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Synthesis of New Haptens for Radioimmunoassay of 2- and 4-Hydroxyestradiol

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For the purpose of obtaining antisera for radioimmunoassay of 2- and 4-hydroxyestradiol, four haptens possessing a carboxyl group have been synthesized. 6-Oxo-2-hydroxyestradiol and 6-oxo-4-hydroxyestradiol were synthesized from estradiol in several steps. The condensation of 6-oxo catechol estrogens with *O*-carboxymethylhydroxylamine provided the desired 6-(*O*-carboxymethyl)oximes as new haptens. The preparation of 2- and 4-hydroxyestradiol 17-hemisuccinates as another type of hapten was also carried out by using succinic anhydride and pyridine.

Keywords——carboxymethyloxime; catechol estrogen; hapten for radioimmunoassay; hemisuccinate; 2-hydroxyestradiol; 4-hydroxyestradiol

The ring A hydroxylation at C-2 or C-4 is a quantitatively major metabolic step of primary estrogen and leads to the formation of 2-hydroxyestrogens and 4-hydroxyestrogens.[1] Evidence for the physiological roles of these catechol estrogens, especially in the regulation of gonadotropin and prolactin secretions, is accumulating.[2] Radioimmunoassay is a method of choice for the measurement of many naturally occurring steroid hormones in body fluids. Since the first report by Yoshizawa and Fishman, several attempts have been made to prepare anti-catechol estrogen antisera for use in radioimmunoassay by coupling the hapten to the carrier protein.[3] In almost all the reported experiments, antigens in which the steroid molecule was coupled to a carrier through the 17-(*O*-carboxymethyl)oxime of ring D were employed for immunization. Therefore, the antisera so far obtained were not fully satisfactory in respect of the specificity. For instance, anti-2-hydroxyestrone antisera showed significant cross-reactivity with 2-hydroxyestradiol. The specificity of such antibodies is strongly influenced by the position on the steroid molecule linked to an immunogenic carrier protein.[4] The requirement for much more specific antisera for the assay of 2- and 4-hydroxyestradiol prompted us to develop new haptens. In this paper we report the syntheses of 6-oxo-2-hydroxyestradiol and 6-oxo-4-hydroxyestradiol 6-(*O*-carboxymethyl)oximes. In addition, in order to investigate the specificity of the antisera produced by another type of hapten-bovine serum albumin (BSA) conjugate, 2- and 4-hydroxyestradiol 17-hemisuccinates were also synthesized.

Our initial effort was directed to the synthesis of 6-oxo-2-hydroxyestradiol 6-(*O*-carboxymethyl)oxime (IIIa) as a new type of hapten of catechol estrogen. For this purpose, 6-oxo-2-hydroxyestradiol (IIa) was prepared from estradiol in several steps employing the methods worked out by Nakagawa *et al.*[5] Condensation of IIa with *O*-carboxymethylhydroxylamine under ascorbic acid protection to avoid oxidative decomposition of the labile catechol estrogen proceeded readily to afford IIIa in a satisfactory yield. The structure of IIIa was confirmed by inspection of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, which showed the methylene protons of the *O*-carboxymethyl group at 4.57 ppm and the aromatic ring protons as two singlets at 6.69 and 7.24 ppm. The results of other instrumental analyses of this material also supported this structure. Next, the preparation of 6-oxo-4-hydroxyestradiol 6-(*O*-carboxymethyl)oxime (IIIb) was carried out in a similar fashion. Recently, a convenient

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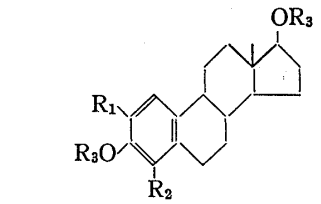
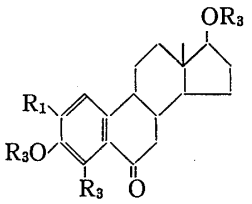
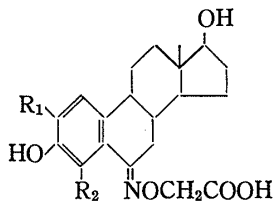


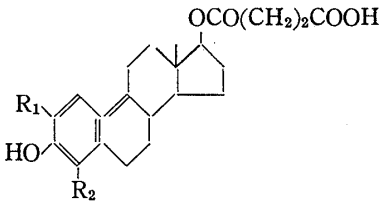
Ia: R_1=OH, R_2=R_3=H
Ib: R_1=R_3=H, R_2=OH
Ic: R_1=H, R_2=OAc, R_3=Ac



IIa: R_1=OH, R_2=R_3=H
IIb: R_1=H, R_2=OAc, R_3=Ac
IIc: R_1=R_3=H, R_2=OH



IIIa: R_1=OH, R_2=H
IIIb: R_1=H, R_2=OH



IVa: R_1=OH, R_2=H
IVb: R_1=H, R_2=OH

Chart 1

method for introducing a hydroxyl group into C-2 or C-4 of the aromatic ring A has been developed by Stubenrauch *et al.*[6] This finding was applied to the synthesis of 4-hydroxyestradiol (Ib) as a starting compound. For the preparation of the 6-keto derivative, protection of the hydroxyl moiety is a prerequisite. When treated with acetic anhydride and pyridine in the usual manner, Ib was transformed into the triacetate (Ic). Oxidation of Ic with chromium trioxide in acetic acid[7] provided the 6-oxo derivative (IIb). Simultaneous removal of the acetyl groups at C-3, C-4, and C-17 by exposure to sulfuric acid in methanol under mild conditions furnished the desired 6-oxo-4-hydroxyestradiol (IIc) in a fairly good yield. Accordingly, IIc was converted to the 6-(*O*-carboxymethyl)oxime (IIIb).

The synthesis of another type of hapten was also undertaken. By refluxing it with succinic anhydride in pyridine,[8] Ia was converted into a mixture of the 2,3,17-trihemisuccinate and 2 (or 3), 17-dihemisuccinate. Isolation of these compounds was unsuccessful because of instability of the succinoyl groups at the C-2 and C-3 positions. After selective saponification of the phenolic succinoyl esters by use of sodium bicarbonate in methanol, the crude product was purified by column chromatography on silica gel loaded with ascorbic acid[9] to furnish 2-hydroxyestradiol 17-hemisuccinate (IVa). In the ^{1}H-NMR spectrum of IVa, the four methylene protons appeared as one singlet at 2.69 ppm, supporting the location of the newly introduced succinoyl moiety at C-17. 4-Hydroxyestradiol 17-hemisuccinate (IVb) was also synthesized by a similar method from 4-hydroxyestradiol (Ib).

Evaluation of the specificity of the antisera raised against these four hapten-BSA conjugates is currently under way. The details of the preparation and antigenic properties of the hapten-BSA conjugates will be reported elsewhere in the near future.

Experimental

All melting points were taken on a Yanagimoto micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO Model DIP-4 digital polarimeter in MeOH unless otherwise specified. Ultraviolet (UV) spectra were obtained on a Hitachi Model 323 recording spectrophotometer and infrared (IR) spectra on a JASCO Model A-102 diffraction grating infrared spectrophotometer. Mass spectral (MS) measurements were run on a JEOL JMS-D 100 instrument. ^{1}H-NMR spectra were recorded using tetramethylsilane as an internal standard on a JEOL FX-100 spectrometer at 100 MHz.

2,3,17β-Trihydroxy-1,3,5(10)-estratrien-6-one 6-(*O*-Carboxymethyl)oxime (IIIa)——*O*-Carboxymethylhydroxylamine·HCl (120 mg) was added to a solution of 6-oxo-2-hydroxyestradiol (IIa) (100 mg) in EtOH

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(6.2 ml) containing ascorbic acid buffer (pH 10.5)[3b] (0.26 ml), and the mixture was refluxed for 1.5 h under nitrogen. The mixture was acidified by adding a few drops of AcOH, then EtOH was removed *in vacuo* and H_2O (2 ml) was added to the oily residue. The reaction mixture was extracted with AcOEt, washed with H_2O and dried over anhydrous Na_2SO_4. After evaporation of the solvent the crude product obtained was recrystallized from MeOH–AcOEt to give IIIa (96 mg) as colorless prisms. mp 155—156°C. $[\alpha]_D^{27}$ +46.7° (c=0.10). *Anal.* Calcd for $C_{20}H_{25}NO_6 \cdot 1/2H_2O$: C, 62.48; H, 6.81; N, 3.64. Found: C, 61.95; H, 6.79; N, 3.44. UV λ_{max}^{MeOH} nm: 272, 312. IR ν_{max}^{KBr} cm^{-1}: 3600—3100 (–OH), 1710 (–COOH), 1590 (>C=N–). ^{1}H-NMR ($(CD_3)_2SO$) δ: 0.64 (3H, s, 18-CH_3), 3.52 (1H, m, 17α-H), 4.57 (2H, s, –OCH_2CO–), 6.69 (1H, s, 1-H), 7.24 (1H, s, 4-H).

1,3,5(10)-Estratriene-3,4,17β-triol Triacetate (Ic)——Treatment of 4-hydroxyestradiol (Ib) (1 g) with Ac_2O (25 ml) and pyridine (25 ml) was carried out in the usual manner and the crude product obtained was chromatographed on silica gel (65 g) using hexane-AcOEt (3: 1). The eluate was recrystallized from MeOH to give Ic (1.1 g) as colorless prisms. mp 202—204°C. $[\alpha]_D^{27}$ +28.6° (c=0.25, $CHCl_3$). *Anal.* Calcd for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.64; H, 7.36. MS m/e: 414 (M^+), 372, 330. UV λ_{max}^{EtOH} nm: 264. IR ν_{max}^{KBr} cm^{-1}: 1770 (>C=O), 1725 (>C=O). ^{1}H-NMR ($CDCl_3$) δ: 0.82 (3H, s, 18-CH_3), 2.06 (3H, s, 17β-$OCOCH_3$), 2.27 (3H, s, 3- or 4-$OCOCH_3$), 2.30 (3H, s, 4- or 3-$OCOCH_3$), 4.69 (1H, m, 17α-H), 6.96 (1H, d, J=8.8 Hz, 2-H), 7.21 (1H, d, J=8.8 Hz, 1-H).

3,4,17β-Trihydroxy-1,3,5(10)-estratrien-6-one Triacetate (IIb)——A solution of CrO_3 (900 mg) in H_2O (0.7 ml)–AcOH (5 ml) was added dropwise to a solution of Ic (1 g) in AcOH (7 ml), and the mixture was stirred at room temperature for 7.5 h. The excess reagent was then reduced with EtOH (2 ml). After dilution with H_2O, the reaction mixture was extracted with AcOEt and the extract was washed with 1% $NaHCO_3$, then dried over anhydrous Na_2SO_4. Removal of AcOEt gave an oily residue, which was chromatographed on silica gel (50 g) using hexane–AcOEt (3: 1). The eluate was recrystallized from MeOH to give IIb (180 mg) as colorless needles. mp 233—236°C. $[\alpha]_D^{27}$ −14.6° (c=0.12, $CHCl_3$). *Anal.* Calcd for $C_{24}H_{28}O_7$: C, 67.27; H, 6.59. Found: C, 66.87; H, 6.56. MS m/e: 428 (M^+), 386, 344. UV λ_{max}^{EtOH} nm: 248, 301. IR ν_{max}^{KBr} cm^{-1}: 1785 (>C=O), 1723 (>C=O), 1680 (>C=O). ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, s, 18-CH_3), 2.06 (3H, s, 17β-$OCOCH_3$), 2.31 (3H, s, 3- or 4-$OCOCH_3$), 2.37 (3H, s 4- or 3-$OCOCH_3$), 4.70 (1H, m, 17α-H), 7.35 (2H, s, 1-H and 2-H).

3,4,17β-Trihydroxy-1,3,5(10)-estratrien-6-one (IIc)——A solution of IIb (120 mg) in MeOH (30 ml) was treated with 13% H_2SO_4 (10 ml) and the mixture was allowed to stand at room temperature for 45 h, then diluted with H_2O saturated with NaCl. MeOH was evaporated off, and the remaining solution was extracted three times with AcOEt. The organic phase was washed twice with 1% AcOH and dried over anhydrous Na_2SO_4. After evaporation of the solvent the crude product obtained was chromatographed on silica gel (42 g) impregnated with ascorbic acid[9] using $CHCl_3$–MeOH–AcOH (99: 1: 1). The eluted product was recrystallized from AcOEt to give IIc (85 mg) as pale yellow needles. mp 194—197°C. $[\alpha]_D^{20}$ −7.5° (c=0.07). *Anal.* Calcd for $C_{18}H_{22}O_4$: C, 71.50; H, 7.33. Found: C, 70.80; H, 7.17. MS m/e: 302 (M^+). UV λ_{max}^{MeOH} nm: 273, 288, 362. IR ν_{max}^{KBr} cm^{-1}: 3550—3300 (–OH), 1605 (>C=O). ^{1}H-NMR ($(CD_3)_2SO$) δ: 0.66 (3H, s, 18-CH_3), 3.53 (1H, m, 17α-H), 6.73 (1H, d, J=8.1 Hz, 1-H), 7.02 (1H, d, J=8.1 Hz, 2-H).

3,4,17β-Trihydroxy-1,3,5(10)-estratrien-6-one 6-(*O*-Carboxymethyl)oxime (IIIb)——*O*-Carboxymethylhydroxylamine·HCl (55 mg) was added to a solution of IIc (45 mg) in EtOH (2.8 ml) containing ascorbic acid buffer (pH 10.5)[3b] (0.12 ml), and the mixture was refluxed for 1.5 h under nitrogen. The reaction mixture was acidified by adding a few drops of AcOH, then EtOH was removed *in vacuo* and H_2O (2 ml) was added to the oily residue. The mixture was extracted with AcOEt, and the extract was washed with H_2O and dried over anhydrous Na_2SO_4. After evaporation of the solvent an oily residue obtained was recrystallized from MeOH–AcOEt to give IIIb (50 mg) as pale yellow needles. mp 226—227°C (dec.). $[\alpha]_D^{18}$ −46.9° (c=0.10). *Anal.* Calcd for $C_{20}H_{25}NO_6 \cdot 1/2H_2O$: C, 62.48; H, 6.81; N, 3.64. Found: C, 62.99; H, 6.66; N, 3.72. MS m/e: 375 (M^+). UV λ_{max}^{MeOH} nm: 273, 331. IR ν_{max}^{KBr} cm^{-1}: 3550—3300 (–OH), 1720 (–COOH), 1600 (>C=N–). ^{1}H-NMR ($(CD_3)_2SO$) δ: 0.64 (3H, s, 18-CH_3), 3.52 (1H, m, 17α-H), 4.73 (2H, s –OCH_2CO–), 6.70 (1H, d, J=8.3 Hz, 1-H), 6.84 (1H, d, J=8.3 Hz, 2-H).

1,3,5(10)-Estratriene-2,3,17β-triol 17-Hemisuccinate (IVa)——Succinic anhydride (300 mg) was added to a solution of 2-hydroxyestradiol (Ia) (100 mg) in pyridine (20 ml), and the mixture was refluxed for 14 h. The reaction mixture was concentrated and then extracted with AcOEt. The organic phase was washed with NaCl-saturated aq. solution and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product obtained was dissolved in MeOH (30 ml) and 5% $NaHCO_3$ (40 ml) was added. The mixture was allowed to stand at room temperature for 15 min, then 10% AcOH (15 ml) was added and most of the MeOH was removed *in vacuo*. This solution was extracted with AcOEt, and the extract was washed with H_2O and dried over anhydrous Na_2SO_4. After usual work-up, an oily product was obtained and chromatographed on silica gel (70 g) impregnated with ascorbic acid using cyclohexane–AcOEt–EtOH–AcOH (65: 32: 2: 1). The eluted product was recrystallized from cyclohexane–AcOEt to give IVa (90 mg) as colorless needles. mp 152—154°C. $[\alpha]_D^{25}$ +52.5° (c=0.29). *Anal.* Calcd for $C_{22}H_{28}O_6$: C, 68.02; H, 7.27. Found: C, 67.99; H, 7.24. MS m/e: 388 (M^+), 288. UV λ_{max}^{MeOH} nm: 289. IR ν_{max}^{KBr} cm^{-1}: 3550—3200 (–OH), 1710 (–COOH, >C=O). ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 2.69 (4H, s, –$CO(CH_2)_2CO$–), 4.75 (1H, m, 17α-H), 6.59 (1H, s, 4-H), 6.79 (1H, s, 1-H).

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1,3,5(10)-Estratriene-3,4,17β-triol 17-Hemisuccinate (IVb)——Succinic anhydride (600 mg) was added to a solution of 4-hydroxyestradiol (Ib) (200 mg) in pyridine (23 ml), and the mixture was refluxed for 7 h. The reaction mixture was concentrated and then extracted with AcOEt. The organic phase was washed with NaCl-saturated aq. solution and dried over anhydrous Na_2SO_4. After evaporation of solvent, the crude product obtained was dissolved in MeOH (50 ml) and 2% $NaHCO_3$ (35 ml) was added. The mixture was allowed to stand at room temperature for 2 h, then 10% AcOH (12 ml) was added and most of the MeOH was removed *in vacuo*. This solution was extracted with AcOEt, and the extract was washed with H_2O and dried over anhydrous Na_2SO_4. After usual work-up, an oily product was obtained and chromatographed on silica gel (50 g) impregnated with ascorbic acid using $CHCl_3$–MeOH–AcOH (99: 1: 1). The eluted product was recrystallized from hexane–AcOEt to give IVb (140 mg) as colorless needles. mp 219—221°C. $[\alpha]_D^{18}$ +34.7° (c=0.18). *Anal.* Calcd for $C_{22}H_{28}O_6$: C, 68.02; H, 7.27. Found: C, 67.45; H, 7.25. MS *m/e*: 388 (M^+), 288. UV λ_{max}^{MeOH} nm: 281. IR ν_{max}^{KBr} cm^{-1}: 3500—3200 (–OH), 1725 (–COOH), 1690 (>C=O). ^{1}H-NMR ($CDCl_3$–CD_3OD (2: 1)) δ: 0.85 (3H, s, 18-CH_3), 2.65 (4H, s, $-CO(CH_2)_2CO-$), 4.68 (1H, m, 17α-H), 6.67 (2H, s, 1-H and 2-H).

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References and Notes

1) P. Ball and R. Knuppen, *Acta Endocrinol. Suppl.*, **232**, 1 (1980).
2) J. Fishman, *J. Endocrinol.*, **89**, 59P (1981).
3) *a*) I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, **32**, 3 (1971); *b*) P. Ball, G. Emons, O. Haupt, H.-O. Hoppen, and R. Knuppen, *Steroids*, **31**, 249 (1978); *c*) S.C. Chattoraj, A.S. Fanous, D. Cecchini, and E.W. Lowe, *Steroids*, **31**, 375 (1978); *d*) D. Berg and E. Kuss, *Hoppe-Seyler's Z. Physiol. Chem.*, **360**, 1683 (1979); *e*) P. Ball, G. Reu, J, Schwab, and R. Knuppen, *Steroids*, **33**, 563 (1979); *f*) G. Emons, C. Mente, R. Knuppen, and P. Ball, *Acta Endocrinol.*, **97**, 251 (1981).
4) T. Nambara, M. Numazawa, Y. Tsuchida, and T. Tanaka, *Chem. Pharm. Bull.*, **24**, 1510 (1976).
5) A. Nakagawa, R. Oh'uchi, and I. Yoshizawa, *Chem. Pharm. Bull.*, **26**, 3567 (1978).
6) G. Stubenrauch and R. Knuppen, *Steroids*, **28**, 733 (1976).
7) B. Longwell and O. Wintersteiner, *J. Biol. Chem.*, **133**, 219 (1940).
8) T. Nambara, M. Takahashi, and M. Numazawa, *Chem. Pharm. Bull.*, **22**, 1167 (1974).
9) H.P. Gelbke and R. Knuppen, *J. Chromatogr.*, **71**, 465 (1972).

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