SYNTHESIS OF NEW STEROID HAPTENS FOR RADIOIMMUNOASSAY. PART IV. 3-O-CARBOXYMETHYL ETHER DERIVATIVES OF ESTROGENS. SPECIFIC ANTISERA FOR RADIOIMMUNOASSAY OF ESTRONE, ESTRADIOL-17β, AND ESTRIOL.

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Received: 12/7/76

ABSTRACT

The syntheses of 3-O-carboxymethyl ether derivatives of estrone, estradiol-17β, and estriol and the preparation of their bovine serum albumin (BSA) conjugates are described. These conjugates were employed for the generation of specific antisera suitable for radioimmunoassay (RIA) of estrone, estradiol-17β, and estriol. The previous concept that specific antisera for estrogens cannot be obtained by employing estrogens derivatized at the 3-position is unfounded.

INTRODUCTION

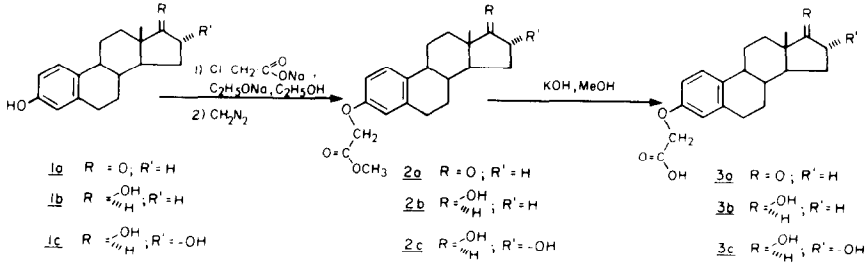
It is well recognized that in order to generate antisera with greater specificity to steroid hormones, it is important to employ conjugates in which the steroid is coupled to carrier protein through a position distal to the sites of unique functional groups (1). The estrogenic hormones estrone (E_1), estradiol-17β (E_2), and estriol (E_3) all contain a phenolic ring A, but differ from each other in having characteristic functional groups in ring D. It appeared possible to generate specific antisera to individual estrogens by employing a C-3 coupled steroid-protein conjugate, which allows the induced antibody to recognize the structural differences present in ring D. Thorneycroft *et al.* (2), as well as Niswender and Midgley (3), observed that antisera raised by employing estradiol-17β-3-hemisuccinyl-BSA conjugate exhibited a high degree of cross-reaction with estrone and estriol. On the other hand, Dray (4) presented data indicating that highly specific antisera for estrone can be obtained by employing estrone-3-hemisuccinyl-BSA conjugate. We found that earlier investigators (3,5) had not completely characterized the estradiol-17β-3-hemisuccinate, and the product they employed could have been an isomeric mixture of 3 and 17β-monohemisuccinates. Use of such a contaminated mixture can lead to nonspecific anti-estradiol-17β serum.

In order to determine how the site of conjugation affects the specificity of antibodies to individual estrogens, we decided to synthesize by an unequivocal method estrogen derivatives with a different "chemical handle" at the C-3 position, prepare their BSA conjugates, and generate antisera for E_1, E_2, and E_3. This communication describes the syntheses of 3-O-carboxymethyl ether derivatives of E_1, E_2, and E_3, preparation of their BSA conjugates, and the generation of specific antisera for the three estrogens.

MATERIALS AND METHODS

Solvents and reagents: All solvents, reagents, and unlabeled steroids were purchased and purified as previously reported in Part I (6). Estrone-[2,4,6,7-^{3}H], estradiol-17β-[2,4,6,7-^{3}H], and estriol-[2,4,6,7-^{3}H], with specific activities of 85-105 Ci/mmole were obtained from New England Nuclear Corp. Mass spectral data were generated with a Finnigan 1015 F-L Quadrapole mass spectrometer or with a Hewlett-Packard 5980A mass spectrometer. All other spectra were obtained as described earlier (6). Dry column chromatography was performed on Woelm silica gel in a nylon column as described by Loev and Goodman (7). The microanalyses were performed by Micro-Tech Laboratories, Skokie, Illinois.

Synthesis of Steroid Haptens



Reaction of estrone (1a) with the sodium salt of chloroacetic acid in the presence of sodium ethoxide in ethanol yielded the 3-O-carboxymethyl ether derivative which, on subsequent esterification with diazomethane, gave the methyl ester (2a) in good yield. We found it advantageous to prepare the methyl ester 2a and then purify it by chromatography. The purified ester 2a was hydrolyzed by refluxing with methanolic potassium hydroxide to give estrone 3-O-carboxymethyl ether (3a). Similarly, reaction of estradiol-17β (1b) and estriol (1c) with sodium chloroacetate in the presence of sodium ethoxide gave the desired 3-O-carboxymethyl ether derivatives which were purified by preparing their methyl esters 2b and 2c. Finally alkaline hydrolysis of the methyl esters 2b and 2c led to estradiol-17β 3-O-carboxymethyl ether 3b and estriol O-carboxymethyl ether 3c, respectively.

Mass spectral data (Table I) provided conclusive evidence and confirmed the structures assigned to the methyl esters 2a, 2b, and 2c as well as the carboxylic acids 3a, 3b, and 3c. As expected, in each case the molecular ion (M^+) is the most intense peak (base peak) and all spectra exhibited characteristic fragment ions corresponding to the loss of ring D (8) as indicated: M-57 for estrone derivatives 2a and 3a; M-59 for estradiol-17β derivatives 2b and 3b; M-75 for estriol derivatives 2c and 3c. The spectra of methyl esters 2a, 2b, and 2c exhibited peaks corresponding to M-73 fragment ion (loss of $CH_2 \cdot COOCH_3$) and the carboxylic acids 3a, 3b, and 3c showed peaks corresponding to M-59 fragment ion (loss of $CH_2 \cdot COOH$).

Table I
Principal Fragmentations in the Mass Spectrum

Compound	Molecular Ion M^+ m/e	Base Peak m/e	M-57 Peak m/e	M-59 Peak m/e	M-73 Peak m/e	M-75 Peak m/e
2a	342	342	285		269	
2b	344	344		285	271	
2c	360	360			287	285
3a	328	328	271	269		
3b	330	330		271*		
3c	346	346		287		271

* The relative intensity of the peak is higher due to the two fragments obtained from loss of ring D as well as CH_2 COOH.

The experimental details for the preparation of the 3-O-carboxymethyl ether derivative of estrone 3a are presented below. By using the same molar quantities of reactants and identical reaction conditions, the 3-O-carboxymethyl ether derivatives of E_2 and E_3 were prepared and their physical constants determined.

Preparation of 3-hydroxyestra-1,3,5(10)-trien-17-one 3-O-methoxycarbonylmethyl ether 2a

To a stirred solution of sodium ethoxide prepared by dissolving sodium (0.23 g, 0.1 mole) in absolute ethanol (35 ml), estrone (1 g, 0.0037 mole) was added and heated under reflux. After 10 minutes, sodium chloroacetate (0.5 g, 0.004 mole) was introduced and the heating continued for 22 hours. The ethanol was evaporated under a stream of nitrogen, the residue diluted with water (50 ml), and extracted with ethyl acetate. From the ethyl acetate extract, unreacted estrone (0.8 g) was recovered. The aqueous solution was acidified with hydrochloric acid to pH 2 and then extracted with ethyl acetate. The ethyl acetate extract was dried over sodium sulfate, filtered, and the solvent evaporated to give the acidic material (100 mg). The residue so obtained was dissolved in methanol (1.5 ml) and esterified by reacting with an ether solution of diazomethane to give the methyl ester 2a. It was then purified on a dry silica gel column (20 X 500 mm) employing ether:hexane (7:3) as the developing solvent and

the chromatographed material crystallized from ether-petroleum ether (b.p. 40–60°C) to yield pure methyl ester 2a (70 mg), m.p. 123–124°C; ν_{max} 1760, 1730, 1607, 1580, 1500, and 1215 cm^{-1}. Elemental analysis: C, 73.63; H, 7.82%. ($C_{21}H_{26}O_4$ requires C, 73.66; H, 7.65%).

3-Hydroxyestra-1,3,5(10)-trien-17-one 3-O-carboxymethyl ether 3a

The methyl ester 2a (70 mg) was dissolved in methanol (20 ml) and an aqueous solution of potassium hydroxide (10% solution, 2 ml) was added and heated under reflux for 1 hour. The methanol was evaporated, the residue diluted with water (10 ml), and acidified with hydrochloric acid to pH 2. The organic material was extracted with ethyl acetate and the ethyl acetate extract washed with brine, dried over sodium sulfate, filtered, and the solvent evaporated to give a solid residue which was purified by crystallization from ether-petroleum ether (b.p. 40–60°C) to give 3a (64 mg), m.p. 208–209°C; ν_{max} 3420, 1730, 1610, 1585, and 1500 cm^{-1}; λ_{max} 278 nm (ϵ 1755), 285 nm (ϵ 1606). Elemental analysis: C, 72.71; H, 7.51%. ($C_{20}H_{24}O_{44} \cdot 1/8\ H_2O$ requires C, 72.65; H, 7.39%).

Estra-1,3,5(10)-triene-3,17β-diol 3-O-methoxycarbonylmethyl ether 2b

Physical constants: m.p. 104-105°C; ν_{max} 3450, 1750, 1610, 1580, 1500, and 1215 cm^{-1}. Elemental analysis: C, 73.11; H, 8.13% ($C_{21}H_{28}O_4$ requires C, 73.23; H, 8.10%).

Estra-1,3,5(10)-triene-3,17β-diol 3-O-carboxymethyl ether 3b

Physical constants: m.p. 192–193°C; ν_{max} 3400, 1730, 1608, 1575, and 1500 cm^{-1}; λ_{max} 278 nm (ϵ 1680), 285 nm (ϵ 1543). Elemental analysis: C, 72.70; H, 8.00% ($C_{20}H_{26}O_4$ requires C, 72.70; H, 7.93%).

Estra-1,3,5(10)-triene-3,16α,17β-triol 3-O-methoxycarbonylmethyl ether 2c

Physical constants: m.p. 137–139°C; ν_{max} 3400, 1760, 1740, 1615, 1580, 1505, and 1220 cm^{-1}. Elemental analysis: C, 69.58; H, 7.84%. ($C_{21}H_{28}O_5$ requires C, 69.98; H, 7.83%).

Estra-1,3,5(10)-triene-3,16α,17β-triol 3-O-carboxymethyl ether 3c

Physical constants: m.p. 243–246°C (dec.); ν_{max} 3360, 1730, 1615, 1580, and 1510 cm^{-1}. λ_{max} 278 nm (ϵ 1701), 285 nm (ϵ 1545). Elemental analysis: C, 68.63; H, 7.61%. ($C_{20}H_{26}O_5 \cdot 1/5\ H_2O$ requires C, 68.62; H, 7.6%).

Preparation of the Steroid-Bovine Serum Albumin Conjugates and Determination of the Moles of Steroid Bound Per Mole of Protein:

The three 3-O-carboxymethyl ether derivatives of E_1 (3a, 0.152 mmoles), E_2 (3b, 0.605 mmoles), and E_3 (3c, 0.866 mmoles) were coupled to BSA (equivalent to 0.003, 0.121, and 0.17 mmoles, respectively, of available amino groups) by the mixed anhydride procedure (9) with our modification described earlier (6).

The number of moles of steroid bound per mole of protein was obtained by ultraviolet absorption spectrometry (9) as well as by determination of free amino groups in the conjugate by a quantitative ninhydrin procedure (10), and the data are presented in Table II.

Table II
Moles of Steroid Per Mole of Conjugate

Conjugate	U.V. Method	Ninhydrin Method
Estrone 3-O-carboxymethyl ether-BSA	23	21
Estradiol-17β 3-O-carboxymethyl ether-BSA	21	20
Estriol 3-O-carboxymethyl ether-BSA	20	17

Immunization procedure and collection of the antibody: A group of five male New Zealand white rabbits (4 months old) was used for immunization with each of the three different conjugates. A primary injection (2 mg) of the conjugate in isotonic saline, mixed with Freund's complete adjuvant (1:1) for a final dilution of 1 mg/ml, was divided into four equal portions and injected intramuscularly into each thigh and below each shoulder blade. Intramuscular injections of 0.5 mg into each thigh were repeated 7, 14, and 21 days after the initial injections, and every 30 days thereafter. Plasma was collected 14 days after the third booster injection and every 30 days thereafter.

Standard curves: A standard curve was established in each case by setting up duplicate centrifuge tubes (3 ml) containing 0, 50, 100, 250, 1000, and 2000 pg of the steroid in a total volume of 0.5 ml of sodium phosphate assay buffer (0.1 M, pH 7, 0.9% NaCl, 0.1% sodium azide). The standards were prepared from a stock solution of unlabeled steroid in absolute ethanol (100 ng/ml). The labeled steroids were prepared in assay buffer at a concentration of 50 pg/0.5 ml. The antiserum was prepared in BSA assay buffer (1 g BSA/1000 ml sodium phosphate buffer) at a concentration equal to five times the final working dilution. The antibody (0.25 ml) and the labeled steroid (0.50 ml) were added to all standard tubes containing 0.5 ml assay buffer; the tubes were mixed and allowed to incubate at 4°C overnight. 0.2 ml of cold γ-globulin dextran-coated charcoal (1 g charcoal, 0.1 g dextran, 0.2 g γ-globulin; 200 ml deionized water), was added to each tube, mixed, and returned to the cold room for 20 minutes. After centrifugation at 2500 rpm for 6 minutes, 0.5 ml of each supernatant was aliquoted into a counting vial. Then 15 ml of scintillation medium (4 g PPO, 50 mg dimethyl-POPOP, 100 ml Bio-Solv BBS-3, 1000 ml toluene) was added to each vial. The samples were counted to a relative standard error of less than 2% in a Packard Model 3320 liquid scintillation counter.

RESULTS AND DISCUSSION

Of the 15 rabbits immunized with various estrogen-BSA conjugates, five died early in the program due to contagious infection. In 3 months' time all the remaining

rabbits produced antisera with usable titers. The titer was determined from the ability of the antibody to bind a constant amount (50 pg) of the labeled steroid as compared to plasma collected prior to the primary injection. All plasma was treated with Rivanol (11) prior to titer assessment. The low titer values observed with anti-E_2 serum did not improve even after prolonged immunization. Individual antiserum from each rabbit differed and exhibited varying degrees of sensitivity and specificity. As a preliminary step, we evaluated the cross reactivity of structurally similar steroids with each antiserum using the method of Abraham (11). With anti-E_1 serum we studied the cross-reactivity of E_2; with anti-E_2 serum we studied the cross-reactivity of E_1 and 2-hydroxy-E_2; with anti-E_3 serum we studied the cross-reactivity of E_1 and E_2. This preliminary evaluation allowed us in each case to select the antiserum which exhibited lowest cross-reactivity with sufficiently high titer and sensitivity for complete characterization. The final working dilution (titer), the antibody production time, and the binding affinity of the three different antisera are presented in Table III.

Table III
Antibody Characterization

Antibody	Titer	Production Time	Binding Affinity
Anti-E_1	1:80,000	3 months	9.24×10^9 L/M
Anti-E_2	1: 1,000	3 months	3.41×10^9 L/M
Anti-E_3	1:10,000	3 months	4.30×10^9 L/M

In establishing the binding affinity, varying nanomolar concentrations of the labeled steroid were incubated with a constant volume and dilution of antibody at 4°C for 18 hours. By varying only the labeled steroid, a relationship between the nanomolar concentration and the bound and free fractions was established and evaluated as a Scatchard plot (12). As seen in Figure 1, the linearity of the Scatchard plots in each case suggests that the antibodies are essentially homogeneous with respect to the binding of the specific estrogen under consideration.

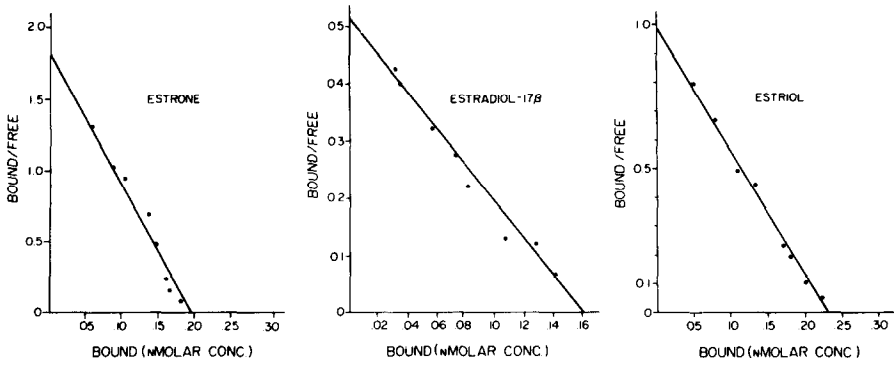


Figure 1. Binding Affinity Determination

The percent cross-reaction of each antibody is presented in Table IV. Standard curves were established over the desired working range and compared to the inhibition curves of pertinent and structurally similar steroids.

Table IV
Cross Reactivity Data

Steroid	Estrone Antibody	Estradiol-17β Antibody	Estriol Antibody
1. Estrone	100.00	0.00	0.00
2. Estradiol-17β	0.00	100.00	0.00
3. Estriol	0.88	0.60	100.00
4. 2-Methoxyestrone	7.30	0.00	0.00
5. 2-Methoxyestradiol-17β	0.00	7.09	0.00
6. 2-Methoxyestriol	0.00	0.00	4.04
7. 2-Hydroxyestrone	3.33	0.00	0.00
8. 2-Hydroxyestradiol-17β	0.00	0.51	0.00
9. 2-Hydroxyestriol	0.00	0.00	0.00
10. 6-Oxoestrone	0.00	0.00	0.00
11. 6-Oxoestradiol-17β	0.00	15.02	0.00
12. 6-Oxoestriol	0.00	0.00	0.00
13. 6β-Hydroxyestradiol-17β	0.00	1.67	0.00
14. 6α-Hydroxyestriol	0.00	0.00	4.34
15. 16α-Hydroxyestrone	0.33	0.00	0.00
16. 16-Epiestriol	0.18	3.50	3.73
17. Estradiol-17α	0.00	0.00	0.00
18. Testosterone	0.00	0.00	0.00
19. Androstenedione	0.00	0.00	0.00
20. Progesterone	0.00	0.00	0.00
21. 17α-Hydroxyprogesterone	0.00	0.00	0.00
22. Cortisol	0.00	0.00	0.00

Anti-estrone serum: The antiserum did not cross-react with estradiol-17β and exhibited less than 1% cross-reaction with estriol. The only other estrogens that showed some cross-reaction included 2-methoxyestrone (7.3%) and 2-hydroxyestrone (3.3%). This is expected because the C-2 substituent in these compounds is too close to the site of conjugation at C-3, and the induced antibody could not clearly distinguish the structural features.

Anti-estradiol-17β serum: The antiserum showed no cross-reaction with estrone and exhibited less than 1% cross-reaction with estriol. Other estrogens that showed some cross-reaction included 6-oxoestradiol-17β (15%), 2-methoxyestradiol-17β (7%), 16-epiestriol (3.5%), and 6β-hydroxyestradiol-17β (1.7%). The particular experimental situation will have to determine whether these cross-reactions affect the measurement of estradiol-17β in a significant manner.

Anti-estriol serum: The antiserum did not cross-react with either estrone or estradiol-17β. Other estrogens which showed slight cross-reaction included 2-methoxyestriol (4%), 6α-hydroxyestriol (4.3%) and 16-epiestriol (3.7%).

The above data clearly demonstrate that the individual antisera obtained for E_1, E_2, and E_3 by employing C-3 coupled steroid-protein conjugates are sufficiently specific and can clearly distinguish the structural features present in ring D. Recently Exley and Woodhams (13) have also come to similar conclusions and demonstrated that the use of highly purified estradiol-17β-3-hemisuccinyl-BSA can lead to specific anti-estradiol-17β serum. After completing our investigations, we noted that Rotti *et al.* (14) have utilized an anti-estriol serum obtained by employing estriol 3-O-carboxymethyl ether-BSA conjugate. However, they did not present data on the steroid hapten or other details concerning the antibody production.

NOMENCLATURE

Trivial Name	
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one
Estradiol-17β	1,3,5(10)-Estratriene-3,17β-diol
Estriol	1,3,5(10)-Estratriene-3,16α,17β-triol
2-Methoxyestrone	2-Methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one
2-Methoxyestradiol-17β	2-Methoxy-1,3,5(10)-estratriene-3,17β-diol
2-Methoxyestriol	2-Methoxy-1,3,5(10)-estratriene-3,16α,17β-triol
2-Hydroxyestrone	2,3-Dihydroxyestra-1,3,5(10)-trien-17-one
2-Hydroxyestradiol-17β	1,3,5(10)-Estratriene-2,3,17β-triol
2-Hydroxyestriol	1,3,5(10)-Estratriene-2,3,16α,17β-tetraol
6-Oxoestrone	3-Hydroxyestra-1,3,5(10)-triene-6,17-dione
6-Oxoestradiol-17β	3,17β-Dihydroxyestra-1,3,5(10)-trien-6-one

NOMENCLATURE
(Continued)

Trivial Name	
6-Oxoestriol	3,16α,17β-Trihydroxyestra-1,3,5(10)-trien-6-one
6β-Hydroxyestradiol-17β	1,3,5(10)-Estratriene-3,6β,17β-triol
6α-Hydroxyestriol	1,3,5(10)-Estratriene-3,6α,16α,17β-tetraol
16α-Hydroxyestrone	3,16α-Dihydroxyestra-1,3,5(10)-trien-17-one
16-Epiestriol	1,3,5(10)-Estratriene-3,16β,17β-triol
Estradiol-17α	1,3,5(10)-Estratriene-3,17α-diol
Testosterone	17β-Hydroxyandrost-4-en-3-one
Androstenedione	Androst-4-ene-3,17-dione
Progesterone	Pregn-4-ene-3,20-dione
17α-Hydroxyprogesterone	17α-Hydroxypregn-4-ene-3,20-dione
Cortisol	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione

ACKNOWLEDGEMENTS

This work was supported by a contract from Schwarz/Mann, a Division of Becton, Dickinson and Company, Orangeburg, New York. The authors wish to acknowledge the skillful assistance of Ms. Robbie A. Johnson and Mr. David Holland. We would like to thank Drs. Arvind T. Modak and Klaus D. Haegele, The University of Texas Health Science Center, San Antonio for the mass spectra.

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