Articles

Organometallic Estrogens: Synthesis, Interaction with Lamb Uterine Estrogen Receptor, and Detection by Infrared Spectroscopy†

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ABSTRACT: As an integral part of the development of a new technique using organometallic markers for the detection of hormone receptors by FT-IR spectroscopy, a series of estradiol derivatives labeled with $Cr(CO)_3$ or $Cr(CO)_2CS$ fragments on the A ring has been synthesized. The stereochemistry of one of these steroids, α-[3-(dimethyl-*tert*-butylsiloxy)-17β-estradiol]dicarbonyl(thiocarbonyl)chromium(0), has been established by X-ray diffraction. The organochromium-labeled steroids are stable in aqueous methanol solution, and their relative binding affinities to estrogen receptor have been determined; these values vary from 0.4 to 28%. The complex exhibiting the strongest affinity, [3-*O*-(3-hydroxypropyl)-17β-estradiol]-chromium tricarbonyl complex, has been prepared in a tritiated form with a high specific activity (4.1 Ci/mmol). This tritiated hormone binds reversibly to the estradiol receptor in lamb uterine cytosol with an affinity (K_d = 0.85 nM) and number of binding sites (n = 770 fmol/mg of protein) close to the values observed for estradiol itself. The level of nonspecific binding is low, and the hormone is not bound significantly to other nontarget tissues. The observation that the binding affinity of the steroid depends on which side of the steroidal A ring the organometallic label is bound demonstrates the nonequivalence of the two sides of the A ring with respect to the receptor site. The FT-IR spectra of the organochromium markers in the ν(CO) region can be used for the detection of the estradiol receptor in lamb uterine cytosol.

Breast cancer is most common for Western women, and about 10% of all women will contract the disease during their lifetime (Salmon et al., 1987). The presence of steroidal hormone receptors in mammary tumors is one of the major conditions necessary to promote response to hormonal stimuli (DeSombre et al., 1979; Clark et al., 1983; Allegra et al., 1979). Estradiol- and progesterone–receptor assays are performed on a large scale in specially equipped hospitals throughout the world (Heuson et al., 1981). these analyses are based on the interaction of a radioactive hormone with its specific receptor. The radioactive hormone used is most frequently labeled with tritium (Hoppen, 1981), but ^{125}I has also been employed (Hochberg, 1979; Hochberg & Rosner, 1980). The advantages of the radiolabeled compounds are well-known; however, there are some drawbacks associated with their use (health hazards, chemical and biochemical instability, limited numbers of isotopes, licensing requirements, etc.) that have prompted the search for nonradioisotopic methods of assay.

The most important developments in this direction have been based on the use of fluorescence-labeled hormones (Lee et al., 1977; Martin et al., 1983; Fevig et al., 1987) or antireceptor antibodies (Pertschuk et al., 1980). Unfortunately, the labeled hormones thus far prepared have a low level of estradiol receptor recognition (Fevig et al., 1987) and a high level of nonspecific binding (Berns et al., 1984) rendering them unsuitable for routine receptor assay. There is a similar lack of specificity for the fluorescence-labeled antibodies (Pertschuk et al., 1980). Other nonradioactive labeling approaches that have recently been employed for the detection of antibody–antireceptor complexes have utilized enzymes (Greene & Jensen, 1982; Perrot-Applanat et al., 1985) or heavy metals such as gold sol (Perrot-Applanat et al., 1986).

We present here a novel procedure of potential utility for assaying steroidal hormones based on the use of transition-metal organometallic carbonyl labels.[1] Metal carbonyl fragments were selected as markers for two reasons. First, a window was anticipated in the protein IR spectra at about 2000 cm^{-1}, and second, this is precisely the region where the stretching modes of terminal CO groups in metal carbonyls absorb strongly. Following interaction with the hormone receptor, the organometallic hormones should be readily identified by detection of their metal carbonyl absorptions with Fourier transform infrared spectroscopy (FT-IR) (Jaouen et al., 1985). With this approach in mind, a series of organochromium-labeled estradiol derivatives was synthesized and tested to determine their ability to bind estradiol receptor. As a crucial part of the work, the [3-*O*-(3-hydroxypropyl)-17β-estradiol]chromium tricarbonyl complex was prepared in a tritiated form with a high specific activity. In this way, it has been possible to monitor directly, for the first time, the interaction of such an organometallic hormone with estradiol receptor.

Experimental Procedures

Chemicals, Materials, and Methods. 17β-Hydroxyestra-1,3,5(10)-triene (E_2, **1**), diethylstilbestrol (DES), other unla-

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[1] Part of this work has been published in preliminary communications; see Jaouen et al. (1984, 1985).

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beled steroids, and protamine sulfate (from salmon, grade X) were purchased from Sigma Chemical Co. Ether, pentane, and acetone were from S.D.S., France. Analytical thin-layer chromatography was performed on 0.2-mm silica gel plates (Merck 5735), and spots were visualized by 254-nm ultraviolet light. Preparative thin-layer chromatography was carried out on 1-mm silica gel plates (Merck 7731) or 0.2-mm silica gel plates (Merck 5735). [6,7-^{3}H]-17β-Estradiol (50–52 Ci/mmol) was from CEA Gif/Yvette, France. Dibutyl ether was dried by distillation from benzophenone–sodium. Syntheses of organometallic complexes were carried out under a dry nitrogen atmosphere. Melting points were determined on a Kofler apparatus and were uncorrected. ^{1}H NMR spectra were taken on a Bruker spectrometer, AM 250. Optical rotations were recorded on a Perkin-Elmer 241 apparatus. Mass spectra were recorded on a Nermag spectrometer. High-performance liquid chromatography (HPLC) was performed on a Beckman gold system with a reverse-phase column (Ultrasphere ODS, 5 μm) and an elution solvent of methanol/water (75/25). Compounds **2–8** were synthesized according to the procedures reported previously (Top et al., 1985).

3-O-(3-Hydroxypropyl)-17β-estradiol (**9**). Estradiol (1.08 g, 4 mmol) was heated under reflux for 15 h with sodium hydroxide (0.320 g, 8 mmol) dissolved in 50 mL of acetone. Bromopropanol (1.4 g, 10 mmol) was then added, and the temperature was maintained for 2 days. After filtration and evaporation of the solvent, the residue was redissolved in CH_2Cl_2. The solution was washed with water until the pH of the washing is neutral and then dried over $MgSO_4$, filtered, and evaporated. The white solid obtained after recrystallization from ether was identified as **9** (0.9 g, 100%): mp 168 °C; $[\alpha]^{21}_D$ +70.6° (CH_2Cl_2, *c* 0.51). Anal. Calcd for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.05; H, 9.17. The mass spectrum showed a peak at *m/z* 330 ([M]$^+$). ^{1}H NMR ([$^2H_6$]acetone): (aromatic ring) δ 7.31 (d, 1), 6.83 (dd, 1), 6.74 (d, 1); (Me-13) δ 0.83 (s, 3); (OCH_2) δ 4.14 (t, 2).

α- and β-[3-O-(3-Hydroxypropyl)-17β-estradiol]tricarbonylchromium (**10** *and* **11**). 3-*O*-(3-Hydroxypropyl)-17β-estradiol (**9**) (0.6 g, 1.8 mmol) and $Cr(CO)_6$ (1.1 g, 5 mmol) were heated under reflux in dibutyl ether (150 mL) for 6 h. After filtration and evaporation of solvent, the resulting yellow oil was chromatographed on silica gel plates with THF/petroleum ether (2/3) as eluent. The fractions were extracted in ether and recrystallized from ether/petroleum ether. The first fraction (yellow crystals) was identified as **10** (0.27 g, 32%): $[\alpha]^{21}_D$ 45.8° (CH_2Cl_2, *c* 1.08). Anal. Calcd for $C_{24}H_{30}O_6Cr$: C, 61.79; H, 6.48. Found: C, 61.30; H, 6.68. The mass spectrum showed peaks at *m/z* 466 ([M]$^+$), 382 ([M − 3CO]$^+$), and 330 ([M − $Cr(CO)_3$]$^+$). ^{1}H NMR ([$^2H_6$]-acetone): (aromatic ring) δ 6.08 (d, 1), 5.40 (dd, 1), 5.34 (d, 1); (Me-13) δ 0.67 (s, 3); (OCH_2) δ 4.00 (t, 2). The second fraction (yellow crystals) was identified as **11** (0.21 g, 25%): $[\alpha]^{21}_D$ 70.0° (CH_2Cl_2, *c* 1.08). Anal. Calcd for $C_{24}H_{30}O_6Cr$: C, 61.79; H, 6.48. Found: C, 61.84; H, 6.60. The mass spectrum showed peaks at *m/z* 466 ([M$^+$]), 382 ([M − 3CO]$^+$), and 330 ([M − $Cr(CO)_3$]$^+$). ^{1}H NMR ([$^2H_6$]-acetone): (aromatic ring) δ 6.00 (d, 1), 5.31 (dd, 1), 5.23 (d, 1); (Me-13) δ 0.78 (s, 3); (OCH_2) δ 4.04 (t, 2).

[^{3}H]-α-[3-O-(3-Hydroxypropyl)-17β-estradiol]tricarbonylchromium(0) ([*^{3}H*]-**10**). This complex was synthesized as described previously (Top et al., 1987). A 10^{-3} M stock solution in absolute ethanol was freshly prepared for each set of experiments and kept in the dark at −20 °C. The specific activity of the solution, determined by ultraviolet spectroscopy and scintillation methods, was 4.1 Ci/mmol.

Structural Determination of **7**. The X-ray crystallographic data for **7** were obtained for small single crystals (0.15 × 0.10 × 0.6 mm) at room temperature on an Enraf-Nonius CAD 4 automatic diffractometer with Mo Kα (0.71069 Å) radiation. $CrSiO_4C_{27}H_{37}$ crystallizes in the $P2_12_12_1$ space group with eight molecules per unit cell. The orthorhombic cell parameters are a = 13.388 (3), b = 32.080 (1), and c = 13.913 (3) Å, V = 5975 (1) Å^3, and D_{calcd} = 1.19 g cm^{-3}. The data were collected by the $\omega - 2\theta$ method with a takeoff angle of 1.0 ± 0.35 tan θ (in degrees); the crystal–counter distance was 173 mm, and the counter slit width was 2.8 ± 0.50 tan θ. Three standard reflections were measured periodically in order to check the crystal orientation and to establish that were no significant variations in the measured intensities. Most of the atoms were located by direct methods and the others from Fourier difference maps. The calculations were performed with the SDP library programs. The crystals are twinned and because of their small dimensions gave weak reflections. A total of 1857 nonzero reflections were collected for 613 variables, assuming anisotropic thermal motions. The two independent molecules in the unit cell were refined separately. The refinements proved difficult, and several of the temperature factors had to be fixed before the solution stabilized at an R factor of 0.098 (R_ω = 0.121).

Animal Tissues. Lamb uteri weighing approximately 7 g were obtained from the slaughterhouse at Mantes-la-Jolie, France. They were immediately frozen and kept frozen in liquid nitrogen prior to use.

Preparation of Lamb Uterine Cytosol. Lamb uteri were thawed and then minced. The resulting tissues were homogenized with an Ultra-Turrax in buffer A (0.05 M Tris-HCl, 0.25 M sucrose, 0.1% β-mercaptoethanol, pH 7.4 at 25 °C). The homogenate was centrifuged at 105000*g* for 60 min in the 52 Ti rotor of a Beckman L5 ultracentrifuge. The protein concentration of the 105000*g* supernatant (cytosol) was determined by the method of Lowry et al. (1951).

Binding Assays. (*1*) *Protamine Sulfate Precipitation Assay*. The technique described by Steggles and King (1970) and modified by Blondeau et al. (1975) was used for the separation of ^{3}H-labeled bound and free steroid. Following incubation of aliquots of cytosol (0.2 or 0.4 mL containing 2 mg of protein/mL) with ^{3}H-labeled steroid, an equal volume of buffer containing 1 mg/mL protamine sulfate was added to each tube. The mixtures were vortexed and allowed to stand at 0 °C for 10 min. The resulting precipitates were filtered on glass fiber paper (Watman GF/C) under a moderate vacuum and washed with 40 mL of ice-cold buffer. The filter papers were then transferred to scintillation vials, and 10-mL portions of scintillation fluid (ACS, Amersham, U.K.) were added. Radioactivity was measured on an LKB-1211 RackBeta counter.

(*2*) *Competitive Binding Assays*. Aliquots of cytosol were incubated for 3 h at 0 °C with ^{3}H-labeled steroids (6×10^{-9} M) in the presence or absence of competing unlabeled steroids. Competitors were added at concentrations ranging from 10^{-10} to 10^{-6} M. The percentage reduction in binding of ^{3}H-labeled steroid (Y) was calculated by use of the logit transformation of Y [logit Y = ln [$Y/(1 - Y)$]] versus the log of the mass of the competing steroid. The concentration of unlabeled steroid required to displace 50% of the bound ^{3}H-labeled steroid (logit Y = 0) was calculated for each steroid tested, and the results were expressed as relative binding affinities (RBA).

(*3*) *Scatchard Analysis*. In order to determine the amount of estrogen-specific binding sites, cytosol was incubated with

varying concentrations of either [^{3}H]-17β-estradiol or [^{3}H]-α-[3-*O*-(3-hydroxypropyl)-17β-estradiol]tricarbonylchromium ([^{3}H]-**10**) with or without a 100-fold excess of unlabeled DES. Estrogen-specific binding of the ^{3}H-labeled steroid is considered to be the difference between binding measurements conducted in the absence and the presence of excess unlabeled E_2. Because the [^{3}H]-17β-estradiolchromium tricarbonyl complex shows higher levels of nonspecific binding than [^{3}H]E_2, it is important to correct for this nonspecific binding in the concentration of the free ligand in binding affinity experiments. A method for making such corrections has been described (Katznellenbogen et al., 1978; Richard-Foy et al., 1978; Blondeau & Robel, 1975). According to these authors, the free ligand concentration which is normally considered to be the difference between total added ligand and the receptor-bound ligand must be corrected by a $1 + nk$ factor. To make these corrections, binding of [^{3}H]E_2 and [^{3}H]-**10** was measured by equilibrium dialysis in 6.3 mm diameter dialysis bags. The binding studies were performed after 18 h at 4 °C with cytosol concentrations of 2.9 mg/mL and ligand concentrations of 37–274 nM for [^{3}H]E_2 and of 100–525 nM for [^{3}H]-**10**. Values for nk were 0.136 mg^{-1} mL^{-1} for [^{3}H]E_2 and 0.82 mg^{-1} mL^{-1} for [^{3}H]-**10**.

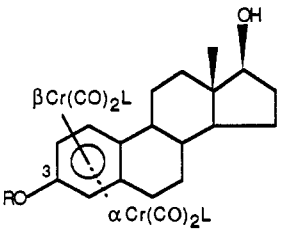
FT-IR Analyses. Varying volumes of cytosol (6–8 mL containing 4 mg/mL proteins) were incubated for 4 h at 0 °C with known concentrations of the organometallic-labeled estradiol derivatives to yield a final concentration of the label in the range 10^{-8}–10^{-6} M. At the end of the incubation period, an equal volume of protamine sulfate solution (2 mg/mL) was added to precipitate the proteins. The precipitates obtained were collected by centrifugation (3300*g*, 15 min). After elimination of the supernatants, the precipitates were washed four times with 5 mL of phosphate buffer (0.05 M, pH 7.4) and twice with 5 mL of distilled water and lyophilized. This procedure yielded white powders which could be used directly for the FT-IR measurements. The receptor concentration in these samples was established by competitive binding assay with [^{3}H]E_2 to be in the range of 300 fmol/mg.

All the solvents used in the IR work either were of spectrograde quality or were distilled under nitrogen immediately prior to use. CsI (gold label 99.999%) was obtained from Aldrich Chemical Co. The solid samples were pressed into 3-mm pellets with a Qwik Handi-Press set, available from Aldrich. When samples were pressed together with CsI, the mixtures were initially ground in stainless steel vials with a Wig-L-Bug (Aldrich). The spectra were recorded on a Nicolet 6000 FT-IR spectrometer equipped with a mercury–cadmium–telluride (MCT) detector (Infrared Associates) and having a beam diameter at the focal point of 5 mm. The pellet holder was mounted on an *X*–*Y* translation stage so that the sample position could be adjusted to maximize the detector response. Large numbers (10 000–20 000) of medium-correlation scans were accumulated and coadded. The mirror velocity was set at 0.640 cm s^{-1} in order to optimize the detector sensitivity. The gain was adjusted until each interferogram reached 50–75% of its height in a background scan recorded for the empty pellet holder. The relative gain of all the points after the first 1024 interferograms was increased by a factor of 8. The coadded interferograms were apodized with the Happ–Genzal function and Fourier transformed with one level of zero filling to yield a resolution of 4 cm^{-1}.

Results and Discussion

In terms of organometallic chemistry, an obvious site for complexation of estradiol derivatives is the six-electron donor, aromatic A ring, and the most compatible metal carbonyl fragment is $Cr(CO)_3$ (Jaouen, 1978). The precursor to this moiety is the parent $Cr(CO)_6$ molecule. Reaction of E_2 (compound **1**) with $Cr(CO)_6$ leads to two diastereomers (Table I) in which the location of the $Cr(CO)_3$ tripod is either on the α (compound **2**) or β (compound **3**) side of the molecule. These diastereomers can be separated by preparative thin-layer chromatography. However, these phenolic complexes rapidly decompose in ethanolic solution to form E_2 and chromium salts. Therefore, they proved unsuitable for our purpose. The modification of the 3-hydroxy function greatly increases the stability; the solid complexes are stable for years in the dark. The relative stabilities of the complexes can be conveniently studied by monitoring their UV spectra in ethanol solution. For example, a band at 319 nm is characteristic of compound **10**. No decomposition of a stock solution (ca. 1×10^{-3} M) of compound **10** occurs when it is kept for 1 week in the dark at −20 °C. In order to minimize the chance of decomposition, a stock solution was freshly prepared for each experiment, and all of the biochemical experiments were performed at 4 °C in the absence of light. We have also checked by HPLC that the complexe does not decompose under the incubation conditions. Finally, it should be noted that the solubility of the hormones in ethanol is greatly increased after complexation (ca. 400 mg/mL in 1 h).

Table I: Characteristic Data of the Estradiol Derivatives



compound	yield	mp (°C)	$[\alpha]^{22}$D (deg)
1, R = H (17β-estradiol)	commercially available		
2, R = H; $\alpha Cr(CO)_3$[a]		dec	+45[b,d]
3, R = H; $\beta Cr(CO)_3$[a]		dec	+54.3[b,d]
4, R = $Si(CH_3)_2tBu$[a]	73	158	+58.5[c,e]
5, R = $Si(CH_3)_2tBu$; $\alpha Cr(CO)_3$[a]	29	220	+38.7[c,d]
6, R = $Si(CH_3)_2tBu$; $\beta Cr(CO)_3$[a]	41.5	179	+62.5[c,e]
7, R = $Si(CH_3)_2tBu$; $\alpha Cr(CO)_2CS$[a]	11	142	+20.8[c,e]
8, R = $Si(CH_3)_2tBu$; $\beta Cr(CO)_2CS$[a]	35	125	+17.7[c,e]
9, R = $HO(CH_2)_3$	100	168	+70.6[c,e]
10, R = $HO(CH_2)_3$; $\alpha Cr(CO)_3$	32	163	+45.8[c,e]
11, R = $HO(CH_2)_3$; $\beta Cr(CO)_3$	25	157	+70[c,e]

[a] Synthesized as described in Top et al. (1985). [b] Solvent: acetone. [c] Solvent: CH_2Cl_2. [d] Concentration from 0.012 to 0.030 g mL^{-1}. [e] Concentration from 0.0005 to 0.002 g mL^{-1}.

The molecular structure of one of the diastereomers of compound **7** was determined by X-ray diffraction. A perspective view of the structure with the ORTEP program is shown in Figure 1. This drawing clearly shows the α disposition of the $Cr(CO)_2(CS)$ tripod with respect to the C(18) atom of the methyl group and that the complexation of the aromatic ring does not modify appreciably the skeleton of the estradiol (Busetta & Hospital, 1972). The most important bond distances and bond angles for compound **7** are listed in Table II. The average C–C distance for the A ring of compound **7** is 1.41 Å, while that for estradiol hemihydrate is 1.39

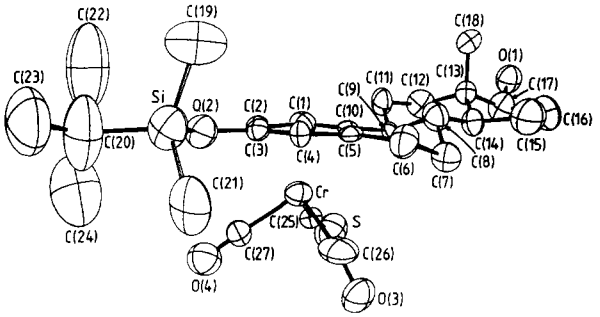


FIGURE 1: An ORTEP view of complex **7** with $Cr(CO)_2CS$ attached to the α-face of the steroid.

Table II: Selected Bond Distances (Å) and Angles (deg) for Compound **7**

Cr–C(1)	2.26_5	C(25)–Cr–C(26)	91
Cr–C(2)	2.21_2	C(25)–Cr–C(27)	89
Cr–C(3)	2.27_6	C(26)–Cr–C(27)	90
Cr–C(4)	2.25_1		
Cr–C(5)	2.14_2		
Cr–C(10)	2.26_3		
Cr–C(25)	1.74_1		
Cr–C(26)	1.78_9		
Cr–C(27)	1.91_9		
C(25)–S	1.49_3		
C(26)–O(3)	1.16_6		
C(27)–O(4)	1.24_3		

Å. The stereochemistries of the other organochromium estradiol derivatives were established by a combination of synthetic and spectroscopic correlations based on the known structure of compound **7**. High-field (500 MHz) NMR spectroscopy was especially useful in differentiating between the α- and β-diastereomers (Top et al., 1985).

The competition curves obtained from a competitive, protein-binding assay using $[^3H]E_2$ as a tracer and seven of the modified estradiol derivatives are shown in Figure 2. As observed previously (Zeelen & Bergink, 1980; Raynaud et al., 1985; Chernayaev et al., 1978), substitution of the C(3) phenolic hydroxyl group invariably decreases receptor binding (see compound **4**). However, a good affinity is regained when the 3-hydroxyl function is located away from the steroid skeleton by means of the spacer chain, $-(CH_2)_3-O-$ (compound **9**; Table III). This increased binding affinity can be explained by folding of the lateral chain which permits the correct positioning of the OH group in the active site (Katzenellenbogen et al., 1978). Among the complexes listed in Table III, it appears that compound **10** with a high affinity value (RBA = 28%), very close to that of the free steroid (**9**, RBA = 37%), would be an excellent choice for further study on the basis of recognition criteria.

In order to determine K_d and the number of binding sites for the receptor and to examine the extent of low-affinity binding, 17α-[^{3}H]-**10** was prepared (sp act. 4.3 Ci/mmol; Top et al., 1987). Figure 3 illustrates the saturation curves obtained after incubation of lamb uterine cytosol in the presence of increasing amounts of [^{3}H]-**10** (1×10^{-10} to 2.5×10^{-8} M) for 1.0–3.5 mg/mL protein concentration range. In each case, [^{3}H]-**10** binds to the estradiol receptor in a saturable and reversible manner. The level of nonspecific binding (i.e., the bound fraction remaining after incubation in the presence of [^{3}H]-**10** and a large excess of nonradioactive E_2) found in uterine cytosol depends on the amount of protein in the incubation medium. For the typical protein concentrations used in this type of saturation study (1.0–1.5 mg/mL), the level of nonspecific binding is quite low. For higher protein concentrations (ca. 3.5 mg/mL), the amount of nonspecific

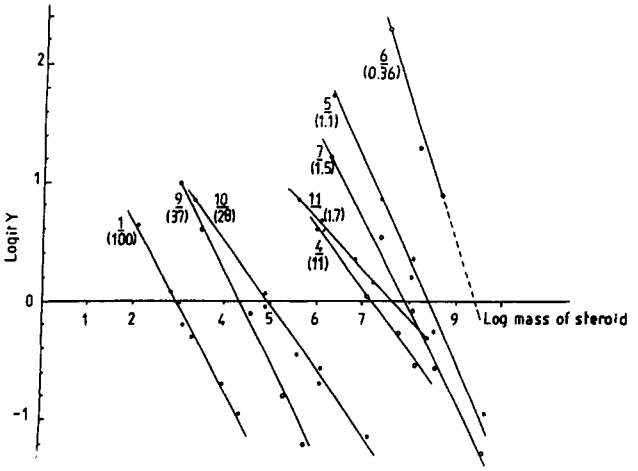


FIGURE 2: Lamb uterine cytosol (0.2-mL fractions containing 8 mg of proteins/mL) was incubated at 0 °C for 3 h with 2 nM [^{3}H]estradiol (Radio Chemical Centre, Amersham, England; sp act. = 58 Ci/mmol) and increasing amounts of competing steroids (10–1000-fold excess; nine concentrations in duplicate). Bound fractions were measured by protamine sulfate precipitation as described under Experimental Procedures. Nonspecific binding was determined with a 500-fold excess of unlabeled estradiol. The binding of labeled hormone in the presence of competitor was expressed as a percentage of its initial value (in the absence of competitor and after deduction of the nonspecific binding), and data were represented by log/logit plots. The relative binding affinity (RBA) of the competitor (number in parentheses) is taken as the ratio of the concentrations of unlabeled estradiol/competitor required to inhibit half of the specific [^{3}H]estradiol binding with the affinity of estradiol set at 100%.

Table III: Receptor Binding Affinity, Nonspecific Binding, and Binding Selectivity Index of the Estradiol Derivatives

compound	receptor binding affinity (RBA)[a]	partition coefficient (log P)[b]	nonspecific binding (NSB)[c]	binding selectivity index (BSI)[c]
1, R = H (17β-estradiol)	100[d]	3.24	1.0[d]	100[d]
4, R = $Si(CH_3)_2tBu$	11	3.26	1.03	11
5, R = $Si(CH_3)_2tBu$; $\alpha Cr(CO)_3$	1.1	2.74	0.42	2.5
6, R = $Si(CH_3)_2tBu$; $\beta Cr(CO)_3$	0.4	2.0	0.12	3
7, R = $Si(CH_3)_2tBu$; $\alpha Cr(CO)_2CS$	1.5	2.6	0.32	5
9, R = $HO(CH_2)_3$	37	2.70	0.39	95
10, R = $HO(CH_2)_3$; $\alpha Cr(CO)_3$	28	2.37	0.22	127
11, R = $HO(CH_2)_3$; $\beta Cr(CO)_3$	1.8	2.04	0.13	13

[a] Values determined as explained in Figure 1. [b] log P = log [1-octanol–water partition coefficient determined experimentally in our laboratory according to the method of Leo et al. (1971)]. [c] NSB = nonspecific binding calculated relative to estradiol from log P with the equation established by Leo et al. (1971): log NSB = 0.751(log P^X – log P^{E2}) where $P^X = P^{E2}$ are the octanol–water partition coefficients of a compound and estradiol, respectively. BSI = RBA/NSB (Katzenellenbogen et al., 1980). [d] Values by definition.

binding increases, but it still remains reasonably low. The nonspecific binding is always appreciably lower than the specific binding, even at high molarities (ca. 2.0×10^{-8} M). This result is in good agreement with the partition coefficients given in Table III which indicate that the complexes are more polar than the parent steroids.

The saturation curve data from Figure 3A and from the saturation curve of E_2 (data not shown) are presented in the form of a Scatchard plot in Figure 4 following correction of the concentrations of free hormone for the low-affinity binding sites. It should be emphasized that the organometallic hor-

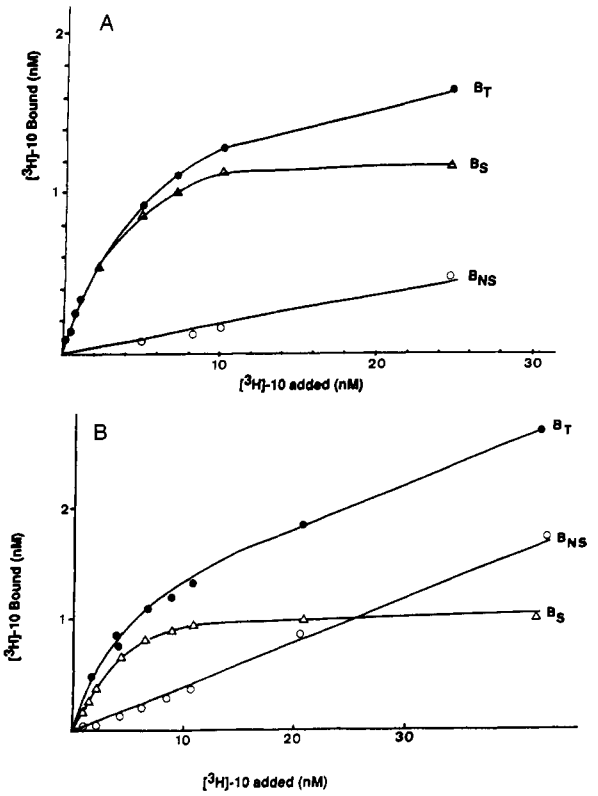


FIGURE 3: Interaction of [^{3}H]-**10** with uterine cytosol estrogen in vitro. Lamb uterine cytosol proteins (1.5 mg/mL for panel A; 4.5 mg/mL for panel B) was incubated with increasing concentrations of [^{3}H]-**10**, alone or together with a 100-fold excess of unlabeled DES [to determine the nonspecific binding (B_{NS})]. Binding equilibrium was achieved by incubating the samples for approximately 3 h at 4 °C. Bound [^{3}H]-**10** steroid was measured by protamine sulfate assay, as described under Experimental Procedures (B_T, total binding; B_S, specific binding).

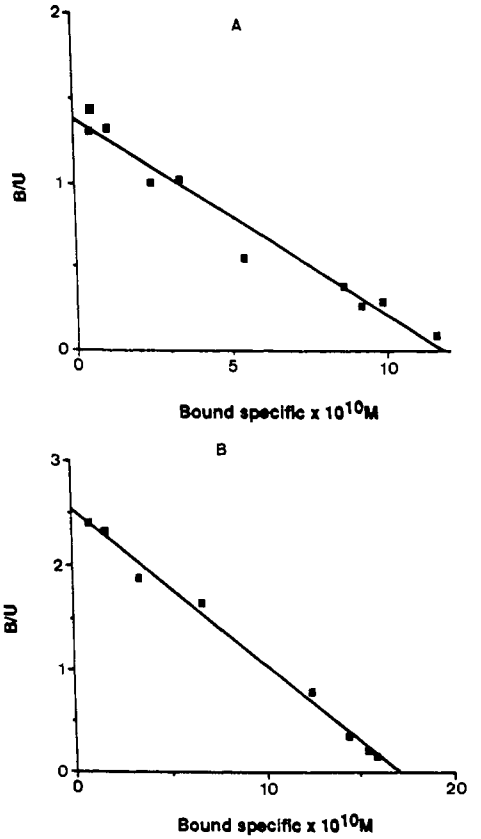


FIGURE 4: Scatchard analysis of the estrogen receptor from lamb uterus. Binding of [^{3}H]-**10** (panel A) and [^{3}H]E_2 (panel B) was meausred after incubation of lamb cytosol proteins (1.54 mg of protein/mL) with increasing concentrations of either [^{3}H]-**10** or [^{3}H]E_2 alone or together with a 100-fold excess of unlabeled DES. In the two panels, the concentrations of free hormone have been corrected for the low-affinity binding ($1 + nk$ = 1.36 for E_2 and 1.82 for **10**; see Experimental Procedures). These results are based on two separate experiments.

Table IV: Binding of [^{3}H]-**10** with Rat Lung[a]

incubation molarity (nM)	[^{3}H]-**10** bound in the absence of DES (nM)	[^{3}H]-**10** bound in the presence of DES (nM)
1.09	0.12	0.11
5.11	0.32	0.31
11.70	0.37	0.37

[a] Fractions of rat lung cytosol (6.5 mg/mL protein) were incubated with increasing amounts of [^{3}H]-**10** either in the presence or in the absence of a 100-fold excess of DES. Bound fractions were measured by protamine sulfate assay (see Experimental Procedures).

mone binds *exclusively* to the high-affinity binding sites of estradiol. The actual binding affinity of [^{3}H]-**10** is very similar to that for E_2 itself [K_d([^{3}H]E_2) = 0.67 nM; K_d([^{3}H]-**10**) = 0.85 nM]. In addition, the number of binding sites for [^{3}H]-**10** is only slightly less (approximately 70–80%) than that for E_2. This observation may not be especially significant since the specific activities were not measured with the same precision for the two hormones.

For nontarget tissue, such as that in rat lung, [^{3}H]-**10** binds very weakly and nonspecifically (Table IV). This provides convincing proof that the organometallic hormones bind specifically for tissue containing estradiol receptors. Moreover, the binding of the hormone is estrogen specific. This result is illustrated by the competition curves (Figure 5) obtained following incubation of lamb uterine cytosol fractions in the presence of 8×10^{-10} M [^{3}H]-**10** and varying concentrations of nonradiolabeled steroids (e.g., estradiol, DES, **10**, testosterone, dihydrotestosterone, and progesterone). Only those steroids having an affinity for estradiol receptor (viz., estradiol, DES, and **10**) displace [^{3}H]-**10** from its binding sites.

The complexation site of the organometallic tripod on the A ring of the steroid strongly discriminates between the α- and β-diastereomers with respect to their receptor recognition properties (Table III). In particular, while the β-isomers (**6**, **11**) show relatively modest affinities for the receptor, the α-isomers (**5**, **7**, **10**) bind with significantly higher affinities. This observation implies a nonequivalence of the two sides of the steroidal A ring with respect to the receptor binding sites. This nonequivalence may be the result of severals factors, e.g., steric effects due to the size of the chromium carbonyl tripod or electronic effects due to its known electron-withdrawing capabilities (Jaouen, 1978). It should be emphasized that the nonequivalence would not have been readily appreciated without the ability of the organometallic label to bond above and below the A ring of the estradiol derivatives.

The advent of FT-IR instrumentation has allowed several orders of magnitude improvement in the signal/noise (S/N) ratio in relatively short times of data acquisition, with detection limits in the picogram range (Holloway et al., 1988), and FT-IR studies of biological samples are now becoming more widespread (Griffiths & de Haseth, 1986). The initial IR studies in our case were for the organometallic-labeled receptor complex formed with compound **5**.

Figure 6 shows the FT-IR spectrum of the cytosol proteins, prepared in the absence of compound **5**, diluted in a CsI matrix. There is no absorption in the region between 2200 and 1800 cm^{-1}. The IR spectrum in the ν(CO) region of **5** in benzene solution is shown in Figure 7A. The greater

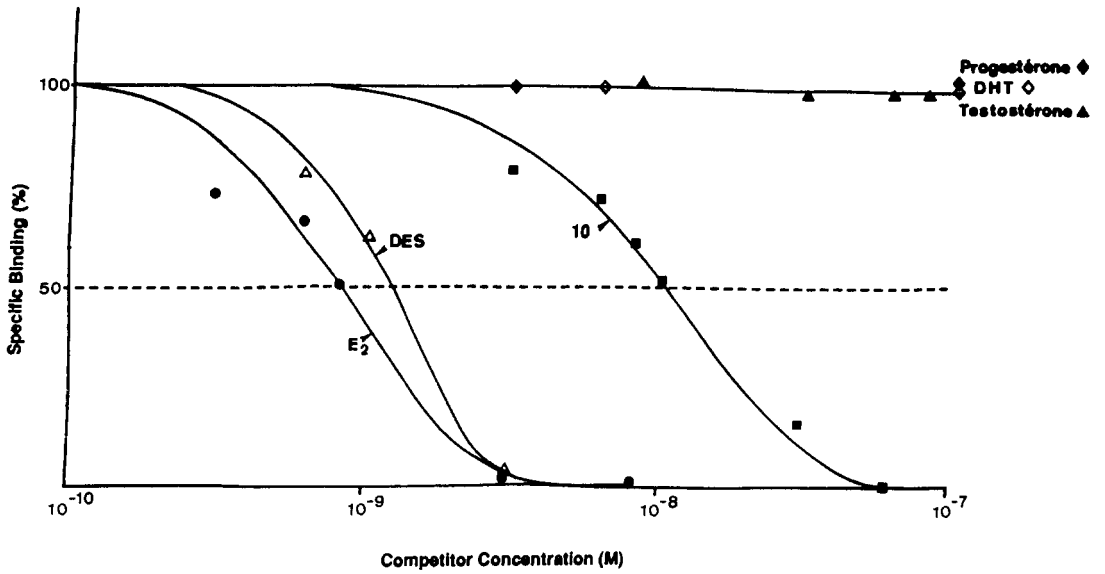


FIGURE 5: Binding affinity assay. Various concentrations of competitor were incubated with 8.8×10^{-9} M [^{3}H]-**10** in lamb uterine cytosol (1.7 mg/mL protein). Bound fractions were measured by protamine sulfate assay as described under Experimental Procedures.

relative intensities of the carbonyl vibrations of the $Cr(CO)_3$ moiety at 1960 (a_1) and 1876 (e) cm^{-1} compared to those of the estradiol backbone vibrations clearly indicate the potential advantage of the $Cr(CO)_3$ label in the detection of low concentrations of steroids by FT-IR spectroscopy. The normal mode assignments indicated in parentheses are based on those given in earlier vibrational work on $(\eta^6\text{-}C_6H_6)Cr(CO)_3$ (English et al., 1981).

Since the ratio of receptor to total precipitated proteins from the cytosol is very low and because dispersion in CsI or dissolution in an organic solvent would dilute the sample (thereby reducing the intensities of the carbonyl peaks in the IR), we decided to press the cytosol proteins directly into 3 mm diameter minipellets. The IR spectrum of a typical pressed pellet of the cytosol proteins following incubation with compound **5** is shown in Figure 6B. The protein absorptions are well off-scale, yet the metal carbonyl region is devoid of any features except for two small peaks. An expansion of this region is illustrated in Figure 7B. The two peaks detected at 1955 and 1881 cm^{-1} correspond reasonably to the ν(CO) modes of compound **5** in a CsI matrix (1953 and 1868 cm^{-1}).

In order to assess the quantitative potential of our IR approach to hormone receptor detection, an investigation was undertaken of the intensity (taken as peak area) of the a_1 ν(CO) IR mode of the $Cr(CO)_3$ label as a function of pellet weight (i.e., amount of protein). The linearity of the resulting graph ($r = 0.98$) is satisfactory in the range of pellet weight from 1.4 to 2.5 mg. Samples weighing less than 1.4 mg proved insufficient to make a satisfactory pellet, while for pellet weights greater than 2.6 mg deviation from linearity occurred, indicating a significant decreases in energy throughput because of the resulting thicker pellets.

The next step in the project involved a similar IR spectral study using the doubly labeled compound, [^{3}H]-**10**. Figure 7C shows the ν(CO) region of the FT-IR spectrum of a sample obtained by in vitro incubation of compound [^{3}H]-**10** with lamb uterine cytosol at approximately the same concentration (ca. 10^{-8} M) as currently used in radiochemical assays with E_2 itself. This concentration is such that the amount of nonspecific binding is quite low. From a visual comparison of Figure 7B,C, it can be seen that the ν(CO) band intensities when [^{3}H]-**10** is used are appreciably lower than those observed when compound **5** is used, indicating less nonspecific binding for compound [^{3}H]-**10** than for compound **5**. Al-

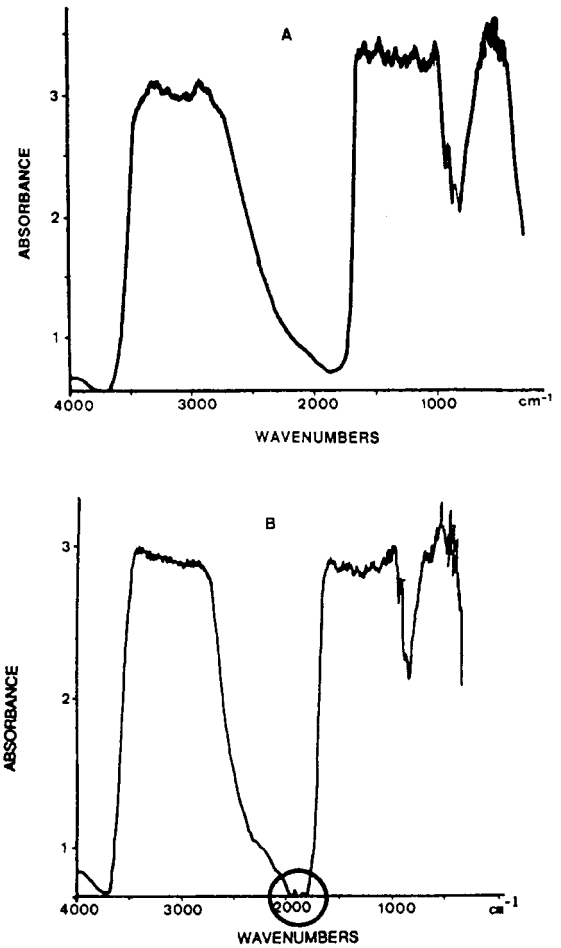


FIGURE 6: FT-IR spectrum of lamb uterine cytosol following incubation with (A) 17β-estradiol and (B) **5** and subsequent precipitation from solution with protamine sulfate (off scale; MCT detector).

though the S/N ratio is high enough in the case of compound [^{3}H]-**10** to discern definitively the presence of the metal carbonyl vibrations, the areas of these peaks could not be meausred with any quantitative accuracy.

The reversibility of the binding between compound [^{3}H]-**10** and the receptor was demonstrated by both FT-IR spectroscopy and radioassay. The IR spectrum of a sample obtained subsequent to the competitive binding experiment with excess

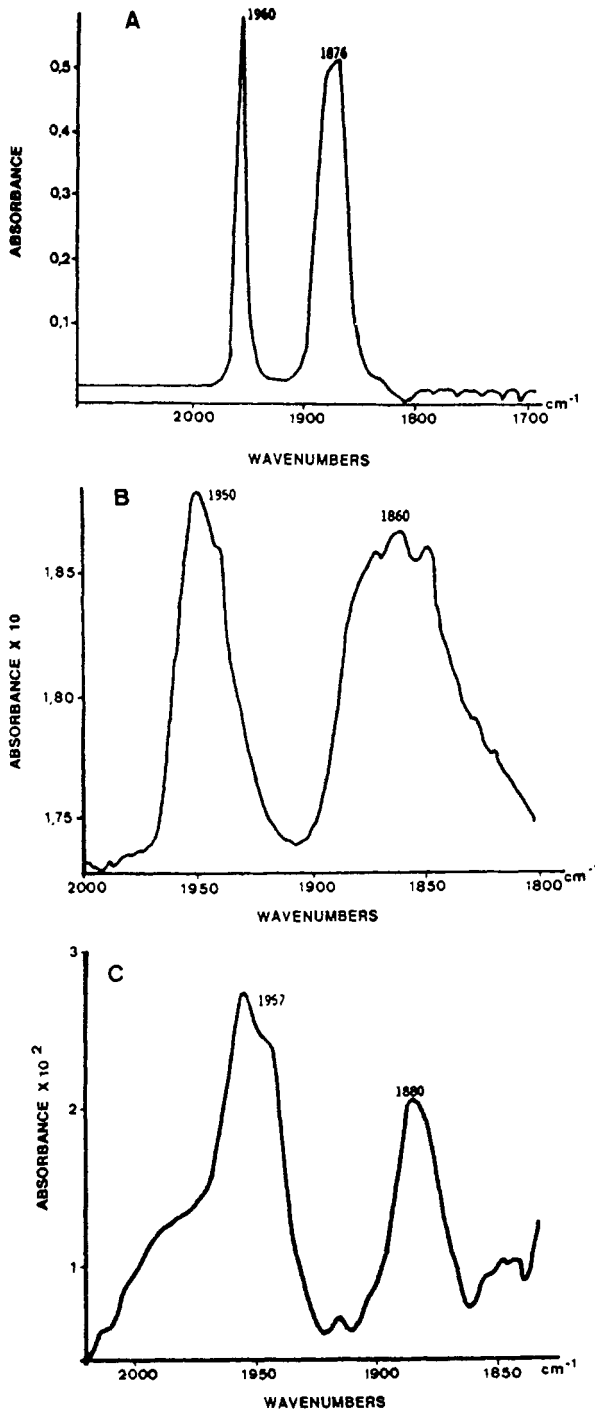


FIGURE 7: (A) ν(CO) section of FT-IR spectrum of complex **5** in benzene solution. (B and C) Expansion of the metal carbonyl area of the IR-FT spectrum of lamb uterine incubated with (B) **5** and (C) **10**.

DES was recorded. The ν(CO) region of this spectrum (Figure 8) reveals the absence of any peaks due to the organometallic-labeled receptor complex, confirming the binding reversibility.

Conclusions

A series of organometallic-labeled derivatives of estradiol has been synthesized. These derivatives are stable in the types of solution encountered in biochemical studies, and some of them have good binding affinities for estradiol receptor. The introduction of an organometallic tripod represents a novel means of exploring the stereochemistry above and below the A ring of estradiol. Furthermore, by virtue of the synthesis

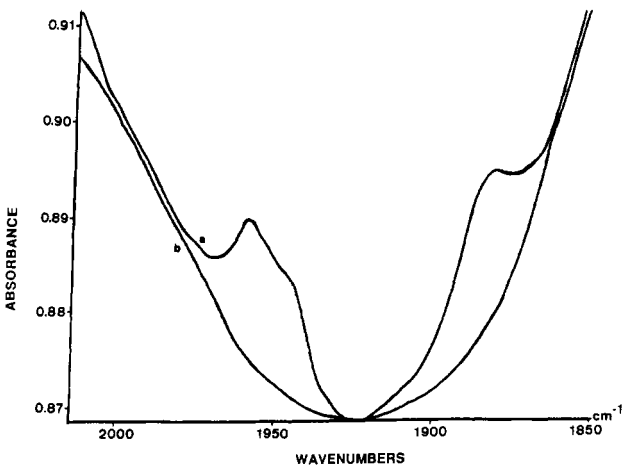


FIGURE 8: FT-IR spectrum (26 000 scans, 4-cm^{-1} resolution) of the metal carbonyl area of lamb uterine cytosol incubated with (a) 10^{-8} M **10** and (b) 10^{-8} M **10** plus a 500-fold excess of DES.

of the radioactive organometallic-labeled hormone, [^{3}H]-**10**, we have been able to show that this modified hormone binds reversibly to estradiol receptor with an affinity close to that of estradiol itself. Clearly, such organometallic-labeled hormones do satisfy the three criteria expected for a good biochemical marker: (i) good stability during the biochemical procedures and a sufficient solubility in solvents such as ethanol, (ii) strong binding to the specific estrogen-binding proteins present in the target tissue, and (iii) low binding to the low-affinity binding sites found in serum and tissues (Katzenellenbogen et al., 1975). Our current research is directed toward increasing the sensitivity of the FT-IR detection so that we can begin testing the quantitative aspects of this new labeling procedure.

Acknowledgments

We thank Dr. J. A. Katzenellenbogen for helpful discussion and A. Cordaville for technical assistance.

Supplementary Material Available

Three tables giving complete structural data for compound **7** (4 pages). Ordering information is given on any current masthead page.

Registry No. **2**, 77109-92-3; **3**, 98757-31-4; **4**, 57441-02-8; **5**, 93036-14-7; **6**, 98757-33-6; **7**, 93173-93-4; **8**, 98757-34-7; **9**, 21830-21-7; **10**, 93061-16-6; [^{3}H]-**10**, 96648-81-6; **11**, 93061-17-7; bromopropanol, 627-18-9.

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