas for 117 hr. After removal of the catalyst by filtration the filtrate was concentrated to give a crystalline product. Recrystallization from MeOH gave IIIb (554 mg) as colorless needles. mp 178–179°. Mixed melting point on admixture with the authentic sample showed no depression. By mass spectrometry the deuterio steroid thus obtained was calculated to consist of 72.2% monodeuterio species along with 7.3% dideuterio and 20.5% normal estradiol. NMR (4% solution in acetone) δ : 0.78 (3H, s, 18-CH₃), 6.48 (1H, s, 4-H), 7.03 (1H, s, 1-H).

Animal—Wistar strain male rats weighing *ca.* 200 g were housed in a cage that was designed to minimize fecal contamination of the urine.

Administration of 2-Deuterioestradiol (IIIb)—Each of five rats was intraperitoneally injected with a suspension of IIIb (50 mg) in Tween 80 and again with 34 mg of IIIb on the 2nd day. Urine was collected for the following 4 days after the initial administration.

Hydrolysis with β -Glucuronidase and Solvolysis—The pooled urine (173 ml) was adjusted to pH 4.5 with 50% H₂SO₄ and then to pH 4.6 with 0.1M acetate buffer (20 ml). After addition of Penicillin G (50000 U) the urine was incubated with beef-liver β -glucuronidase (Tokyo Zōkikagaku, Co.) (51900 Fishman U) at 37° for 5 days. The hydrolyzate was then brought to 2N H₂SO₄ solution with 50% H₂SO₄, saturated with NaCl (20 g/100 ml) and extracted with AcOEt. The organic phase was separated and allowed to stand at 37° for 12 hr. The extract was washed with 5% NaHCO₃, H₂O and dried over anhydrous Na₂SO₄. After evaporation of solvent a gummy residue was submitted to preparative TLC using benzene-ether (4:1) as developing solvent. The adsorbent corresponding to the spot (*R_f* 0.13) was eluted with CHCl₃-MeOH (10:1). The eluate was again chromatographed on TLC plate with use of hexane-CHCl₃-AcOH (2:2:1). Elution of the zone corresponding to the spot (*R_f* 0.60) gave 2-hydroxyestrone (VI) as colorless amorphous substance (5 mg). When trimethylsilylated in the usual manner and then submitted to gas chromatography using 3% SE-30 on a support of Chromosorb W (60–80 mesh) as a stationary phase, the metabolite showed the identical retention time (10.0 min) with that of the authentic sample. The gas chromatographic conditions employed were as follows: the column, injection chamber and detector were kept at 215°, 250° and 250°, respectively, and the flow rate of carrier gas (N₂) was 60 ml/min.

Acknowledgement The authors are indebted to all the staffs of central analytical laboratory of this Institute for elemental analyses, mass and nuclear magnetic resonance spectral measurements. Thanks are also due to Showa Denko Co., Ltd. for generous supply of deuterium oxide.