The Ferrocenylethynyl Unit: a Stable Hormone Tag

by **Domenico Osella***[a]), **Carlo Nervi**[b]), **Francesco Galeotti**[b]), **Giorgio Cavigiolio**[a]), **Anne Vessières**[c]), and **Gérard Jaouen***[c])

[a]) Dipartimento di Scienze e Tecnologie Avanzate, Università del Piemonte Orientale "A. Avogadro", C.so T. Borsalino 54, I-15100 Alessandria

[b]) Dipartimento di Chimica IFM, Università di Torino, via P. Giuria 7, I-10125 Torino

[c]) Ecole Nationale Supérieure de Chimie de Paris, Laboratoire de Chimie Organométallique, UMR 7576, 11 Rue Pierre et Marie Curie, F-75231 Paris Cedex 05

We prepared the organometallic complex 17α-(ferrocenylethynyl)estradiol (= [(3,17β-dihydroxyestra-1,3,5(10)-trien-17α-yl)ethynyl]ferrocene; FcEE; **1**) by a novel synthetic method. This metallocene possesses sufficient stability in aqueous media to permit the study of its biological properties. Thus, we were able to show that, despite the addition of a bulky substituent at the 17α position of the steroid, the metallocene is still well-recognized by an antibody specific to estradiol ($CR = 40\%$) and by both subtypes (ERα, ERβ) of the estrogen receptor (at 0°, $RBA = 28$ and 37%, resp.). A DCI-MS study of the stability of the carbocation $[FcEE - OH]^+$ showed moderate stabilization of the carbocation, in agreement with the pK_{R^+} value of -0.72 found for the metallocene by means of *Deno*'s method. The presence of the ferrocene allows the electrochemical detection of FcEE (**1**) by HPLC-ED, with a detection limit of *ca.* 1 nM, suitable for quantitative pharmacological analysis.

1. Introduction. – Measuring the circulating levels of estradiol is an important task for assessing ovarian function and monitoring follicular development for assisted-reproduction protocols. Quantification of this hormone in blood was first performed by radioimmunoassay techniques, which are well-known for their specificity and sensitivity [1]. These methods, based on a typical immunological by radioactive labelling, are nowadays being displaced by photochemical sensor-based assays. A number of automated methods using chemiluminescence tracers [2] and enzymatic immunoassay (ELISA: enzyme-linked immunosorbent assay) systems [3][4] have recently been developed and successfully employed for routine analysis. The crucial point in the development of new techniques is the broad sensitivity required for analysis, since serum levels of estradiol in humans range between 30 and 1500 pM. From the analytical and clinical point of view, estradiol is also used to quantify the level of specific estrogen receptors (ER) localized in target organs such as the uterus, as well as in certain breast cancers [5]. Assessment of receptor levels in these tumors is important for diagnostic purposes, facilitating the choice of treatment for a given case [6]. In general, analysis is based on binding of a tracer to ER. Irrespective of whether the assay of estradiol levels *in vivo* is to determine the concentration of the circulating hormone or the hormone bound to its receptor, the analysis always requires the labelling of the steroid with a sensor that can be detected at the pM or, for receptors, even at the fM level.

Electrochemical detection (ED) coupled with an HPLC system is a simple analytical technique that approaches this sensitivity, provided that the substrate contains a redox system or can be converted to a redox-active product by pre- or postcolumn reactions [7]. The use of this technique for the assay of estradiol requires a synthetic

modification of the estrogen that enhances its electrochemical response. Provided that this can be achieved and that the labelled estrogen retains an acceptable degree of recognition for its specific antibodies or receptor, assays are performed through a competitive reaction between the natural hormone and the modified estradiol.

In previous papers, we have reported attempts to employ various organometallic substructures as redox-active markers for estradiol. The [$Co_3(CO)_9C$] cluster works as a good electroreducible marker [8]; however, the set up of the HPLC-ED analysis with cathodic potentials proved to be not straightforward. Attempts to obtain an estradiol molecule marked with a ferrocenyl fragment (a mononuclear oxidizable organometallic marker) led to the synthesis of 17α-ferrocenylestradiol (FcE) [9]. This compound proved to be quite suitable for analytical detection enhancement *via* the HPLC-ED technique [10]. Relative-binding-affinity (*RBA*) measurements show that the modified hormone is still able to interact with the estrogen receptor. Since the ferrocenium derivatives are well-known for exhibiting antitumor activity [11][12], the estradiol unit of FcE could act as a carrier for the potentially active ferrocene unit, transferring it to DNA [13] *via* the estrogen-receptor system. If Fe^{II}/Fe^{III} oxidation occurs naturally during cell metabolism, this molecule could be employed as a selective antineoplastic agent against estrogen-responsive tumors [9]. Unfortunately, the complex is not completely stable under physiological conditions, and decomposition occurs during the time required for biological treatments [14]. This seriously complicates the possibility of employing such a compound both in therapy and in immunological labelling. The aim of this work is to provide a more-stable hormone derivative containing the ferrocene unit.

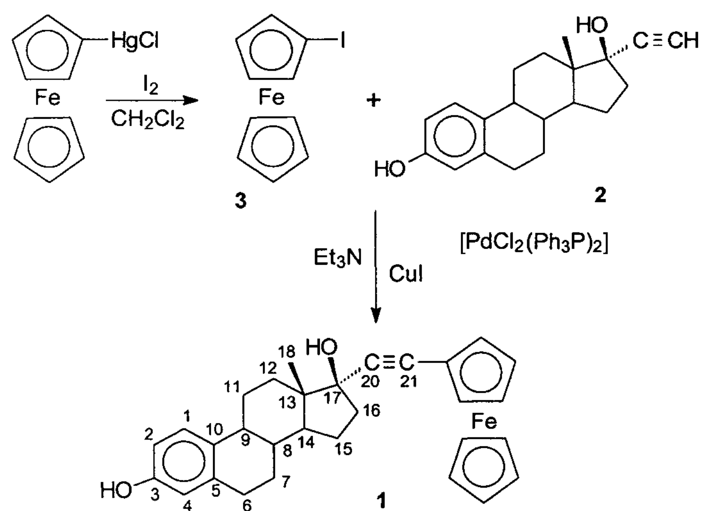
2. Results and Discussion. – 2.1. *Synthesis.* Our previous attempt to link the [$Co_3(CO)_9C$] moiety directly to ethynylestradiol (HC≡C−E) did not produce the desired compound [$Co_3(CO)_9C$−C≡C−E], due to its instability [15]. However, the insertion of a spacer between the estradiol and the organometallic core gave positive results [8], yielding a more stable metal-attached hormone. A similar approach has been employed to stabilize the ferrocenyl derivative of estradiol (FcE).

The complex 17α-(ferrocenylethynyl)estradiol (= [(3,17β-dihydroxyestra-1,3,5(10)-trien-17α-yl)ethynyl]ferrocene; FcEE; **1**) has been previously synthesized [16] starting from estrone. However, this procedure requires the protection of the phenolic OH group of estrone and involves epimerization at C(17). Since the affinity to the receptor is dependent on the free phenolic OH group and the OH at C(17), in β-position [17], we developed a different procedure.

Model reactions with 1-ethynyl cyclohexanol revealed the best route to ferrocenyl derivatives [18–20], and consequently were employed to prepare FcEE (**1**); *i.e.* a modified Pd-catalyzed *Sonogashira* reaction [21] between 17α-ethynylestradiol (**2**) and iodoferrocene (**3**), furnished FcEE (**1**) in 38% yield (*Scheme 1*).

The characterization of FcEE (**1**) was performed by comparing the ^{1}H- and ^{13}C-NMR spectra with known ethynylestradiol derivatives [22]. We obtained crystals of **1** apparently suitable for X-ray determination; however, molecular disorder on the ethynylferrocene unit did not allow proper refinement of the structure. The leitmotif of the crystal cell is based on H-bonds and is similar to that found for the analogous FcE [23] (*Fig. 1*).

Scheme 1



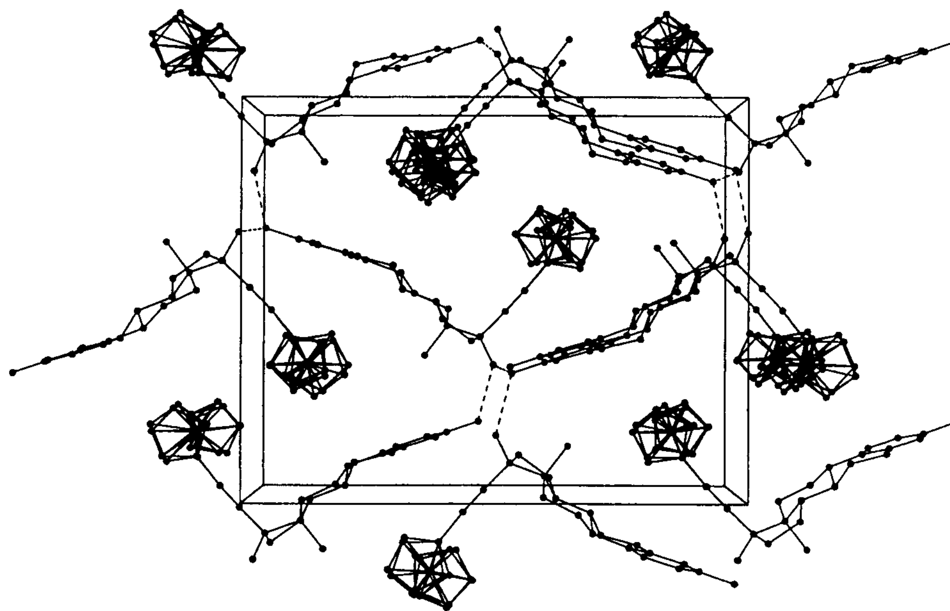


Fig. 1. *Unit-cell representation of FcEE* (**1**). Dashed lines indicate intermolecular H-bonds.

Probably by virtue of the presence of the rigid C≡C spacer group between the steroid and the metallocene moiety, FcEE (**1**) exhibits remarkable stability compared to FcE in physiological medium.

2.2. *Electrochemistry.* The cyclic voltammetry (CV) of an MeCN solution of FcEE (**1**) at a glassy carbon electrode reveals a first reversible oxidation process followed by a

second irreversible anodic step (*Fig. 2*). The irreversible wave, located at high potentials (> +1.2 V), is likely due to oxidation of the OH groups of the steroidal skeleton [24]. The reversible 1e$^-$ oxidation process, at $E^0 = +0.51$ V *vs.* SCE, can be easily assigned to the Fc (0/1 +) redox couple. This oxidation potential is 130 mV more anodic with respect to the oxidation of FcE. This shift is similar to that observed between ferrocene and ethynylferrocene [25], thus ascribing the effect to the presence of acetylene moiety.

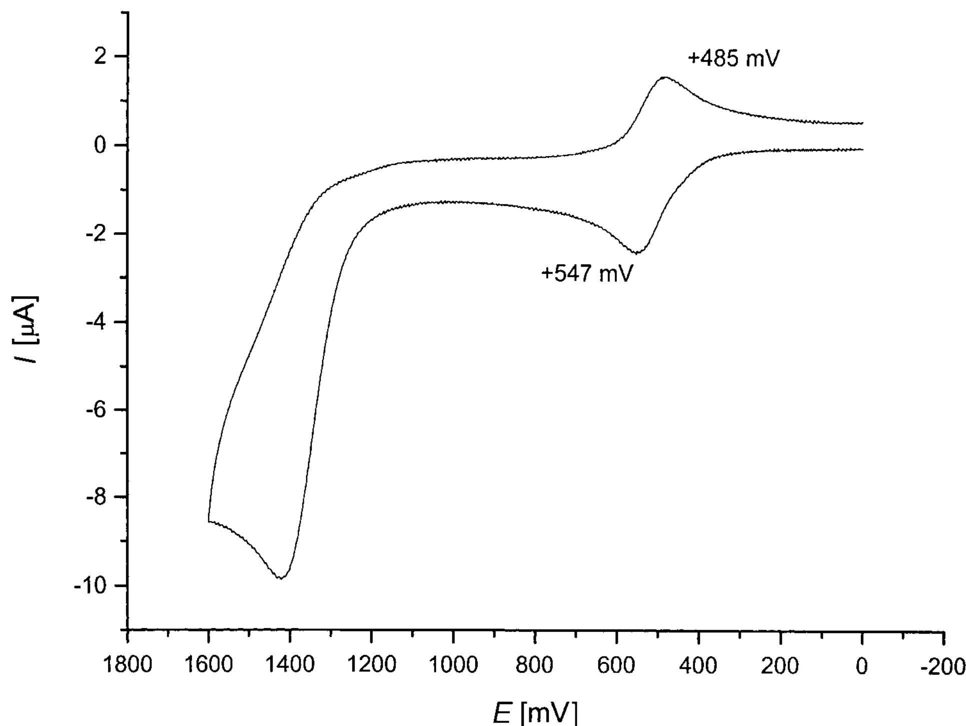


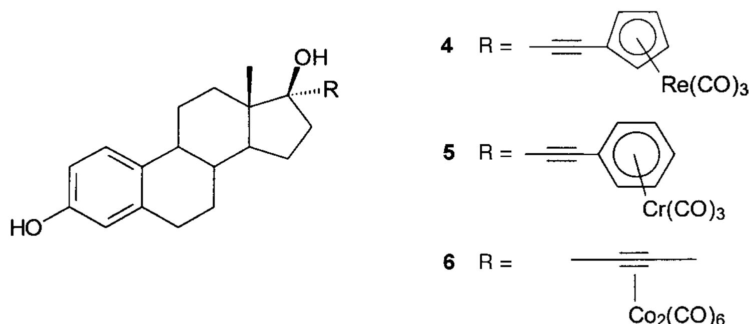
Fig. 2. *Cyclic voltammetric* (CV) *response of an acetonitrile solution of FcEE* (**1**; 1.0 mM) vs. *SCE*. Supporting electrolyte, 0.1M; $LiClO_4$ working electrode, glassy carbon; scan rate, 200 mV s^{-1}.

2.3. *Biochemical Results.* The biochemical properties of the FcEE (**1**) complex were evaluated both for their recognition of antibodies specific to estradiol and for their affinity for the two subtypes, α and β, of the estrogen receptor.

Cross-Reaction (CR) *Rate of FcEE* (**1**) *for Antibodies Specific to Estradiol Measured by Radioimmunoassay (RIA)*. To use the organometallic complex FcEE (**1**) as a tracer in an immunoassay, the CR rate of this complex for antibodies specific to estradiol must be determined by RIA. The CR value (see *Exper. Part*) found for **1** is 40%, showing that the complex is still well-recognized by the specific antibodies.

Determination of Relative Binding Affinities (RBA) *of FcEE* (**1**) *for the* α *and* β *Subtypes of the Estrogen Receptor*. The RBA of FcEE (**1**) for the two subtypes of the estradiol receptor were measured. The estradiol receptor identified by *Jensen* in the 1960s [5] is now known as ERα since the recent discovery in other tissues, including

ovary and prostate, of a second subtype designated ERβ [26]. The *RBA* values of FcEE (**1**) obtained with ERα (28%) and ERβ (37%) at 0° are high, indicating that the modified hormone retains very good affinity for the receptor, despite the high degree of encumbrance caused by addition of the ferrocenylethynyl entity. This value is higher than that found for a similar carbonyl rhenium complex (see **4**; RBA = 16%), but close to that of the corresponding benzenetricarbonylchromium complex **5** (*RBA* = 24%) [27].



This confirms the previously noted tolerance of the 17α position for the addition of bulky substituents. The RBA value found for ERα rises to 39% after incubation at 25°, but only if FcEE (**1**) is dissolved in DMSO. If **1** is dissolved in EtOH, the RBA to ERα drops to 4%. A temperature-induced rise in the RBA value is usually attributed to slowing or even blocking of the dissociation of the bound hormone [28].

2.4. *Carbocation Stability.* Thermodynamic stability of the carbocation generated from FcEE (**1**) by loss of the OH group in the 17β position, has been evaluated by two different methods; *i.e.* by the classical approach of *Deno et al.* [29] and by a new procedure based on DCl mass spectrometry [22]. The former method provides a value of pK_{R^+} = −0.72 (see *Exper. Part*), while the latter gives a value of 1.6 for the ratio $[M-\mathrm{OH}]^+/[\mathrm{M}+\mathrm{H}]^+$. These values are in accordance with a moderately stabilized but reasonably reactive carbocation [30]. The interposed triple bond delocalizes the positive charge to the ferrocenyl unit (the ferrocenium cation is stable) (*Scheme 2*).

Knowing this pK_{R^+} value, it is of interest to establish whether or not this compound induces inactivation of the receptor. This requires measurement of its receptor inactivation ratio (RI). The ratio of 25.5% found for FcEE (**1**) is clearly lower than the 80% obtained for hexacarbonyl (propynylestradiol)dicobalt ([$Co_2(CO)_6$(PE)]; **6**), the best affinity marker so far synthesized in the series of organometallic estradiol complexes [27]. This moderate ratio, associated with the increase in RBA observed when the incubation temperature is raised from 0 to 25°, suggests a slow dissociation of the hormone-receptor complex, rather than the formation of a covalent hormone-receptor bond as observed, *e.g.*, with [$Co_2(CO)_6$(PE)] (**6**).

2.5. *HPLC Tests.* We studied the reversed-phase HPLC behavior of FcEE (**1**) in MeCN, in comparison with the behavior of pure ethynylestradiol. We employed both a UV/VIS and an electrochemical detector connected in series. With a flow rate of 1.0 ml/min, the retention times t_R of 2.52 and 3.27 min are recorded for EE (**2**) and FcEE (**1**), respectively (*Fig. 3*). As previously observed with other organometallic complexes, the

Scheme 2

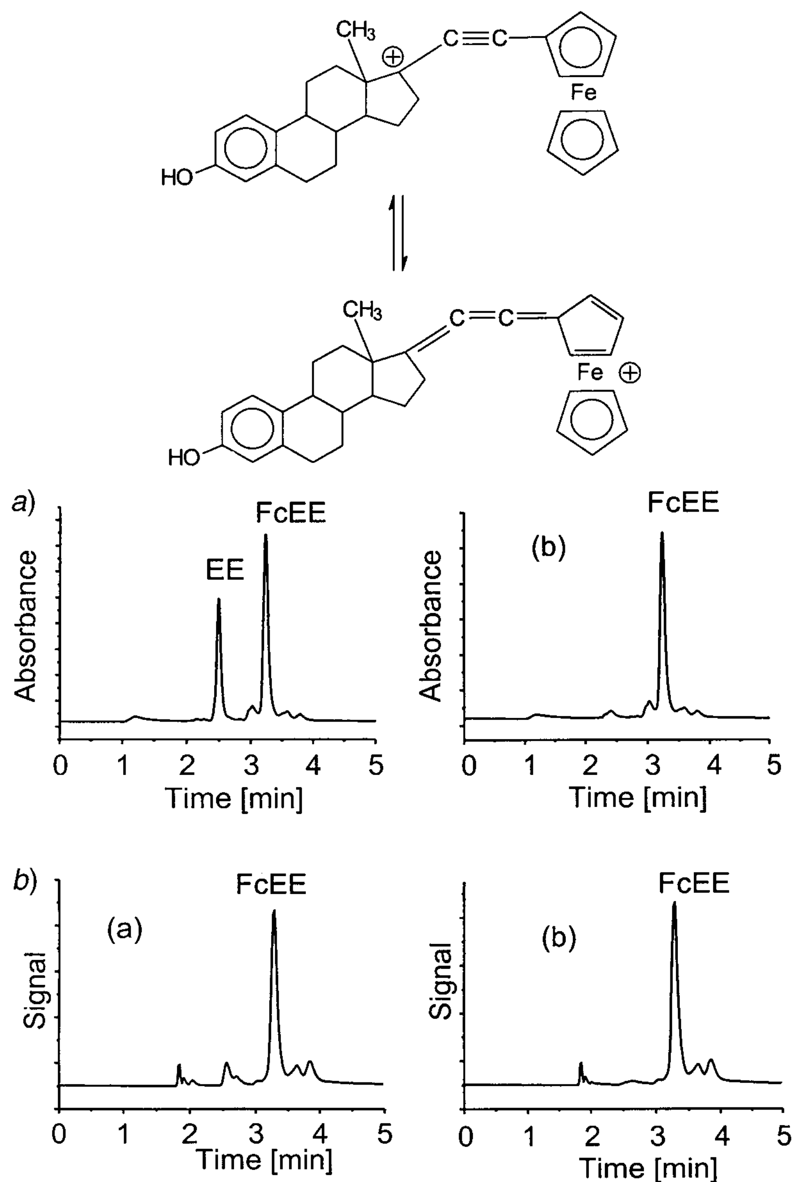


Fig. 3. a) *Reversed-phase HPLC profiles with UV* (254 nm) *and* b) *ED responses* (guard cell, +250 mV; analytical cell, +550 mV) *of an equimolar mixture of EE* (**2**)/*FcEE* (**1**) (left) *and of a pure FcEE* (**1**) *solution* (right). Column, *Merck Lichrosphere 100 RP-18*; solvent, MeCN containing 0.01M $LiClO_4$; flow rate, 1 ml min^{-1}.

retention time of the complex on the column is higher than that of the corresponding estradiol. This can be explained by the log $P_{o/w}$ value of 4.22 found for FcEE (**1**). This value, higher than that found for estradiol (3.3) shows an increase in the lipophilicity of

the complex compared to estradiol, resulting in a longer retention time when the reversed-phase HPLC technique is employed.

Since the ED cell employs a Pd pseudoreference electrode, to determine the optimum potential to apply, the hydrodynamic voltammogram (HDV) is obtained by repeated injection of solutions of FcEE (**1**) at different ED potentials [10]. A fixed difference of *ca.* 300 mV between the potentials of the guard and the analytical cell allows us to increase the S/N ratio during the measurements. According to the HDV, the analytical cell is set at +0.55 V *vs.* the Pd pseudoreference. At this potential, free estradiol, as well as most of the other substances that might cause interference, do not undergo any oxidation process; this enhances the selectivity of the method. The electrochemical detector shows a linear dependence in response/concentration of the sample, and the minimum detectable quantity (*MDQ*) is about 1 nM under these experimental conditions. This limit is fully adequate for pharmacological analyses [31][32], while for physiological analyses, a preconcentration step is still required [7]. A purification/preconcentration step is still frequently performed in any case to avoid interference between compounds present in complex matrices such as blood and urine [33].

3. Conclusion and Outlook. – The ethynylestradiol derivative **1** (FcEE) carrying a redox-active ferrocenyl unit was prepared by a novel synthetic method. The stability of **1** in aqueous media is sufficent to permit investigations of its biological properties. We found that this metallocene retains a satisfactory affinity for an antibody specific to estradiol and that it remains well-recognized by the two natural estrogen receptor subtypes, ERα and ERβ. Under the conditions described, the minimum detectable quantity by HPLC-ED was 1 nM. The study of the potential of metallocene **1** as a tracer for biological analysis will be carried out subsequently and will require a preconcentration step. It will also be of interest to study the proliferative/antiproliferative effects of **1** on hormone-dependent cell lines derived from breast cancers, such as the MCF 7 cell line.

Experimental Part

1. *General.* All reactions were carried out under N_2. A three-necked flask was fitted with a pressure-equalizing dropping funnel, and gas inlet/outlet. Reagents were purchased from *Sigma-Aldrich*, except for iodoferrocene (**3**), which was prepared starting from FcHgCl as previously described [34] and used immediately after synthesis. Solvents were dried and purified before use. Electrochemical measurements were performed using an *EG & G-PAR-273* electrochemical analyzer interfaced to a personal computer (PC), equipped with *PAR M270* electrochemical software. A standard three-electrode cell, designed to allow measurements to be carried out under Ar in anh. deoxygenated MeCN, was employed. The working electrode was glassy carbon, and potentials were referred to the standard calomel electrode (SCE). The supporting electrolyte was 0.1M $LiClO_4$. HPLC: *Kontron* HPLC system (pump model *420*, UV detector model *742*) and an *ESA Coulochem II* electrochemical detector (ED) were interfaced to a PC with the *Perkin-Elmer* 'Turbochrome IV' software; the ED consists of a high-efficiency (70%) amperometric cell (*ESA* model *5011*) equipped with a Pd pseudo-reference electrode and two porous graphite electrodes (guard and anal.). *Merck Lichrosphere 100-RP-18* column (5 μm, 250 × 4 mm) with removable guard column; injection volume 20 μl; eluent, MeCN (flow rate 1.0 ml/min) distilled over CaH_2 before use, and 0.010M $LiClO_4$ as supporting electrolyte; flow rate 1.0 ml/min; the detectors being set in series, the dead volume between the UV (closest to the column) and the ED causes a delay (*ca.* 1 s) between the two responses. NMR Spectra: *JEOL EX-400*; deuterated solvent as internal lock; δ in ppm rel. to $SiMe_4$ ($\delta = 0.00$ ppm). DCI-MS: *Finnigan MAT-95Q* instrument with both magnetic and

electrostatic analyzers [35]; initial ionization of the reagent gas isobutane (at a pressure of 50 Pa) by electron impact (EI), yielding the stable ^{t}Bu$^+$, followed by ionization of the analite (M) by proton transfer from ^{t}Bu$^+$ generating the quasi-molecular ion $[M+H]^+$, which possesses lower energy than that generated by conventional EI and, therefore, undergoes less fragmentation; m/z (rel. %).

2. *[(1-Hydroxycyclohexyl)ethynyl]ferrocene.* A soln. of 1-ethynylcyclohexanol (248 mg, 2 mmol) and iodoferrocene (**3**; 624 mg, 2 mmol), in Et_3N (50 ml) containing cat. amounts of $[PdCl_2(Ph_3P)_2]$ (7 mg) and CuI (5 mg) was stirred for 4 h with heating at 90° under N_2 (slight change from yellow to light orange). The mixture was then cooled to r.t., hydrolyzed with deionized H_2O, and extracted with CH_2Cl_2 (3 ×). After evaporation, the solid product was washed with small amounts of petroleum ether: 277 mg (45%). Orange powder. ^{1}H-NMR ($CDCl_3$): 4.41, 4.18 (*m*, subst. Cp); 4.20 (*s*, Cp); 2.18 (*s*, OH); 2.00–1.26 (*m*, 10 H). ^{13}C-NMR ($CDCl_3$): 88.9 (C≡); 82.7 (≡C); 71.3, 68.4 (subst. Cp); 69.7 (Cp); 64.8 (C–OH); 40.1; 25.1; 23.4.

3. *[(3,17β-Dihydroxyestra-1,3,5(10)-trien-17α-yl)ethynyl]ferrocene* (FCEE; **1**). As described in *Exper. 2*, from 17α-ethynylestra-1,3,5(10)-triene-3,17β-diol (**2**; 2 mmol, 593 mg) and iodoferrocene (**3**; 624 mg, 2 mmol). The CH_2Cl_2 extract was filtered over $MgSO_4$ and evaporated and the residue dried under reduced pressure and chromatographed (silica-gel column, petroleum ether/acetone 3:2), which resolved three bands: a yellow band (residual **3**), an orange band, and a colorless band (unreacted **2**). After evaporation of the eluted orange band, its residue was dried under vacuum and crystallized from CH_2Cl_2: 365 mg (38%) of **1**. ^{1}H-NMR ((D_6)acetone): 8.03 (*s*, OH–C(3)); 7.11 (*d*, H–C(1)); 6.59 (*dd*, H–C(2)); 6.52 (*d*, H–C(4)); 4.33 (*s*, OH–C(17)); 4.39, 4.18 (2*m*, subst. Cp); 4.19 (*s*, Cp); 0.91 (*s*, Me(18)). ^{13}C-NMR ((D_6)acetone): 155.3 (C(3)); 137.7 (C(5)); 131.2 (C(10)); 126.4 (C(1)); 115.3 (C(4)); 113.0 (C(2)); 90.4 (C(20)); 88.6 (C(17)); 83.0 (C(21)); 69.8 (Cp); 68.5–71.3 (subst. Cp); 47.7 (C(13)); 12.7 (Me(18)); 49.5–22.7 (all the remaining resonances). DCI-MS: 481 (43, $[M+H]^+$), 463 (72, $[M-OH]^+$), 308 (30, $[M-FeCp]^+$), 291 (76, $[M-FeCpOH]^+$), 279 (100, $[M-FeCp_2OH]^+$).

The same synthesis performed in Et_2NH led to a lower yield and several secondary products.

4. *Cross reactivity* (CR) *of FcEE* (**1**) *for Specific Antiestradiol Antibodies, as Determined by RIA*. The antibody specific to estradiol used was a polyclonal antibody from sheep and was a gift from the *Société Medgenix* (Fleurus, Belgium). The injected antigen was 3-(carboxymethyl)estradiol oxime synthesized by the method of *Erlanger* [36]. Fractions (500 μl) containing 50 μl of antibody (final dilution 1/1000), 10 μl of [^{3}H]estradiol (0.17 pmol/tube), and increasing amounts of either estradiol or FcEE (**1**; ranging from 10 to 200 pg/tube) in phosphate buffer were incubated for 3 h at r.t. At the end of the incubation, the free and bound fractions were separated by dextran-coated charcoal (final concentration 1.25% of charcoal, 0.125% of dextran T70). The supernatant liquids containing the [^{3}H]estradiol-bound fractions were transferred into vials containing BCS (*Amersham*) scintillation fluid, and the radioactivity was counted in an *LKB-1211 Rackbeta* counter. The CR is equal to the ratio of the quantity of FcEE (**1**) displacing 50% of [^{3}H]estradiol *vs.* the quantity of nonradioactive estradiol displacing 50% of [^{3}H]estradiol. By definition, the CR value of estradiol is equal to 100%.

5. *Relative Binding Affinity* (RBA) *of FcEE* (**1**) *for the Estrogen Receptors α and β*. Sheep uterine cytosol prepared as previously described [37] was used as the source of ERα. The source of ERβ was the human recombinant produced by *PanVera* from baculovirus-infected insect cells. Aliquots (200 μl) were incubated for 3 h at 0° with $2 \cdot 10^{-9}$ M of [^{3}H]estradiol in the presence of nine different concentrations of unlabelled estradiol or FcEE (**1**). The final dilutions of the hormones were made from a $1 \cdot 10^{-3}$ M stock soln. in DMSO with a final percentage of DMSO in the incubation medium of 5%. The RBA of **1** was the concentration of the unlabelled estradiol/FcEE required to inhibit half of the specific [^{3}H]estradiol binding, with the affinity of estradiol set by definition at 100%.

6. *Receptor Inactivation* (RI) *of FcEE* (**1**). Aliquots (200 μl) of sheep uterine cytosol were incubated with 100 nM of FcEE (**1**) for 2.5 h at 0°. The unbound fraction of the hormone was removed by treatment with dextran-coated charcoal (DCC). The remaining reversibly bound hormone was measured after exchange in the presence of [^{3}H]estradiol for 19 h and separation of the free and bound fractions of the tracer by protamine sulfate precipitation. The *RI* value is obtained by taking as 100% the binding value obtained for estradiol itself [38].

7. pK_{R^+} *Value of FcEE* (**1**). The pK_{R^+} value of a compound is related to the stabilization of the carbenium ion generated from the corresponding alcohol. The position of the alcohol-carbenium-ion equilibrium $R^+ + H_2O \rightleftharpoons ROH + H^+$ was studied as a function of sulfuric acid concentration, with the empirical acidity function (C_0) proposed by *Deno et al.* [29]: $pK_{R^+} = C_0 + \log([R^+]/[ROH])$. FcEE (**1**) was dissolved in MeCN ($1 \cdot 10^{-1}$ M soln.). A 50-μl aliquot of this soln. was then added to 450 μl of various concentrations of H_2SO_4 (ranging from 8.8 to 12.3%). The UV measurements were performed at 445 nm after 10 min incubation at r.t. Final calculations were performed on the acidity function C_0.

We thank Dr. *Hans Pritzkow*, University of Heidelberg, for the unit-cell determination and Professor *Marco Vincenti*, University of Torino, for recording DCI mass spectra. We are indebted to *MURST* (COFIN), Rome, for financial support, and to the EU for a mobility grant allocated by the *COST Action* (D5 and D15). We are indebted to a reviewer for having greatly improved the manuscript.

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Received March 13, 2001