Affinity Labelling of Estradiol Receptor by Ferrocenyl Tagging of Estradiol 17α-Position

Anne Vessières, Colette Vaillant, Michel Gruselle, Dominique Vichard, and Gérard Jaouen

Ecole Nationale Supérieure de Chimie de Paris, UA CNRS 403, 11, rue Pierre et Marie Curie, 75 231 Paris Cedex 05, France

The introduction of a ferrocenyl moiety at the 17α-position of estradiol brings about a dramatic change in the behaviour of the hormone at the receptor binding site since an irreversible binding takes place; this unusual reactivity may help to elucidate the steroid's mode of action.

Inorganic and organometallic complexes represent an arsenal of substances whose potential as biomedical agents[1] is being increasingly explored. Tuning such an agent to target a particular biological site is one of the most challenging aspects of these compounds.[2] Ferrocene derivatives constitute a series of organometallic species of particular interest since the stability of the metal complex renders it compatible with almost any biochemical treatment. The efficiency of the easily prepared ferricinium ions has been proved in the suppression of several types of tumours,[3] while biomedical tracers are

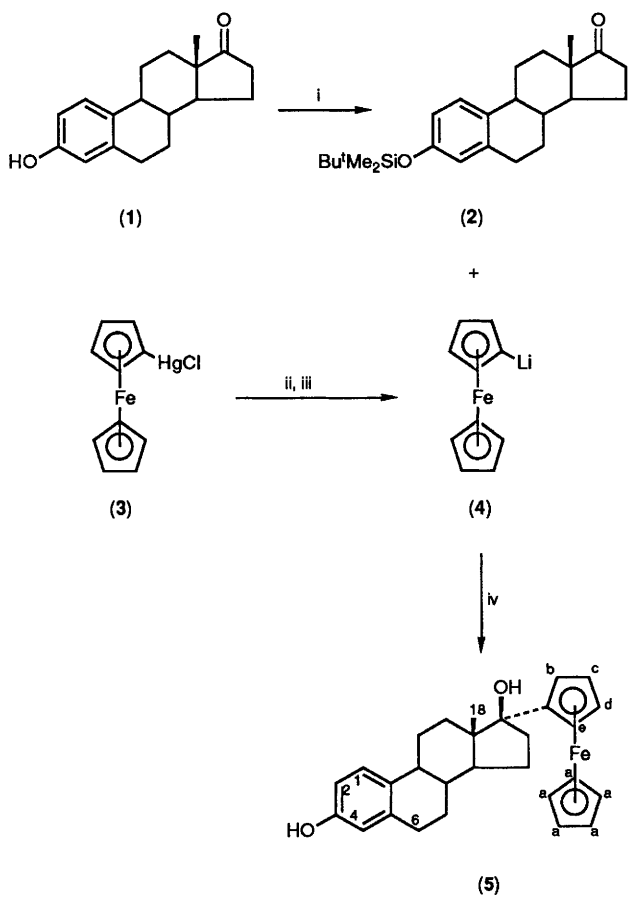
readily accessible from ferrocenes by substituting the iron atom with radioactive ruthenium isotopes.[4] We show here that modification of estradiol by a ferrocenyl unit at the 17α-position [compound (**5**); Scheme 1] causes a dramatic change in the chemical behaviour of the organometallic hormone with respect to that of estradiol itself at the estradiol receptor binding site. This new type of binding site directed reagent may help to identify the effective amino acids implicated in the molecular recognition between estradiol and its receptor. The quantification of the estradiol receptor is currently performed, for prognostic and therapeutic reasons, for a very common hormono-dependent cancer, namely breast cancer,[5] but the role of the steroid in the protein activation process is almost completely unknown.[6] Therefore, the establishment of a covalent bond between modified estradiol and its receptor, *i.e.*, affinity labelling, is of crucial interest,[7] since the equilibrium conditions which take place with natural estradiol preclude the accurate study of the receptor.

Compound (**5**) was prepared in 29% yield by reaction at −50 °C of the protected estrone (**2**) with ferrocenyl lithium obtained from bromoferrocene as shown in Scheme 1. This product has been fully characterized by spectroscopic techniques and elemental analysis.† The stereoselective introduction of the bulky ferrocenyl group at the 17α position is due to the presence of the methyl at the 13β position; this type of stereochemical control is quite general for steroids.[8]

Interestingly, the presence of the 17α-ferrocenyl group does not dramatically hinder the recognition properties of (**5**) for the estradiol receptor since its apparent relative binding affinity (RBA) was found to be 15%, with estradiol taken as 100%.‡

Furthermore, while estradiol shows a reversible binding for its receptor, the organometallic modification of (**5**) brings about an irreversible binding. The irreversible nature of the interaction of compound (**5**) with the receptor was shown as follows. Fractions of lamb uterus nuclear extracts§ were first incubated in the presence of varied quantities of complex (**5**). At the end of the incubation, free tracer was removed by charcoal dextran treatment.[10] The surviving reversible estrogen binding activity (which indicated the quantity of non-covalent bound receptor) was measured after exchange with tritiated oestradiol ([^{3}H]-E_2; 10^{-8} M) for 16 h at 25 °C.[10] Protamine sulphate precipitation was used to determine oestradiol receptor concentration.[9] Under optimised conditions [incubation: 25 °C, 1 h, in the presence of (**5**) (10^{-8} M)] the maximum labelling efficiency achieved is 80% (average value of seven experiments). These data clearly indicate that compound (**5**) inactivates the receptor by irreversibly binding the estradiol receptor present in nuclear extracts.

In recent studies[10,11] the acidic cysteine #530 flanked by lysines #529 and #531 in the human estradiol receptor



Scheme 1. *Reagents*: i, dimethylformamide, Bu^{t}Me$_2$SiCl, imidazole; ii, *N*-bromosuccinimide, tetrahydrofuran (THF); iii, BuLi, ether; iv, Bu_4NF, THF.

sequence,[12] has been suspected to be the key amino acid implicated in the recognition process of cycle D 17β-OH. This hypothesis is in agreement with our results. We have checked that methyl methane thiosulphonate (MMTS), a specific thiol reagent,[13] efficiently inactivates the nuclear estradiol binding sites. For example, 84% inactivation is obtained when lamb uterus nuclear extracts are preincubated for 1 h in the presence of MMTS (10^{-4} M) at 0 °C followed by incubation with [^{3}H]-E_2 (10^{-8} M).

It is well documented in ferrocene series that α-carbenium ions, easily generated in a weakly acidic medium, are stabilized by the metallic moiety;[14] the pK(R^+) value for $FcCH_2^+$ [Fc = $(C_5H_5)_2Fe$] is about −1.2[15] and this type of carbenium ion is able to alkylate a range of nucleophiles. In addition, the pK of the thiol group is known to be sensitive to the local structure through 'medium effects.' For proteins, the –SH group of a cysteine residue surrounded by basic amino acid side chains is considerably more acidic than the –SH group in the cysteine residue surrounded by acidic amino acid side chains.[16] Furthermore, it has been reported that reaction of ferrocenyl ethanol with thioglycolic acid gives rise to (1-ferrocenylethyl)thioglycolic acid[17] in 92% yield with retention of configuration *via* the ferrocenyl ethyl carbenium ion. Therefore, the occurrence of an alkylation reaction with (**5**) in the estradiol binding site can be postulated in terms of generation of a carbenium ion-like intermediate by interaction with cysteine #530, followed by establishment of a covalent bond with a nearby nucleophile. Modelling studies of hormone receptors based on hydrophobic cluster analysis (HCA)[18] have shown that cysteine #530, which is surrounded

† *Selected data* for (**5**): ^{1}H NMR (250 MHz, $CDCl_3$) δ 1 [3H, s, H(18)], 2.77 [2H, m, H(6)], 4.22 [5H, s, H(a)], 4.02, 4.16, 4.24, 4.29 [4H, 4m, H(b,c,d,e)], 6.53, 6.58 [2H, 2m, H(2), H(4)], 7.03 [1H, d, H(1)]; m.p. 175 °C; cyclic voltammetry, ferrocene/ferricinium, $E_{1/2}$ 0.515 V.

‡ RBA was determined as follows: lamb uterine cytosol (0.2 ml fractions containing 4 mg protein ml^{-1}) was incubated at 0 °C for 3 h with [^{3}H]-17β-oestradiol (2 nM) and an increasing amount of competing steroids. Bound fractions were measured by protamine sulphate precipitation.[9] The RBA of the competitor is taken as the ratio of the concentrations of unlabelled oestradiol/competitor required to inhibit half of the specific [^{3}H]-17β-oestradiol binding, with the affinity of oestradiol set at 100%.

§ Prepared as described in ref. 10 and containing about 0.3 nM of specific binding sites.

by two lysines, is located in a turn region near the entrance of the hormone binding channel.[19,20] This situation correlates with the above results and suggests primary regulation of hormone receptors *via* the selection of the appropriate steroids at the orifice of the activating canal.

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