Journal of Organometallic Chemistry, 160 (1978) 223—230

METALLOIMMUNOASSAY

II *. IRON-METALLOHAPTENS FROM ESTROGEN STEROIDS **

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(Received May 26th, 1978)

Summary

Ferrocenyl-containing derivatives of estrone, estradiol and estriol have been synthesised and used to demonstrate the feasibility of metalloimmunoassay (MIA), a novel non-isotopic system with potential applications in immunochemical studies.

Introduction

Biologically active substances are often effective in microgram, nanogram or even smaller quantities. Accurate quantitative monitoring of specific compounds in the presence of a multitude of other substances occurring in biological fluids has become one of the most important auxilliary tools in pharmacokinetic studies, chemotherapeutic treatment of diseases, forensic toxicology and various aspects connected with the widespread use of drugs of abuse. One of the prominent techniques practiced today for the determination of biologically-active molecules and their metabolites in physiological fluids is radioimmunoassay (RIA), which makes use of radioactive isotopes in combination with immunochemical methods. In a recent communication [1] we reported briefly on the feasibility of a novel non-isotopic system, designated metalloimmunoassay (MIA), in which a variety of metal atoms, in the form of their organometallic or coordination complexes, replaced radioisotopes as the labelling agents. In this paper we wish to present in some detail the principles of MIA, with particular reference to those results in which iron, in the form of its ferrocene derivatives, has been used as a labelling agent.

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* Part I see ref. 1.

** Dedicated to Professor Ernst Otto Fischer on the occasion of his 60th birthday on November 10, 1978.

Principle of the method

For the benefit of readers of this Journal, who may not be familiar with immunology nomenclature, a few brief definitions follow: Antigens (Ag) are substances which, upon introduction into the body of an animal species, trigger the formation of, and react with, antibodies. Antibodies (Ab) are glycoproteins formed in pertinent cells of the organism on intrusion of antigenic substances. Antisera are sera that contain antibodies as a result of the intrusion of antigens into the organism. Haptens are small molecules (mol. wt. < 5000) which exhibit antigenic properties only when covalently bonded to a macromolecule carrier. Antigens (and/or haptens) labelled with a metal-containing compound have been called [1] metallo-antigens (and/or metallohaptens), (Ag—M).

The specific recognition and binding of an antigen by an antibody with sufficient binding affinity forms the basis of the immunological reaction equilibrium (eq. 1):

$$Ab + Ag \rightleftharpoons Ab \cdot Ag \qquad (1)$$

If the antigen Ag is replaced by a metal-labelled antigen, Ag—M it is possible that a similar equilibrium (eq. 2) will be established, provided the metal-labelling does not cause immunological changes in the antigen, i.e. the antibody (Ab)

$$Ab + Ag—M \rightleftharpoons Ab \cdot Ag—M \qquad (2)$$

exhibits equal recognition (or very nearly so) of both the unlabelled antigen (Ag) and the metalloantigen (Ag—M). In this case, the binding constant (or affinity constant), K is the same (or very nearly so) for the complexes Ab · Ag and Ab · Ag—M. If mixtures of a variable amount of antigen (Ag) and a constant amount of metalloantigen (Ag—M) are allowed to compete for a limited and constant concentration of antibody binding sites, the reaction mixture, upon equilibration will consist of a "free" antigen phase (unbound Ag and Ag—M) and a "bound" phase of antibody—antigen complexes (Ab · Ag and Ab · Ag—M). After separation of the two phases, the amount of metal present in either the "bound" or "free" phase can be determined by suitable analytical methods such as emission, absorption and fluorescence spectrometry, electrochemical methods, neutron activation, etc. Preparation of a calibration curve plotted for standardized amounts of metalloantigen and unlabelled antigen provides the means of determining the quantity of analysed substance in unknown samples.

In general, the major components in the development of a metalloimmunoassay are: (1) production of specific antibodies; (2) synthesis of metalloantigens (metallohaptens); (3) techniques for separation of free, unbound antigen from antibody—antigen complex and (4) choice of analytical method to determine metal concentration.

In what follows, we shall limit ourselves to report some results on our approach to the synthesis of metallohaptens, with specific reference to estrogen steroid hormones incorporating the ferrocenyl moiety.

Synthesis of metallohaptens

The principle of MIA as described in the previous section is different from RIA [2] mainly with respect to the labelling agents used. Although high sensi-

tivity can be achieved by the use of radioisotopes, RIA suffers from a number of disadvantages such as high costs of isotopes, health hazards in preparation and use of isotope-labelled antigens, very limited variety of useable isotopes (mainly ^{3}H, ^{14}C and ^{125}I), short shelf-life of the labelled antigens and synthesis difficulties of introducing the radioactive isotope on to the antigen molecule. Hopefully, the metal-labelling of antigens, which is the most important component of MIA, should provide a way to overcome most of the disadvantages enumerated above.

The considerable research activity in the fields of coordination and organometallic chemistry during the past three decades has produced a wealth of information which is pertinent to the subject in hand and is readily available in the scientific literature. This should provide a rich and versatile source for the synthesis of "tailor-made" metallo-antigens.

Two general strategies for metal-labelling antigens can be envisaged. One approach would be the introduction of metal atoms directly into the antigen, provided that the chemical structure of the latter is suitable for reaction with metals. In the other approach, one would plan the synthesis of a functionalized metal-containing reagent which could react with the antigen (or a suitable antigen derivative) to produce the desired metallo-antigen. We have employed both these pathways [1] but only the second approach will be illustrated here using as substrates the estrogen hormones, estrone, estradiol and estriol, Ia—Ic respectively (Fig. 1).

A readily available, stable, non-toxic and versatile metallo-reagent for labelling antigens is ferrocene. Functionalization of ferrocene can be suitably carried out to obtain the required derivatives for reaction with antigens. Therefore, it was convenient to work with this reagent in the initial stages of our work in order to test feasibility of the MIA concept.

Since the low molecular weight steroids are not antigenic, the 3-*O*-carboxymethyl ether derivatives IIa—IIc (Fig. 1) were first synthesised. This provided a suitable handle for reaction with the protein carrier, bovine serum albumin (BSA) to produce the protein-steroid conjugates VIa—VIc (Fig. 2). These conjugates were then used for animal immunization and the production of antisera. The

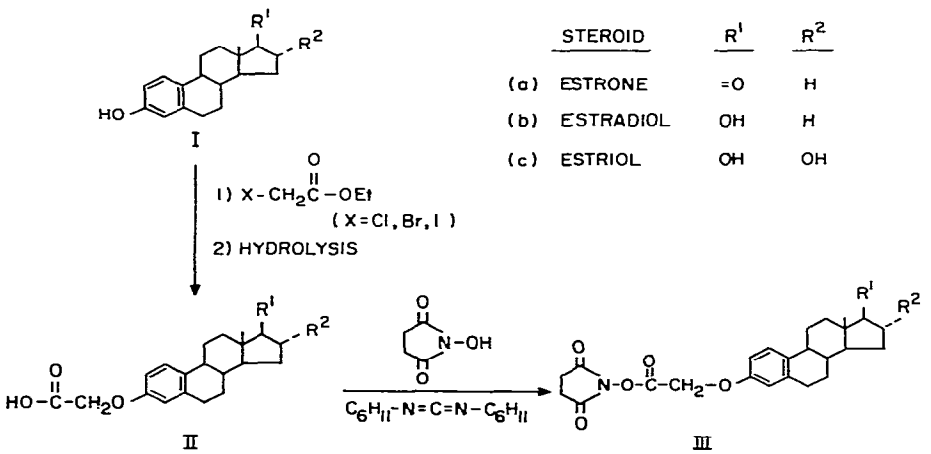

Fig. 1. Synthesis of 3-*O*-substituted functionalized estrogens.

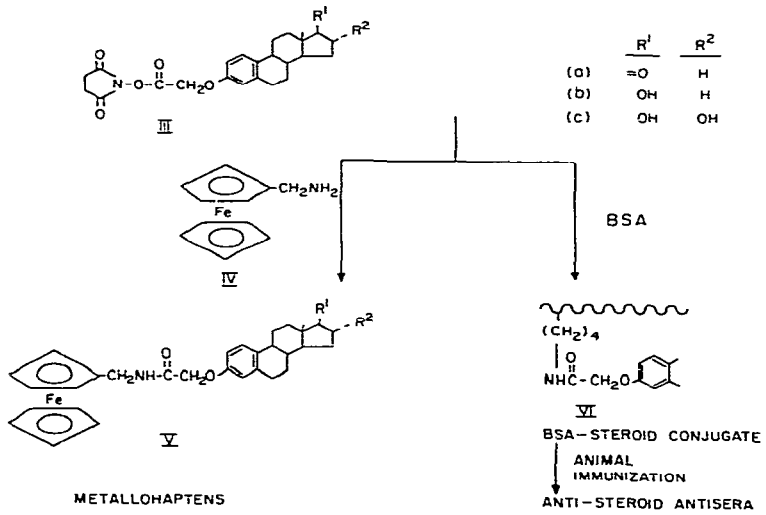

Fig. 2. Synthesis of 3-*O*-ferrocenyl-containing estrogens and bovine serum albumin (BSA)-estrogen conjugates for production of antisera.

carboxymethyl derivatives, IIa—IIc were reacted with *N*-hydroxysuccinimide to form the "active" esters IIIa—IIIc which upon reaction with aminomethylferrocene (IV) yielded the estrogen metallohaptens, Va—Vc (Fig. 2).

Another way of functionalizing estradiol (Ib) and estriol (Ic) was to prepare estradiol-17β-hemisuccinate (VIIa) and estriol-16α,17β-bis(hemisuccinate) (VIIb). These derivatives were used both for making BSA-steroid conjugates for animal immunization [3] and in the reaction with aminomethylferrocene (IV) to yield the iron-metallohaptens VIIa, VIIb (Fig. 3).

In a similar fashion we used other ferrocene derivatives (carboxyferrocene,

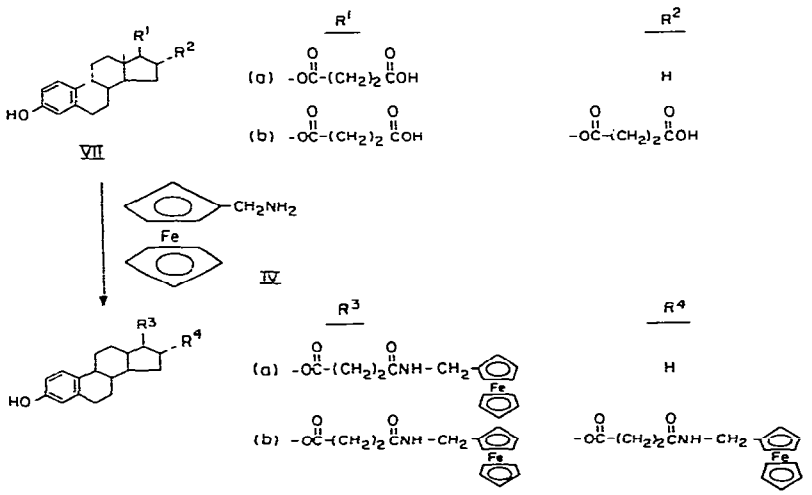

Fig. 3. Synthesis of ferrocenyl-containing estradiol and estriol substituted in positions 17β and 16α,17β respectively.

diferrocenylcarbinol, β-ferrocenylethylamine and 4-ferrocenyl-4-oxobutanoic acid) for Fe-labelling of haptens and high molecular weight antigens.

Assay procedure

The iron-labelled metallohaptens described in the previous paragraphs have been tested in the development of procedures for a metalloimmunoassay. Only a brief description of one of the procedures can be given here, with fuller details to be published elsewhere. In order to facilitate the separation step between the "free" and "bound" phases (vide supra), *anti*-estradiol-17β-hemisuccinate antibody was covalently bound to a water-insoluble polymer by reaction with cyanogen bromide-activated Sepharose 4B. [4]. Standardised solutions of the iron-containing estradiol-17β-hemisuccinate metallohapten (VIIIa) were prepared in 0.01 *M* phosphate buffer (pH 7.3) with 15% dimethylformamide and the iron concentration was determined by flameless atomic absorption spectrometry [5] to establish a calibration curve in the concentration range 0—300 ng Fe ml^{-1} (0—5.4 × 10^{-6} *M*). A fixed amount of the Sepharose-bound *anti*-estradiol-17β-hemisuccinate antibody was titrated with standard solutions of metallohapten VIIIa, and incubated at room temperature for 30 minutes with occasional mixing. Upon centrifugation, the Sepharose-bound antibody was precipitated carrying with it the antibody-Fe-metallohapten complex ("bound" phase). Aliquots (30 ml) from the supernatant were injected into the graphite furnace of the atomic absorption spectrometer to measure (with help of the calibration curve) the amount of "free" Fe-metallohapten, Ag—M. The results from this experiment established the concentration of antibody binding sites (5.01 × 10^{-6} *M*) available on the Sepharose-bound antisera and the affinity constant *K* (3.44 × 10^{5} M^{-1}) for this antibody-Fe-metallohapten complex.

A similar experiment was then carried out using a fixed amount of Sepharose-bound antisera, a constant concentration of the Fe-metallohapten VIIIa (2.75 × 10^{-6} *M*) and increasing amounts of unlabelled estradiol-17β-hemisuccinate, VIIa (in the range 0.36 × 10^{-6} *M* to 15 × 10^{-6} *M*). It was expected that the higher the concentration of unlabelled hapten, relative to the constant Fe-metallohapten concentration, the greater would be the competition for the limited (fixed) concentration of antibody binding sites. This would result in augmented inhibition of metallohapten binding. This was shown to be so by the increasing Fe-concentrations found in the supernatant aliquots injected in the graphite furnace of the atomic absorption spectrometer. With the particular reagents used in this experiment it was found that a molar ratio unlabelled steroid VIIa to metallohapten VIIIa of 1.3/1 was required to obtain about 44% inhibition of metallohapten binding.

From the outset, it was clear to us that the concentration range used in the above experiments would not be suitable for the direct measurement of estradiol in serum. However, the above results demonstrated the feasibility of the MIA concept and at the same time indicated some of the problems to be overcome in order to arrive at a viable, practical assay. In addition to work directed towards optimization of assay conditions, we are currently investigating the synthesis of a variety of metalloantigens as well as other facets and possibilities which are inherent in MIA. We hope that this concept, which incorporates an interdiciplin-

ary approach by combining immunochemistry, organic, organometallic and coordination chemistry as well as aspects of analytical instrumentation, will stimulate the interest of other researchers in these fields.

Experimental

Infrared spectra were recorded on a Perkin—Elmer model 257 grating spectrometer, NMR on a Varian T60 and mass spectra on a Varian model 711. Atomic absorption measurements were carried out on a Perkin—Elmer model 403 spectrometer fitted with a flameless graphite furnace model HGA-70 and deuterium background corrector. Estrone, estradiol and estriol (Merck, Damstadt) were used without further purification in the synthesis of the 3-*O*-carboxymethyl ether derivatives (IIa—IIc) (details will be published elsewhere) and of estradiol-17β-hemisuccinate (VIIa) and estriol-16α,17β-bis(hemisuccinate) (VIIb) [3]. Aminomethylferrocene was prepared by known procedures [6]. *N,N'*-Dicyclohexylcarbodiimide (puriss) and *N*-hydroxysuccinimide (purum) were purchased from Fluka. Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia. The buffer solutions, 0.1 *M* PBS and 0.01 *M* PBS were prepared [7] with A.R. grade reagents. Solvents, unless analytical grade, were dried and distilled.

Preparation of 3-hydroxyestra-1,3,5(10)-trien-17-one-3-O-ferrocenylmethyl-aminocarbonylmethyl ether (Va)

A solution of the 3-*O*-carboxymethyl ether derivative of estrone (IIa) (493 mg, 1.5 mmol) in freshly distilled THF (30 ml) was allowed to stir for 15 min in a cold room (4°C). To this solution was then added *N*-hydroxysuccinimide (191 mg, 1.66 mmol) followed by *N,N'*-dicyclohexylcarbodiimide (437 mg, 2.12 mmol) and stirring at 4°C was continued for 20 h. The white precipitate formed in the reaction was removed by filtration. To the filtrate, containing the active ester derivative IIIa, (as indicated by TLC) there was added a solution of aminomethylferrocene (380 mg, 1.77 mmol) in THF (5 ml) and excess triethylamine (1 ml). Stirring in the cold room (4°C) was continued for 20 h and then at room temperature for 4 h. After evaporation of the solvent, the remaining solid was washed with water (3 × 5 ml) dried in vacuo and then chromatographed on a silica gel column. Elution with chloroform/benzene (3/2) yielded 579 mg (~81%) of the TLC pure iron-metallohapten Va. IR ($CHCl_3$): ν(ketone-C=O) 1735vs; ν(amide-C=O) 1670vs, 1605m cm^{-1}. NMR ($CDCl_3$) (δ, ppm): 7.3—6.8 (multiplet, aromatic 3H); 4.5 (singlet, 3-O—CH_2—); 4.2 (broad singlet, ferrocenyl 9H and ferrocenyl ring—CH_2—N); 0.9 (singlet, angular CH_3). Molecular ion (high resolution mass spectrum) *m/e* 525.1929; $C_{31}H_{35}O_3NFe$ calcd.: mol. wt. 525.44.

Preparation of estra-1,3,5(10)-trien-3,17β-diol-3-O-ferrocenylmethylaminocarbonylmethyl ether (Vb)

The same molar quantities of reactants and similar reaction conditions as above were used with the 3-*O*-carboxymethyl ether derivative of estradiol (IIb) to obtain, after column chromatography (elution with chloroform/benzene 9/1) 588 mg (70%) of the iron-metallohapten Vb. IR ($CHCl_3$): ν(amide-C=O) 1670vs and 1610m cm^{-1}. NMR ($CDCl_3$) (δ, ppm): 7.3—6.8 (multiplet, aromatic 3H);

4.6 (singlet, 3-O—CH_2); 4.2 (broad singlet, ferrocenyl 9H and ferrocenyl ring—CH_2—N); 0.8 (singlet, angular CH_3). Molecular ion (high resolution mass spectrum) *m/e* 527.2204; $C_{31}H_{37}O_3NFe$ calcd.: mol. wt. 527.44.

Preparation of estra-1,3,5(10)-trien-3,16α,17β-triol-3-O-ferrocenylmethylaminocarbonylmethyl ether (Vc)

Employing the same molar quantities and similar procedure as in the previous experiments, the 3-*O*-carboxymethyl ether derivative of estriol, IIc, yielded after column chromatography (elution with 4% methanol in chloroform) 669 mg (~85%) of the iron-metallohapten, Vc. IR ($CHCl_3$): ν(amide-C=O) 1680vs and 1610m cm^{-1}. NMR ($CDCl_3$) (δ, ppm): 7.3—6.8 (multiplet, aromatic 3H); 4.6 (singlet, 3-O—CH_2); 4.2 (broad singlet, ferrocenyl 9H and ferrocenyl ring—CH_2—N); 0.9 (singlet, angular CH_3). Molecular ion (high resolution mass spectrum) *m/e* 543.2114; $C_{31}H_{37}O_4NFe$ calcd.: mol. wt. 543.44.

Preparation of estra-1,3,5(10)-trien-3,17β-diol-17β-O-[3-(N-ferrocenylmethylaminocarbonyl) propionate] (VIIIa)

N,N'-Dicyclohexyldiimide (186 mg, 0.9 mmol) was added to an ice-cold solution of estradiol-17β-hemisuccinate, VIIa, (200 mg, 0.75 mmol) and *N*-hydroxysuccinimide (90 mg, 0.78 mmol) in freshly distilled THF (10 ml). The reaction mixture was stirred for 3 h at 0°C and then for 20 h at room temperature. After filtration to remove the precipitated *N,N'*-dicylohexylurea (177 mg) the filtrate was evaporated to dryness to yield 328 mg of a white solid which was shown by IR and NMR to be the expected active ester and was used in the next step without further purification. A solution of the active ester (120 mg, 0.25 mmol) and aminomethylferrocene (55 mg, 0.25 mmol) in freshly distilled THF (4 ml) was stirred at room temperature for 20 h. The mixture was filtered to remove some precipitated material and the filtrate was evaporated to dryness. The solid residue was taken up in ethyl acetate (10 ml) and the solution washed with water (2 × 10 ml). The organic solvent phase was dried ($MgSO_4$) and evaporated to dryness to yield 160 mg of a solid residue. This was purified by column chromatography on a 10% deactivated basic alimina (7 g). Elution with benzene/chloroform (4/1) yielded 55 mg (40%) of pure (TLC) iron-metallohapten, VIIIa, m.p. 140—143°C. The lower yield in this and the following experiment, is ascribed to the absence of triethylamine from the reaction [8]. IR ($CHCl_3$): ν(ester-C=O) 1720vs; ν(amide-C=O) 1660vs; ν(ferrocene) 1100 and 1000 cm^{-1}. NMR ($CDCl_3$) (δ, ppm): 7.3—6.8 (multiplet, aromatic 3H); 4.3 (broad singlet, ferrocenyl 9H); 0.8 (angular methyl, 3H). Molecular ion (high resolution mass spectrum) *m/e* 569.2238; $C_{33}H_{39}O_4NFe$ calcd.: mol. wt. 569.53.

Preparation of estra-1,3,5(10)-trien-3,16α,17β-triol-16α-O-17β-O-bis[3-(N-ferrocenylmethylaminocarbonyl) propionate] (VIIIb)

By following the same procedure as in the previous experiment, estriol-16α, 17β-bis(hemisuccinate) (VIIb) (496 mg, 1.0 mmol), *N*-hydroxysuccinimide (230 mg, 2.0 mmol) and *N,N'*-dicyclohexylcarbodiimide (450 mg, 2.2 mmol) yielded 550 mg (81%) of the expected bis-"active" ester, m.p. 90—95°C (without purification).

In the next step, the bis-ester (130 mg, 0.19 mmol) and aminomethylferro-

cene (81 mg, 0.38 mmol) were reacted as described in the previous experiment to yield, after purification by column chromatography and then by preparative TLC, 60 mg (30%) of the iron-metallohapten, bis-amide VIIIb. IR ($CHCl_3$): ν(ester-C=O) 1730vs; ν(amide-C=O) 1615vs; ν(ferrocene) 1110, 1000 cm^{-1}. NMR ($CDCl_3$) (δ, ppm): 7.3—6.7 (multiplet, aromatic 3H); 4.26 (broad singlet, bisferrocenyl, 18H); 0.8 (angular methyl, 3H).

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