ELSEVIER

Journal of Organometallic Chemistry 512 (1996) 79–84

Journal of Organometallic Chemistry

Metal-marked steroids of the estrane group from the reaction of steroidal functional groups with arene–iron(Cp) complexes

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Received 31 May 1995; in revised form 24 August 1995

Abstract

Reaction of (η^6-p-chlorotoluene)(η^5-cyclopentadienyl)iron(II) hexafluorophosphate with the phenolic hydroxy group of estrane steroids, under mild conditions, leads to the formation of 3-O-p-tolyl ethers of steroids, with an iron(Cp) marker. Employing this reaction, estrone, estradiol and 17-α-ethynylestradiol have been marked in high yield. The utility of such marked steroids in metalloimmunoassay has been examined. Steroids marked in such a way show no measurable affinity for the estradiol receptor. The 3-O-p-tolylestrone complex is stable at room temperature for 24 h, in water solutions of buffers at pH 2.0 and 7.41, as well as in a THF solution in the presence of pyrrolidine. The marker fragment may be removed at room temperature upon the action of pyrrolidine in a 1:1 water–THF solution or potassium hydroxide in water for 24 h with the efficient recovery of steroid. Treatment of marked estrone with an excess of potassium t-butoxide leads to demetallation and the formation of 3-O-p-tolylestrone. Attempts to mark estrone and testosterone via the carbonyl group or estradiol and cholesterol via the secondary hydroxyl group, under mild conditions, have been unsuccesful.

Keywords: Steroids; Metalloimmunoassay; Cyclopentadienyls; Arene complexes; Iron complexes

1. Introduction

The concept of metalloimmunoassay was introduced by Cais et al. [1] in 1977, and since that time the technique has become an important tool in molecular biology [2,3]. Biologically important molecules, including steroids, are usually marked with a metal moiety using one of two strategies: complexation of the unsaturated fragment of the molecule with the metal, or formation of a bond between the functional group of the compound with a suitably functionalized metal complex. In the area of steroids containing an aromatic ring, the formation and assay of η^6 complexes prepared from estradiol and chromium hexacarbonyl has been extensively studied by Jaouen and coworkers [4–10]. Chromium tricarbonyl complexes of estrane steroids were also synthesized by Gill et al. [11] as well as Künzer and Thiel [12,13]. Synthesis of analoguous Cp–ruthenium and pentamethylCp–ruthenium complexes has been communicated by Moriarty and coworkers [14–17], Vichard et al. [18] and Urbanos et al. [19] respectively, while Sweigart and coworkers [20] studied manganese tricarbonyl complexes.

Among the steroids marked through the bonding to the steroidal functional group, Cais et al. [21] have reported on the formation and assay of the estrane type of steroids bonded to ferrocene through an additional bifunctional chain. Mahaffy and Pauson [22] reported synthesis of 3-O-phenylcholesterol with the phenyl ring complexed by chromium tricarbonyl moiety, while Jaouen and coworkers [23–32] reported on numerous η^2-metal–ethyne complexes of the steroids possesing a triple bond in the structure [23–32]. The last group of complexes (usually containing Co, Mo and Re carbonyl fragments in the marker) exhibits relatively high affinity towards estradiol receptors, equal to that of free steroids. Carbonyl groups of their metal marker may be easily detected even at very low concentrations using FTIR. Taking advantage of these properties, Jaouen and coworkers [33–35] have developed a "carbonylmetal-

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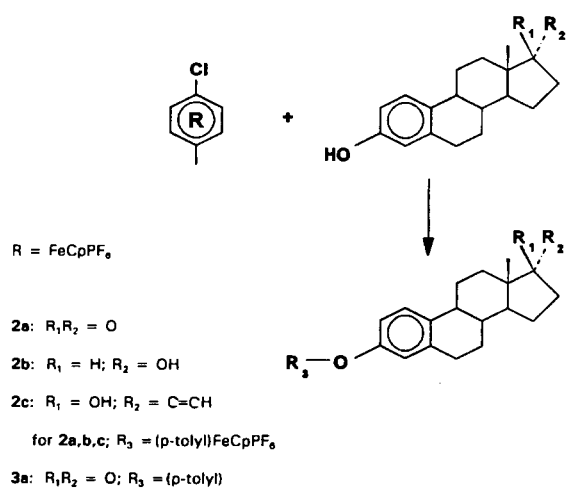
SSDI 0022-328X(95)05915-6

loimmunoassay" (CMIA) procedure to study the interaction of receptors with biomolecules. In this contribution, we present the results of our attempts at marking steroids with an arene–iron(Cp) moiety and evaluation of the binding ability of such molecules to estrogen receptors.

2. Results and discussion

Complexes of 3-O-p-tolyl ethers of estrone (**2a**), estradiol (**2b**) and 17-α-ethynylestradiol (**2c**) have been prepared in good to high yield under mild conditions (Scheme 1). In the best case, a mixture of the steroid (5% excess) and the p-chlorotoluene complex (**1**) was stirred for 4–6 h at ca. 55°C in the presence of potassium carbonate in DMF or a 9:1 THF–DMSO solution. Neutralization of the reaction mixture with 10% hydrochloric acid, followed by evaporation of the volatile solvent under reduced pressure, extraction of the residue with the dichloromethane or 4:1 dichloromethane–nitromethane and then evaporation of the solvents afforded complexes **2** contaminated with an unreacted steroid. Chromatographic purification on a short alumina column led to removal of impurities and unreacted steroid with carbon tetrachloride and chloroform and to recovery of the complex **2** with dichloromethane–chloroform mixtures. After evaporation of the solvents, pure complexes were obtained. The complexes **2** are dense, orange–brown oils, solidifying under reduced pressure or upon extended exposure to air. The structures of the products were confirmed by analytical results. Interestingly, our study indicates that it is advantageous to use a small excess of the steroid, which is the more expensive reagent, in experiments. We have found that it is very easy to remove unreacted steroid. In the other case, the excess of p-chlorotoluene complex may be removed from the steroidal product by a lengthy and laborious procedure with the loss of ca. 15% of the product.



Scheme 1.

When potassium t-butoxide was used as a catalyst in the synthesis of the complex **2a**, the yield of the product was significantly lower than for potassium carbonate. Additionally, prolonged heating (12 h) resulted in complete demetallation and 3-O-p-tolylestrone (**3a**) was obtained as the only product of the reaction, in a 72% yield. This compound has not been reported previously and its complete analytical data are given in the Experimental section. A demetallation reaction also occurred when the complex **2a** was gently refluxed overnight in THF, in the presence of potassium t-butoxide. The yield of the steroid **3a** in this case was 70%. Similar base-catalyzed demetallation reactions were reported by Roberts and coworkers [36].

We found that under the action of a strong base, at elevated temperature, the metal-marked steroid may be efficiently demetallated, however, it is obvious that such conditions will not be found in vivo. At the same time, the question arises as to whether steroids marked as described would be stable under conditions found in living systems, since such a problem has been encountered by other researchers (see Ref. [29] and references cited therein). To answer this question, we studied the stability of the complex **2a** under different conditions during a 24 h period. We found that the complex is stable at room temperature in 1:1 solutions of THF–water buffer at pH 2.0 and 7.41; over 88% of the starting complex was recovered in each case. Similarly, the complex remained unchanged and was recovered in 86% yield from a reaction in the THF solution with a five-fold excess of pyrrolidine. When pyrrolidine was used in a 1:1 THF–water system, ca. 60% of the marked steroid (2a) was converted to the p-N-pyrrolidinyltoluene complex and free steroid. A full conversion of the complex **2a** to η^5-cyclohexadienone iron(Cp) and free estrone (77% recovery) has been observed in a 1:1 THF–20% KOH/water. These results can be rationalized on the basis of our earlier studies on nucleophilic aromatic substitution for arene–iron(Cp) complexes [37].

In a series of contributions, McGlinchey and coworkers [16,38,39] analysed both the proton and carbon-13 NMR spectra of η^6 metal-complexed steroids. They found that the proximity of a metal strongly influences chemical shifts of atoms. Signals of aromatic ring protons are shifted significantly upfield; generally, for the other signals, a downfield shift is observed for the protons located on the side of the metal moiety and an upfield shift for protons located on the other side of the plane. Signals of more remote protons are usually not affected. All those changes were explained in terms of diamagnetic anisotropy of the metal moiety and changes in the aromatic ring current resulting from complexation [39]. In the carbon-13 spectra of such complexes, a

significant upfield shift is observed for the carbon atoms of a complexed aromatic ring, while changes of smaller and decreasing magnitude (with increasing distance from a complexed aromatic ring) are observed for the other carbon atoms of the steroidal skeleton.

In the case of complexes **2**, a less pronounced effect of the distant metal moiety on the chemical shifts might have been expected. This has been observed both in the proton and in the carbon-13 spectra. Usually downfield shifts are observed and, with the exception of the aromatic proton (shifts of 0.4–0.6 ppm) and the aromatic carbon (shifts of 2–10 ppm) signals, they are not significant. Additionally, at a frequency of 300 MHz, aliphatic proton signals overlap considerably, precluding precise analysis of the high field part of the spectrum (as has been presented earlier [16,38,39]). Despite this restriction, NMR spectra may serve as an efficient analytical tool for confirmation of the metal marker–steroid bonding and to indicate the purity of such complexes. A full assignment of carbon-13 spectra for estrane compounds and complexes is presented in the Experimental section.

Reactions described here are efficient if a steroid possesses a phenolic hydroxy group. Under mild conditions, our attempts to complete similar reactions utilizing a secondary aliphatic hydroxy group of cholesterol or estradiol were unsuccessful. We did not attempt this reaction under the harsher conditions, reported earlier by Mahaffy and Pauson [22], for the chromium tricarbonyl complex. Similarly, our attempts to mark the steroids possesing a keto function were unsuccessful. Thus reactions of testosterone and estrone with either p-tolylhydrazine or p-toluidine(Cp)iron complex led only to the recovery of unreacted steroids. Also, under similarly mild conditions, hydrazones of both ketosteroids did not react with the p-chlorotoluene complex that might have led to the attachment of a metal marker to the steroid through a hydrazo fragment. The only product isolated in these reactions was the 3-O-p-tolylestrone complex **2a**. Apparently, hydrolysis of the hydrazone and reaction of estrone via its 3-hydroxy group with the p-chlorotoluene complex occurred.

3. Study of the relative binding affinity

The relative binding affinity (RBA) values of new complexes **2** for a uterine estradiol receptor have been measured by a competitive method [40]. These complexes have no measurable affinity for the estradiol receptor (they are unable to displace the tritiated estradiol bound to its receptor). Such a result is not very surprising for the estrone derivative, since the RBA value for estrone itself is only 24%. For estradiol (RBA = 100%) and ethynylestradiol (RBA = 70%) one could expect a value close to that found by Chernayaev et al. [41] for 3-deoxyestradiol (RBA = 1.7%). Our results show that replacement of the 3-hydroxyl group by a bulky substituent has a more negative effect for the binding than the simple suppression of the 3-OH group, which is in line with earlier observations on the importance of the 3-hydroxy group of steroids in interaction with receptors [42].

4. Experimental

The ^{1}H NMR (300.133 MHz) and ^{13}C NMR (75.469 MHz) spectra were recorded on a Bruker AM 300 spectrometer in acetone-d_6 solutions; all chemical shifts are given in δ, ppm scale (internal TMS for proton; calculated from the solvent signal in carbon-13 spectra) where $\delta_{TMS} = 0$. The preparation of p-chlorotoluene and p-toluidine iron(Cp) complexes was described previously [43,44]. A complex of p-toluenehydrazine was obtained as an orange–yellow solid in 55% yield, following a procedure similar to that described for the o-chlorophenylhydrazine complex [45]. Anal. Found: C, 37.25; H, 3.8. $C_{12}H_{15}F_6FeN_2P$. Calc.: C, 37.1; H, 3.9%. ^{1}H NMR: δ 6.26 (2H, d, $J = 6.9$ Hz, ArH) and 6.18 (2H, d, $J = 6.9$ Hz, ArH); 4.96 (5H, s, Cp) and 2.45 (3H, s, Me); ^{13}C NMR: δ 121.25 (quaternary ArC), 97.94 (quaternary ArC), 87.04 (2C, ArC), 69.86 (2C, ArC), 77.04 (5C, Cp), 19.78 (Me).

Steroids were purchased from the Aldrich Chemical Co. while buffers at pH 2.00 and 7.41 were obtained from the Micro Essential Laboratory (Brooklyn, NY). Hydrazones of estrone and testosterone were obtained following the procedure published earlier [46] and their identity was confirmed by analytical data. Estrone hydrazone had been obtained as a white solid, m.p. 243–244°C (Ref. [45] 244–246°C). Anal. Found: C, 76.3; H, 8.5. $C_{18}H_{24}N_2O$. Calc.: C, 76.0; H, 8.5%); MS, m/z 284 (M^+, 100%); ^{1}H NMR: δ 7.23 (2H, s, NH_2), 7.14 (3H, m, NH and ArH, overlapping), 6.62 (2H, d, $J = 6.0$ Hz, ArH), 6.57 (1H, d, $J = 2.3$ Hz, ArH), 3.5 (1H, bs, OH), 2.83–2.80 (2H, m), 2.38–1.83 (8H, m's), 1.60–1.32 (5H, m's), 0.95 (3H, s, CH_3); ^{13}C NMR: δ 161.03 (C 17), 154.49 (C 3), 137.67 (C 5), 131.12 (C 10), 126.22 (C 1), 115.32 (C 4), 112.95 (C 2), 52.25 (C 14), 44.79 (C 13), 43.94 (C 9), 38.18 (C 8), 33.94 (C 16), 29.48 (C 12), 27.37 (C 6), 26.20 (C 7), 24.64 (C 11), 22.88 (C 15), 16.82 (C 18). Testosterone hydrazone had been obtained as a white solid, m.p. 117–118°C (Ref. [46] 118–120°C). Anal. Found: C, 75.5; H, 10.2. $C_{19}H_{30}N_2O$. Calc.: C, 75.4; H, 10.0%. MS, m/z 302 (M^+, 100%). ^{1}H NMR: δ 7.44 (2H, s, NH_2), 6.33 (1H, s, NH), 5.92 (1H, s, H 4), 3.64 (2H, t, $J = 8.4$ Hz), 2.91 (1H, d, $J = 16.8$ Hz, d, $J = 3.5$ Hz), 2.6 (1H, bs, OH), 2.4–2.2 (2H, m), 2.14–1.98 (2H, m), 1.93–1.78 (4H, m), 1.62–1.24 (4H, m), 1.10 (3 H, s, 18-CH_3), 1.09–0.84 (5H, m), 0.79 (3H, s, 19-CH_3); ^{13}C NMR: δ

160.08 (C 5), 157.81 (C 3), 121.14 (C 4), 81.02 (C 17), 53.77 (C 9), 50.44 (C 14), 42.56 (C 13), 37.89 (C 10), 36.39 (C 12), 35.60 (C 8), 35.12 (C 1), 32.30 (C 6), 31.61 (C 7), 29.92 (C 16), 23.14 (C 15), 21.61 (C 2), 20.71 (C 11), 17.48 (C 18), 10.86 (C 19).

4.1. General procedure for the synthesis of complexes 2

A mixture of the steroid (3.15 mmol), potassium carbonate (0.75 g, 5.4 mmol) and (p-chlorotoluene) (cyclopentadienyl) iron hexafluorophosphate (1.18 g, 3 mmol) in 10 ml of N,N-dimethylformamide was stirred at ca. 55°C for 4–6 h. The mixture was cooled to room temperature and treated with 10 ml of 10% hydrochloric acid followed by 0.5 g (ca. 3 mmol) of ammonium hexafluorophosphate. The volume of the solution was then halved by means of evaporation under reduced pressure, and the solution was extracted with dichloromethane or a 4:1 dichloromethane–nitromethane mixture. After drying (anhydrous sodium sulfate), the solvents were evaporated and the crude product was purified on a short column (ca. 5 cm, alumina delivered by Alcoa Chimica, deactivated by 48 h exposure to air). Impurities and unreacted steroid were removed upon washing with carbon tetrachloride and chloroform. The pure product was recovered from the column by elution with a mixture of dichloromethane and chloroform of varying polarity. Evaporation of the solvents gave a dense, orange–brown oil which, in each case, solidified upon storage in a vacuum dessicator. Reactions may be carried out using a 9:1 mixture of tetrahydrofuran and dimethylsulfoxide instead of dimethylformamide. Similarly, potassium t-butoxide may be used as a catalyst, giving yields lower by around 5–10% than those observed for potassium carbonate. Reaction with those replacement solvents and catalyst, whilst carried out overnight, led to the isolation of 3-O-p-tolylestrone (**3a**) in 72% yield as the only product (isolated as described above). Demetallation of complex **2a**, upon gentle overnight refluxing under similar conditions, led to the isolation of **3a** in 70% yield.

4.1.1. 3-O-[(η^6-p-tolyl)(η^5-cyclopentadienyl) iron] estrone hexafluorophosphate (2a)

This complex was obtained as a microcrystalline orange–brown solid (78%). Anal. Found: C, 57.2; H, 5.2. $C_{30}H_{33}F_6FeO_2P$. Calc.: C, 57.5; H, 5.3%. ^{1}H NMR: δ 7.50 (1H, d, $J = 8.2$ Hz, ArH), 7.11 (1H, d, $J = 8.2$ Hz, ArH), 7.07 (1H, s, ArH), 6.37 (2H, d, $J = 6.4$ Hz, complexed ArH), 6.29 (2H, d, $J = 6.4$ Hz, complexed ArH), 5.21 (5H, s, Cp), 2.99–2.96 (2H, m), 2.56–2.38 (7H, s and m), 2.12–2.04 (2H, m), 1.89 (1H, m), 1.73–1.41 (6H, m), 0.93 (3H, s, 18-CH_3); ^{13}C NMR: δ 219.54 (CO), 151.70 (C 3), 140.32 (C 5), 138.82 (C 1′), 133.39 (C 10), 128.21 (C 1), 121.29 (C 4), 118.43 (C 2), 101.02 (C 4′), 87.47 (2C, 3′, 5′), 78.16 (5C, Cp), 76.49 (2C, 2′, 6′), 50.74 (C 14), 48.12 (C 13), 44.70 (C 9), 38.52 (C 6), 35.91 (C 16), 32.29 (C 12), 30.05 (C 8), 26.88 (C 7), 26.27 (C 11), 21.89 (C 15), 19.63 (C 7′), 13.96 (C 18).

4.1.2. 3-O-[(η^6-p-tolyl)(η^5-cyclopentadienyl) iron] β-estradiol hexafluorophosphate (2b)

The complex was obtained as a microcrystalline orange–brown solid (77%). Anal. Found: C, 56.9; H, 5.3. $C_{30}H_{35}F_6FeO_2P$. Calc.: C, 57.3; H, 5.6%; ^{1}H NMR: δ 7.48 (1H, d, $J = 8.2$ Hz, ArH), 7.06 (1H, d, $J = 8.2$ Hz, ArH), 7.04 (1H, s, ArH), 6.36 (2H, d, $J = 6.5$ Hz, complexed ArH), 6.28 (2H, d, $J = 6.5$ Hz, complexed ArH), 5.20 (5H, s, Cp), 3.70 (1H, m), 3.60 (1H, bs, OH), 2.94–2.90 (2H, m), 2.49 (3H, s, CH_3–Ar), 2.42–2.37 (1H, m), 2.32–2.25 (1H, m), 2.06–1.92 (3H, m), 1.58–1.21 (6H, m), 0.81 (3H, s, 18-CH_3); ^{13}C NMR: δ 151.39 (C 3), 140.19 (C 5), 139.15 (C 1′), 133.20 (C 10), 128.11 (C 1), 121.07 (C 4), 118.19 (C 2), 100.82 (C 4′), 87.28 (2C, 3′, 5′), 81.32 (C 17), 78.00 (5C, Cp), 76.28 (2C, 2′, 6′), 50.44 (C 14), 44.58 (C 13), 43.57 (C 9), 39.02 (C 8), 37.22 (C 12), 30.66 (C 16), 29.72 (C 6), 27.36 (C 7), 26.65 (C 11), 23.47 (C 15), 19.51 (C 7′), 11.50 (C 18).

4.1.3. 3-O-[(η^6-p-tolyl)(η^5-cyclopentadienyl) iron] 17-α-ethynylestradiol hexafluorophosphate (2c)

The complex was obtained as a microcrystalline orange–brown solid (77%). Anal. Found: C, 59.0; H, 5.3. $C_{32}H_{35}F_6FeO_2P$. Calc.: C, 58.9; H, 5.4%. ^{1}H NMR: δ 7.49 (1H, d, $J = 8.4$ Hz, ArH), 7.08 (1H, d, $J = 8.4$ Hz, ArH), 7.04 (1H, s, ArH), 6.36 (2H, d, $J = 6.6$ Hz, complexed ArH), 6.28 (2H, d, $J = 6.6$ Hz, complexed ArH), 5.21 (5H, s, Cp), 3.44 (1H, bs, OH), 3.01 (1H, s, C≡;CH), 2.95–2.91 (2H, m), 2.50 (3H, s, CH_3–Ar), 2.48–2.42 (1H, m), 2.33–2.20 (2H, m), 2.06–1.92 (2H, m), 1.84–1.74 (3H, m), 1.57–1.37 (5H, m), 0.93 (3H, s, 18-CH_3); ^{13}C NMR: δ 151.68 (C 3), 140.39 (C 5), 139.22 (C 1′), 133.48 (C 10), 128.32 (C 1), 121.25 (C 4), 118.39 (C 2), 101.06 (C 4′), 88.94 (C 17), 87.53 (2C, 3′, 5′), 79.50 (*C*≡CH), 78.19 (5C, Cp), 76.55 (2C, 2′, 6′), 74.58 (C≡*C*H), 50.11 C 14), 47.60 (C 13), 44.55 (C 9), 39.79 (C 8), 39.73 (C 12), 33.46 (C 16), 29.94 (C 6), 27.58 (C 7), 26.90 (C 11), 23.28 (C 15), 19.62 (C 7′), 13.09 (C 18).

4.1.4. 3-O-p-tolylestrone (3a)

The compound was obtained as a white solid, m.p. 128–130°C (72%). Anal. Found: C, 83.1; H, 7.9. $C_{25}H_{28}O_2$. Calc.: C, 83.3; H, 7.8%. ^{1}H NMR: δ 7.27 (1H, d, $J = 8.4$ Hz, ArH), 7.16 (2H, d, $J = 8.2$ Hz, ArH), 6.87 (2H, d, $J = 8.2$ Hz, ArH), 6.73 (1H, d, $J = 8.4$ Hz, d, $J = 2.4$ Hz, ArH), 6.68 (1H, s, ArH), 2.87–2.82 (2H, m), 2.48–2.44 (2H, m), 2.30 (3H, s, CH_3–Ar), 2.15 (1H, m), 2.10–1.98 (3H, m), 1.85 (1H, m), 1.69–1.38 (6H, m's) and 0.89 (3H, s, Me); ^{13}C

NMR: δ 219.60 (CO), 155.94 (C 1'), 155.62 (C 3), 138.63 (C 10), 134.99 (C 4'), 132.81 (C 5), 130.62 (2 C, 3', 5'), 127.07 (C 1), 119.13 (2C, 2', 6'), 118.86 (C 2), 116.28 (C 4), 50.71 (C 14), 48.10 (C 13), 44.52 (C 9), 38.69 (C 6), 35.86 (C 16), 32.14 (C 12), 29.83 (C 8), 26.81 (C 7), 26.27 (C 11), 21.84 (C 15), 20.58 (C 7'), 13.94 (C 18).

Reactions of the estradiol complex **2b** or cholesterol with the p-chlorotoluene(Cp)iron complex were attempted under conditions described above. In any case, we did not find a trace of a complex with the marker attached through the secondary hydroxyl group. Reactions of testosterone and estrone with p-toluidine or p-toluenehydrazine(Cp)iron complexes, as well as the p-chlorotoluene(Cp)iron complex with hydrazones of estrone and testosterone were attempted under the same conditions and the complex **2a** has been isolated in ca. 60% yield as the only metal-containing product of these reactions.

4.2. Study of the stability of complex 2a

The stability of the complex **2a** has been studied in the following solutions: (i) water solution of buffers at pH 2.0 and 7.41; (ii) water solution of pyrrolidine (5 mmol); (iii) a 1:1 solution of 20% potassium hydroxide in water–tetrahydrofuran; and (iv) a 1:1 solution of water–tetrahydrofuran with pyrrolidine (5mmol). Typically, 1 mmol of the complex was introduced to 25 ml of reagent solution and, protected against the light, was stirred at room temperature for 24 h. The mixture was then extracted with chloroform (for the potassium hydroxide reaction) or with dichloromethane and analyzed using ^{1}H NMR spectroscopy. The steroid or complex was isolated using methods described above. Under conditions (i) and (ii) over 80% of the starting complex was recovered. Under condition (iii) ca. 60% of the complex was converted into a p-N-pyrrolidinyltoluene (Cp)iron complex identical with the original sample [47] (on the basis of NMR spectra and recovery of unchanged starting complex). Under condition (iv) the complex **2a** was converted into estrone (isolated in 77% yield) and the (η^5-4-methylcyclohexadienone)(Cp)iron complex identical with the original sample [48].

4.3. Study of the RBA of complexes 2

The RBA values for the estradiol receptor (ER) were measured in a routine screening assay, as described previously [40], using lamb uterine cytosol as a source of ER and [^{3}H]-estradiol as a marker. Cytosol was incubated for 3 h at 0°C. Protamine sulfate precipitation assay was used for the separation of the [^{3}H]-bound and the free steroid [49]. The RBA value of estradiol for ER was taken to be equal to 100.

Acknowledgements

Financial support given for this study to RGS by the Natural Sciences and Engineering Research Council of Canada, and by the Centre National de la Recherche Scientifique to AV-J and GJ is gratefully acknowledged. We also wish to thank Mme Annie Cordaville for technical assistance.

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