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The $Co_3(CO)_9C$ moiety acts as an electroreducible marker for estradiol detection enhancement in the HPLC-ED technique

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Dedicated to Professor F. Calderazzo on the occasion of his 70th birthday.

Abstract

The tricobalt enneacarbonyl–ethynylestradiol derivative $Co_3(CO)_9C–C\equiv C–E$ (E = estradiol) proved to be elusive, due to the strong electron-withdrawing properties of both the 17β OH and the $Co_3(CO)_9C$ groups. However, the advantage of introducing the Co_3C core, which undergoes a reversible 1 e reduction, can be achieved by the interposition of a spacer arm, namely $–C(O)NHC_6H_4–$, generated in situ during the synthesis in the appropriate experimental conditions. The corresponding bioorganometallic product $Co_3(CO)_9C–C(O)NHC_6H_4–C\equiv C–E$ retains an acceptable relative binding affinity for estradiol receptor affording a new electrochemical marker suitable for biochemical analyses. © 2000 Elsevier Science S.A. All rights reserved.

Keywords: Bioinorganic chemistry; Cobalt carbonyls; Steroids; Immunoassay; Electrochemistry

1. Introduction

The assay of circulating hormones and of their specific receptors, which can be involved in certain pathologies (e.g. hormone-dependent tumours) [1], is commonly performed by an immunoassay technique, by virtue of its specificity and sensitivity. Radioisotopic labelling represents the base of the widely used radioimmunoassay (RIA) methods [2]. However, the cost and problems related to the use of radioactive materials encouraged the investigation of alternative, non-isotopic (cold) procedures [3–5]. The labelling of biological molecules by means of inorganic fragments yielded new immunoanalytical protocols. The acronyms metalloimmunoassay (MIA) and carbonylmetalloimmunoassay (CMIA) have been coined by Cais [6] and by Jaouen [7], respectively. Such methods are potentially interesting from the practical point of view, provided that an acceptable degree of molecular recognition between the marker and the specific target (receptor, antibody or enzyme) is retained.

Biological derivatives of transition metal complexes offer detection enhancement in several commonly employed analytical techniques, such as atomic absorption spectroscopy (AAS) and electrochemistry. HPLC is recognised as an ideal tool for the separation and determination of bio-molecules in biological fluids [8,9]. By means of a reverse-phase HPLC separation and a conventional fixed-wavelength UV detector ($\lambda = 254$ nm) we observed that the detection sensitivity of several metal-marked estradiols is increased several times, due to their intense $d(M) \rightarrow \pi^*(CO)$ metal-to-ligand charge transfer (MLCT) transitions [10]. The electrochemical detector (ED) coupled with HPLC shows an even higher sensitivity, provided that the substance under analysis is electroactive, or can be converted into a reducible/oxidisable product by pre- or post-column reaction. The co-ordination of an organometallic fragment generally adds an independent redox process to the overall electrochemical behaviour of the bioorganometallic molecules. We successfully employed

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the ferrocenyl fragment, which exhibits a fully reversible 1 e oxidation process at a low anodic potential, as an electroactive marker for the analytical detection enhancement of steroids [11]. The $Co_3(CO)_9C$– moiety undergoes a reversible 1 e reduction process at a low cathodic potential, representing a complementary approach for HPLC-ED.

Our previous attempt to directly link $Co_3(CO)_9C$– to ethynylestradiol (HC≡C–E) did not give the desired derivative $Co_3(CO)_9C$–C≡C–E (**1**) [12]. The goal of the present work is to provide an alternative synthetic route to this electro-reducible bioorganometallic marker.

2. Experimental

2.1. General remarks

All reactions were carried out under nitrogen. A three-necked flask was fitted with a pressure-equalising dropping funnel, and gas inlet/outlet. Reagents were purchased from Sigma-Aldrich; solvents were dried and purified before use. $Co_3(CO)_9CBr$ was prepared as previously described [13]. NMR and IR spectra were recorded on a JEOL-EX 400 and on a Bruker FT-IR Equinox 55 spectrometer, respectively. All NMR spectra were recorded in $CDCl_3$ solutions, using the deuterated solvent as an internal lock, the chemical shifts are reported downfield positive with respect to TMS (δ 0.00). DCI-MS spectra were recorded on a Finningan MAT 95Q instrument with both magnetic and electrostatic analysers [14]. In DCI technique, the reagent gas (isobutane at a pressure of 0.5 mbar) is initially ionised by electron impact (EI), then yields the stable *tert*-butylcarbenium ion. Ionisation of the analite (M) takes place by proton transfer from the carbocation generating the quasi-molecular ion $[M+H]^+$. Besides proton transfer, capture of thermal electrons generate $[M]^-$ ions. Both ions posses lower energy with respect to those generated by conventional EI, and therefore undergo lower fragmentation. Positive and negative ions were collected. Electrochemical measurements were performed using an EG&G PAR 273 electrochemical analyser 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 anhydrous deoxygenated solvents was employed. The working electrode was glassy carbon, and potentials were referred to the standard calomel electrode (SCE). The supporting electrolyte was $LiClO_4$ 0.1 M. Ferrocene (Fc) was added as an internal standard; the Fc(0/1+) formal potential was evaluated in the same experimental conditions to be +0.46 V versus SCE in acetone.

2.2. $Co_3(CO)_9C$–C≡C–E (**1**), $Co_3(CO)_9C$–C(O)NHCH$(CH_3)_2$ (**2**), $[Co_3(CO)_9C$–C≡C(OH)$C_6H_{10}][Co_2(CO)_6]$ (**3**), and $Co_3(CO)_9C$–C≡C(OH)C_6H_{10} (**4**)

A solution of $Co_3(CO)_9CBr$ (2.1 mmol) in THF (30 ml) was added dropwise to a THF solution containing the appropriate terminal acetylene ligand, HC≡C–E or HC≡C(OH)C_6H_{10} (2 mmol), CuI in a catalytic amount (4–5%) and isopropylamine (2.4 mmol). The reactions were carried out at 26 ± 1°C in a nitrogen atmosphere for several hours. The mixtures were filtered through cellulose, concentrated under reduced pressure, and then chromatographed on silica gel column. A *n*-hexane/CH_2Cl_2 gradient (0–50% CH_2Cl_2) resolved several bands. From both reactions the violet $Co_3(CO)_9C$–C(O)NHCH$(CH_3)_2$ (**2**) was isolated (yield ca. 40%); Anal. Found: C, 32.0; H, 1.48; Co, 33.0. $C_{14}H_8O_{10}NCo_3$ Calc.: H, 1.53; C, 31.91; N, 2.66; O, 30.36; Co, 33.55%; ν_{CO} (hexane) 2109w, 2064vs, 2044s, 2033sh cm^{-1}; ν_{NH} (CCl_4) 3441 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 5.7 (1H, s, br, NH), 4.22 (1H, m, CH), 1.27 (6 H, d, CH_3, $^3J_{HH}$ 7.3 Hz); m/z 528 $[M+H]^+$.

The reaction with HC≡C(OH)C_6H_{10} gave a further green band identified as $[Co_3(CO)_9C$–C≡C(OH)-$C_6H_{10}][Co_2(CO)_6]$ (**3**) (yield ca. 30%); Anal. Found: C, 34.1; H, 1.32; Co, 34.5. $C_{24}H_{11}O_{16}Co_5$ Calc.: H 1.30 C 33.91 O 30.12 Co 34.67%; ν_{CO} (hexane) 2106w, 2080s, 2064vs, 2057vs, 2044sh, 2030sh, 2026s, 2022sh cm^{-1}; ν_{OH} (CCl_4) 3607 cm^{-1}; ^{1}H-NMR: δ 6.07 (1H, s, OH), 1.8–1.4 (10H, m). ^{13}C-NMR: δ 199.38 (CO), 118.23 (C≡C), 110.8 (C≡C), 74.8 (C–OH), 39.7, 24.8, 22.0.

Violet compounds (**1**) or (**4**) were isolated in trace amounts from both reactions and showed the typical pattern of the tricobalt carbonyl unit, ν_{CO} (CH_2Cl_2) 2109 (w), 2064 (vs), 2043 (s).

2.3. Synthesis of $Co_3(CO)_9C$–C(O)NH(C_6H_5) (**5**)

A solution of $Co_3(CO)_9CBr$ (2 mmol) in THF was added dropwise to a THF solution containing aniline (1.8 mmol) and a catalytic amount of CuI (30 mg). It is convenient to use a slight excess of $Co_3(CO)_9CBr$, in order to completely consume the ligand. The reaction was stirred for 3–4 h at 29°C under nitrogen. The mixture was worked-up as above. The violet band, corresponding to unreacted $Co_3(CO)_9CBr$ is eluted first. The brown band corresponds to complex (**5**) (yield ca. 45%): Anal. Found: C, 36.41; H, 1.09; Co, 31.5. $C_{17}H_6O_{10}NCo_3$ Calc.: H, 1.08; C, 36.39; N, 2.50; O, 28.52; Co, 31.51%; ν_{CO} (CH_2Cl_2) 2108 (m), 2064 (vs), 2045 (s, sh) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 7.69 (1H, s, br, NH), 7.61 (2H, d, *o*-H), 7.38 (2H, t, *m*-H), 7.17 (1H, t, *p*-H); ^{13}C-NMR ($CDCl_3$): δ 267.7 C(methylidyne), 198.5 (CO), 176.0 (C(O)), 137.6 (C25) *ipso*, 129.1 (C24), 124.6 (C22), 119.8 (C23); m/z 562 $[M+H]^+$.

2.4. Synthesis of (1,4-C_6H_4)[$Co_3(CO)_9C$–C(O)NH]$_2$ (6)

This dimeric complex (yield ca. 30%) was prepared according to the method described for (**5**), with a slight excess of $Co_3(CO)_9CBr$, using 1,4-phenylenediamine as ligand. Anal. Found: C, 32.3; H, 0.57; Co, 33.8. $C_{28}H_6O_{20}N_2Co_6$ Calc.: H, 0.58; C, 32.21; N, 2.68; O, 30.65; Co, 33.87%; ν_{CO} (CH_2Cl_2) 2109 (w), 2065 (vs), 2046 (s) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 7.70 (2H, s, br, NH), 7.63 (4H, s, –C_6H_4–): ^{13}C-NMR ($CDCl_3$): δ 198.44 (CO), 120.48 (–C_6H_4–). We were unable to observe the resonances of methylidyne, *ipso* and carboxamide carbon atoms, due to the low solubility of (**6**) in $CDCl_3$; m/z 1016 $[M–CO]^-$.

2.5. Synthesis of $NH_2(C_6H_4)$–C≡C–E (7)

An equimolar mixture of 17α-ethynylestradiol (2 mmol) and 4-iodoaniline (2 mmol), were dissolved into 50 ml of diethylamine containing catalytic amounts of $(Ph_3P)_2PdCl_2$ (7 mg) and CuI (5 mg). The mixture was stirred for 3 h at room temperature, under nitrogen atmosphere. The solution was first hydrolysed with distilled water, and then extracted with benzene and dichloromethane. The organic solution was dried under vacuum. The crude compound (**7**) was obtained (95% yield) as a pale-yellow fine powder, and used as obtained. Anal. Found: C, 80.6; H, 7.55. $C_{26}H_{29}O_2N$ Calc.: H, 7.54; C, 80.59; N, 3.61; O, 8.26%; ^{1}H-NMR (acetone-d_6): δ 8.05 (1H, s, C3–OH), 7.25–6.60 (4H, m, -C_6H_4–, AA′, BB′ system), 7.16 (1H, d, C1–H), 6.64 (1H, dd, C2–H), 6.57 (1H, d, C4–H), 5.31 (1H, s, br, C17–OH), 3.84 (2H, s, br, NH_2), 2.80/1.18 (all remaining, partially overlapped, resonances), 0.93 (3H, s, C13–Me). ^{13}C-NMR ($CDCl_3$): δ 153.4 (C3), 138.1 (C5), 132.9 (C24), 132.2 (C10), 126.4 (C1), 115.2 (C4), 114.6 (C23), 112.6 (C2), 87.4 (C17), 79.8 (Cα), 74.0 (Cβ), 47.0 (C13), 12.6 (C18); $m/z = 388$ $[M + H]^+$.

2.6. Synthesis of $Co_3(CO)_9C$–C(O)NH(C_6H_4)–C≡C–E (8)

The reaction was carried out as for (**5**). The brown band was recrystallised from a hexane/CH_2Cl_2 mixture and identified as (**8**) (yield ca. 35%); Anal. Found: C, 52.0; H, 3.27; Co, 20.59. $C_{37}H_{28}O_{12}NCo_3$ Calc.: H, 3.30; C, 51.95; N, 1.64; O, 22.44; Co, 20.67%; ν_{CO} (CH_2Cl_2) 2110 (w), 2065 (vs), 2046 (s) cm^{-1}; ^{1}H-NMR (acetone-d_6): δ 8.10 (1H, s, C3–OH), 7.75 (1H, s, br, NH), 7.60–7.42 (4H, m, –C_6H_4–, AA′, BB′ system), 7.17 (1H, d, C1–H), 6.66 (1H, dd, C2–H), 6.58 (1H, d, C4–H), 5.58 (1H, s, br, C17–OH), 2.81 ÷ 1.27 (all remaining, partially overlapped, resonances), 0.94 (3H, s, C13–Me). ^{13}C-NMR ($CDCl_3$): δ 198.3 (CO), 176.2 (C26), 153.5 (C3), 138.0 (C5), 137.5 (C25), 132.5 (C24), 132.2 (C10), 126.4 (C1), 119.3 (C23), 115.2 (C4), 112.6 (C2), 85.5 (C17), 80.3 (Cα), 73.8 (Cβ), 47.5 (C13), 12.8 (C18); m/z 856 $[M + H]^+$.

2.7. Study of the RBA of complex 8

The Relative Binding Affinity (RBA) for the estradiol receptor (ER) were measured in a routine screening assay, as described previously [15], 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. A protamine sulphate precipitation assay was used for the separation of the free and bound fractions of the tracer.

2.8. HPLC

A Kontron HPLC system (pump model 420, UV detector model 742) and an ESA Coulochem II electrochemical detector (ED) were interfaced to a PC employing the Kontron Integration Pack software. The ED consists of high-efficiency (70%) amperometric cell (ESA model 5011) equipped with a palladium pseudo-reference electrode and two porous graphite electrodes (guard and analytical). The column was Merck Lichrosphere 100 RP-18 5 μm, 250 × 4 mm diameter with removable guard column. The eluent (acetone, flow rate of 0.7 ml min^{-1}) was distilled over molecular sieves before use, and $LiClO_4$ 0.010 M was used as supporting electrolyte.

3. Results

3.1. Attempts to synthesise $Co_3(CO)_9C$–C≡C–E (1)

Recently, we showed that the widely employed synthesis of tricobalt–enneacarbonyl derivatives, which involves the hydrogenation of $Co_2(CO)_6$(H–C≡C–R) in the presence of $Co_2(CO)_8$, followed an unexpected pathway when the terminal alkyne is ethynylestradiol [12]. In fact, instead of obtaining the expected $Co_3(CO)_9C$–CH_2–E derivative, elimination of the 17β-OH group in the original molecule occurs affording $Co_3(CO)_9$(dehydroxyethynylestradiol). The loss of 17β-OH group which is essential for the recognition of the hormone by its receptor causes a dramatic decrease of its affinity towards ER (RBA ≈ 0.4%). However $Co_3(CO)_9$(dehydroxyethynylestradiol) is still recognised by estradiol specific polyclonal antibodies and could be used as a tracer for the immunoassay of this hormone [12].

We attempted the synthesis of $Co_3(CO)_9C$–C≡C–E (**1**) via a different pathway, namely the copper-catalysed Cadiot–Chodkiewicz reaction, a well-known method for controlled coupling between terminal alkynes and $Co_3(CO)_9CBr$ [16]. Such a reaction affords a plethora of products in trace yields, although only the

carboxamido cluster $Co_3(CO)_9C–C(O)NHCH(CH_3)_2$ (**2**) has been isolated in reasonable yields and fully characterised. A similar compound, namely $Co_3(CO)_9C–C(O)NHCH_2CH_3$, has been reported by Robinson et al. [16]. We were unable to fully characterise the violet compound $Co_3(CO)_9C–C\equiv C–E$, due to its extreme instability. Internal acetylenes having both substituents with electron-withdrawing properties are kinetically unstable [17]. In order to better understand the reaction pathway, 1-hydroxy-1-ethynylcyclohexane was employed as model for ethynylestradiol, since this alkyne affords compounds that are more soluble and can be used in larger amount. The above-said Cadiot–Chodkiewicz reaction between $Co_3(CO)_9CBr$ and this alkyne model gave a similar pattern of products, but the new cluster $[Co_3(CO)_9C–C\equiv C(OH)C_6H_{10}][Co_2(CO)_6]$ (**3**) resulted as the main product (Scheme 1).

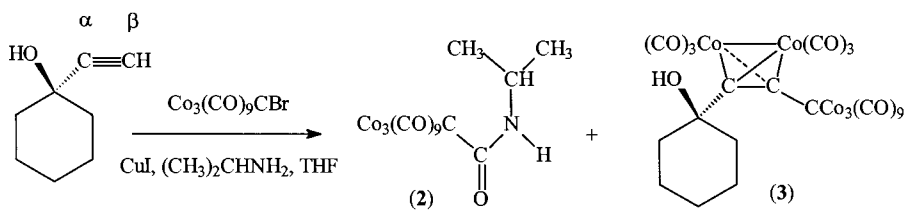
The infrared spectrum of (**3**) in the carbonyl region is a superposition of the idealised band spectrum of the tricobalt enneacarbonyl unit and the dicobalthexacarbonyl fragment. In the ^{1}H-NMR spectrum the singlet due to the –OH (at 6.07 δ) disappears on addition of D_2O, while the signals in the region 1.8/1.4 δ strongly overlap and are assigned to the remaining ten hydrogen atoms. The $^{13}C\{^1H\}$-NMR spectrum of (**3**) showed an intense resonance at 199.38 δ, typical of the carbonyls of the $Co_3(CO)_9C$ fragment undergoing rapid scrambling [18]. The two resonances of C(α) and C(β) in the alkynic region were shifted from 71.9 and 68.2 in free $HC\equiv C(OH)C_6H_{10}$ to 118.2 and 110.8 δ in (**3**), respectively. This downfield shift is stronger than that expected for a simple co-ordination of the $Co_2(CO)_6$ moiety; for instance, the corresponding derivative $[HC\equiv C(OH)C_6H_{10}][Co_2(CO)_6]$ shows C(α) and C(β) resonances at 105.7 and 73.6, respectively (Table 1).

This indicates that the electron-withdrawing properties of the $Co_3(CO)_9C$ unit propagate through the carbon atom chain, shifting the acetylenic carbon resonances. We were also unable to unambiguously characterise $Co_3(CO)_9C–C\equiv C(OH)C_6H_{10}$ (**4**). Confirmation of its transient formation comes from the isolation of its $Co_2(CO)_6$– derivative (**3**). There are several examples in the literature of further co-ordination of triple bonds attached to $Co_3(CO)_9C$ units by $Co_2(CO)_6$ fragments [19,20]. The existence of recombining $Co_2(CO)_6$ fragments has been demonstrated by spectroelectrochemistry [21]. Furthermore, when a triple bond in $Co_3(CO)_9C–C\equiv C–C\equiv C–CCo_3(CO)_9$ is co-ordinated to a $Co_2(CO)_6$ moiety, the electronic delocalisation of the π-conjugated carbon chain is interrupted [22]. Therefore, the co-ordination of the $Co_2(CO)_6$ moiety to the triple bond of the elusive complex (**4**) is able to afford the stable derivative (**3**). In the reaction with ethynylestradiol, the bulky estradiol skeleton inhibits the further co-ordination of the triple bond by a $Co_2(CO)_6$ fragment.

3.2. The amido approach

Having established that an insulating arm is needed to separate the Co_3C from the triple bond, we focused our attention on the major product obtained from the reaction with isopropylamine (added as auxiliary bases) and ethynylestradiol (Scheme 1), and optimised a pathway for the model carboxamido complex (**5**) (Scheme 2). It is important to notice that complex (**5**) was reported by Seyferth et al. [23] first and in higher yields. However we have followed the amido approach because the Seyferth's acilium route is not suitable for the 1,4-(C_6H_4)–aminoethynylestradiol intermediate, since the two hydroxyl groups of such a hormone could undergoes nucleophilic addition as well [23].

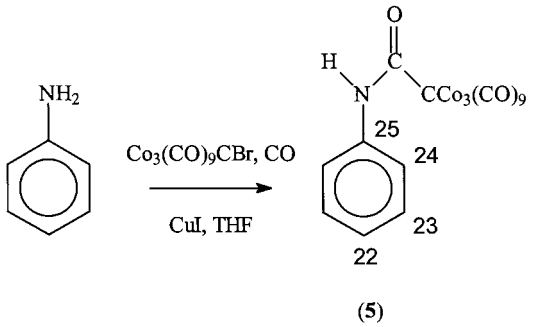
The intramolecular CO insertion giving (**5**) is consistent with a normal coupling mechanism catalysed by organocopper complexes; when the reaction is carried out in an atmosphere of CO the yields increase [24].



Scheme 1.

Table 1
Effect of the Co_3C group on the ^{13}C chemical shifts of α and β acetylenic carbon atoms

^{13}C resonances	(**3**)	HC≡C–E [10]	$[Co_2(CO)_6]$	$HC\equiv C(OH)C_6H_{10}$	$[Co_2(CO)_6]$ $[HC\equiv C(OH)C_6H_{10}]$
C(α)	118.2	80.0	103.7	71.9	105.7
C(β)	110.8	74.9	73.5	68.2	73.6



(5)

Scheme 2.

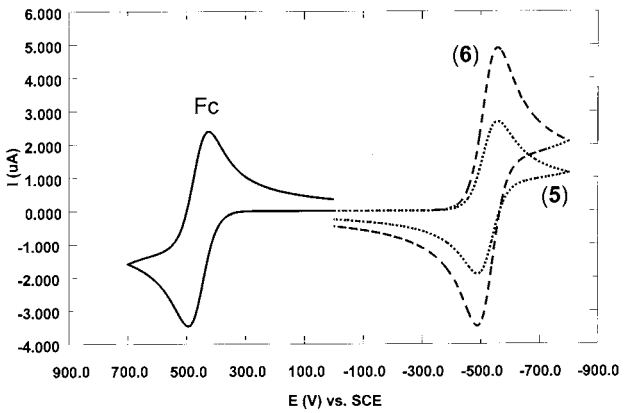


Fig. 1. CV response at a glassy carbon (GC) electrode in acetone of equimolar solutions (1.0 mM) of ferrocene (solid line), monomer (**5**) (dotted line), and dimer (6) (dashed line), scan rate 200 mV s^{-1}.

Derivative (**5**) is stable as opposed to (**1**) and (**4**), thus the interposition of the phenylcarboxamide unit as a spacer decreases the electron-withdrawing effect of the Co_3C core.

In order to check the conductive properties (via inductive and/or mesomeric effects) of the phenylcarboxamide spacer, we synthesised the corresponding dimer

$Co_3(CO)_9C–C(O)NH–C_6H_4–NHC(O)–CCo_3(CO)_9$ (**6**).

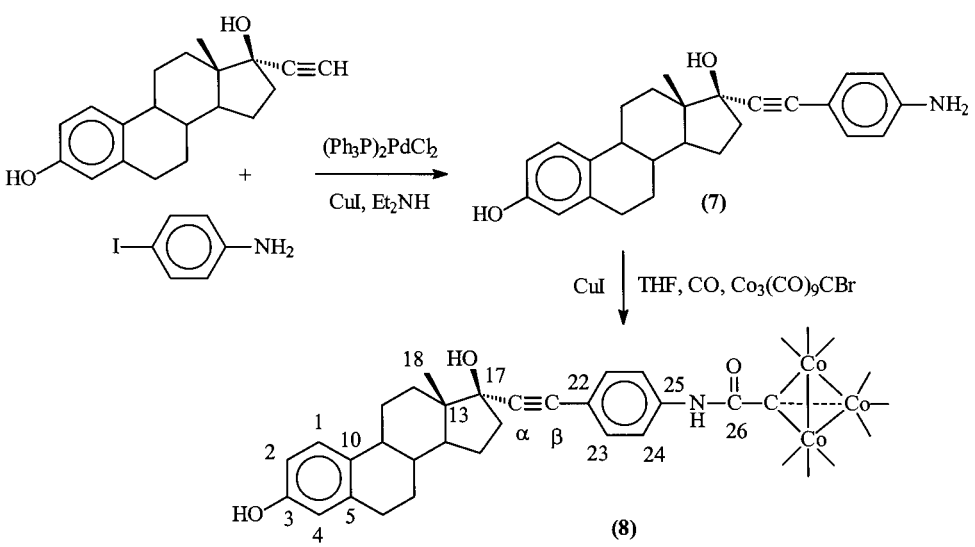
The simplest way to check electronic conducting properties is by the electrochemistry of compounds, in which the spacer under investigation links two equivalent redox centres [25,26]. If the spacer is an insulator the two redox centres are not perturbed and a single (double in height) wave/peak is observed [27]. If moderate to strong electronic coupling between the redox centres is operative, the electrochemical response consists of two resolved waves/peaks.

The cyclic voltammetric response of (**5**) and (**6**) are similar to that previously reported for other $Co_3(CO)_9CR$ analogues [28]; in acetone solution both show a single reduction at the same formal potential $E^{\circ\prime} = -0.52$ V. Both compounds show a ΔE_p value (ca. 60 mV) typical of a single electron Nernstian process, but the reduction current of the dimer (**6**) is double that of an equimolar solution of the monomer (**5**) (Fig. 1). This result confirms that the spacer –C(O)NH–C_6H_4–NHC(O)– acts as an insulator.

3.3. Linking the Co_3C unit to ethynylestradiol

With the aim to insert the desired phenyl–amino group into the ethynylestradiol, we employed the known palladium catalysed coupling reaction between an aromatic halide and a terminal acetylene [29] (Scheme 3).

The new intermediate ligand (**7**) has been characterised by comparison with the ^{1}H- and ^{13}C-NMR data of the ethynylestradiol [10,30] (see Table 2). Compound (**8**) has been characterised by ^{13}C-NMR spectra (Table 2). We were unable to observe the resonance of the apical methylidyne carbon-capping group.



Scheme 3.

Table 2
Comparison of selected ^{13}C-NMR chemical shift

Compound	C(1)	C(2)	C(3)	C(4)	C(5)	C(10)	C(13)	C(17)	C(18)	C(α)	C(β)	C(23)	C(24)	C(25)	C(26)
HC_2E	126.5	112.8	153.4	115.3	138.2	132.7	47.1	87.6	12.7	80.0	74.9				
(**5**)												119.8	129.1	137.6	176.0
(**7**)	126.4	112.6	153.4	115.2	138.1	132.2	47.0	87.4	12.6	79.8	74.0	114.6	132.9		
(**8**)	126.4	112.6	153.5	115.2	138.0	132.2	47.5	85.5	12.8	80.3	73.8	119.3	132.5	137.5	176.2

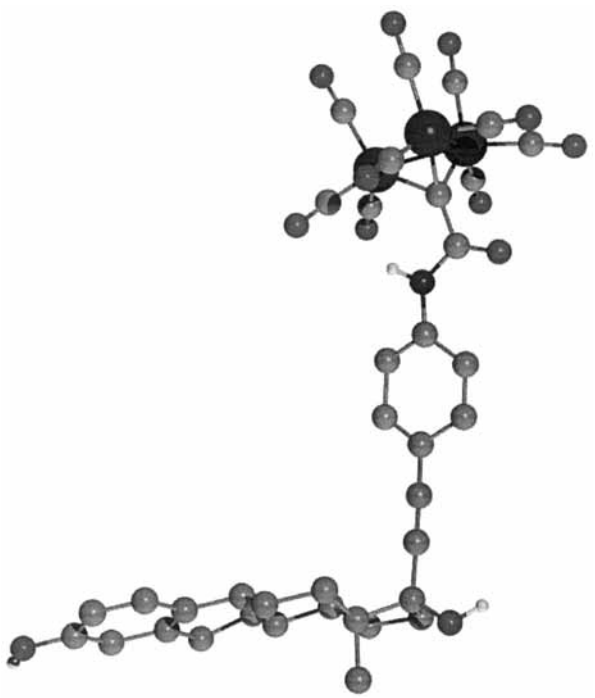


Fig. 2. Graphical molecular (Moldraw [36]) representation of (**8**).

3.4. Biochemical results

The relative binding affinity (RBA) value of the new complex (**8**) for estradiol receptor (ER) has been measured by a competitive method [15] using lamb uterine cytosol as a source of ER. The RBA value found for (**8**) is 3.0% (the RBA value of estradiol itself being equal by definition to 100%). This RBA value is 8 times higher than the one found for the corresponding dehydroxylated hormone, i.e. $Co_3(CO)_9$(dehydroxyethynylestradiol) [12]. This result confirms that the 17α modification of estradiol by a rigid –C≡C– spacer attached to a bulky organometallic group is tolerant in maintaining an acceptable recognition for the estradiol receptor. This could be the sign that the active site of the receptor in its monomeric form is not deeply buried into the protein but is most likely located not too far from the surface. Complex (**8**) then appears as good candidate to be used as tracer in bioanalyses.

3.5. Spatial structure of (*8*)

We were unable to obtain suitable crystals of (**8**) for an X-ray diffraction study. However, a reasonable rep-

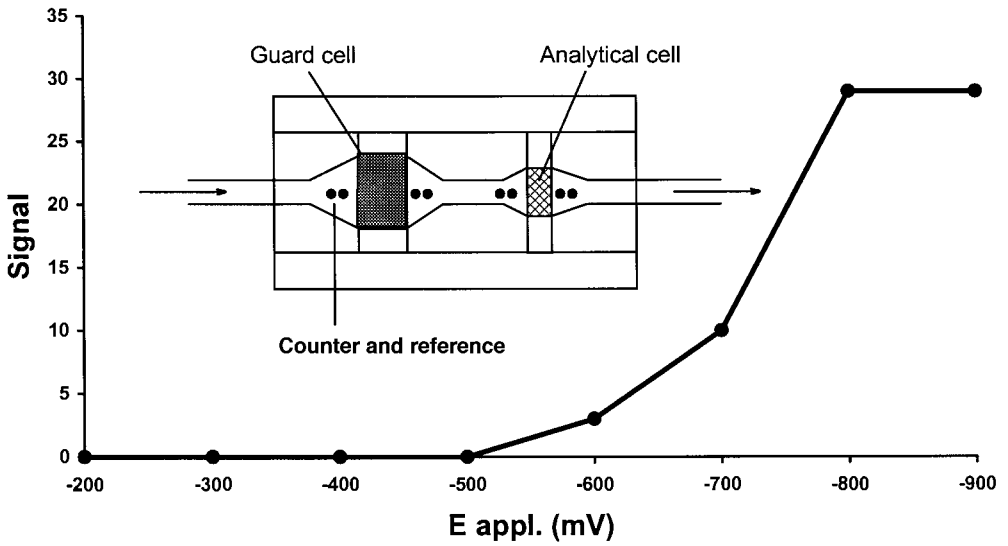


Fig. 3. Hydrodynamic voltammogram (HDV), recorded at the dual-cell ED (sketched), obtained by repeated injection containing 1.7 μg of (**8**) (i.e. 20 μl of 0.1 mM solution). Mobile phase: acetone containing $LiClO_4$ (0.01 M), room temperature, flow rate: 0.7 ml min^{-1}.

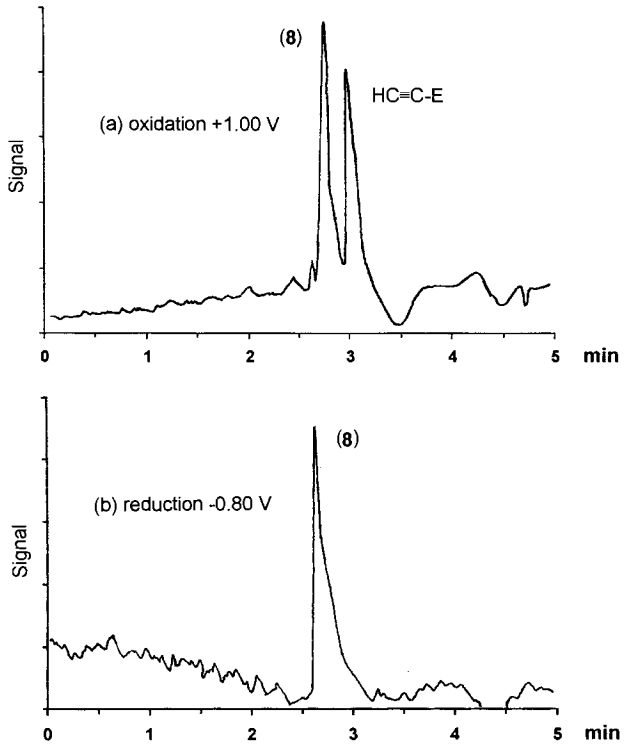


Fig. 4. ED responses of an equimolar solution of ethynylestradiol and (**8**) at (a) +1.00 V (oxidation) and at (b) −0.80 V (reduction).

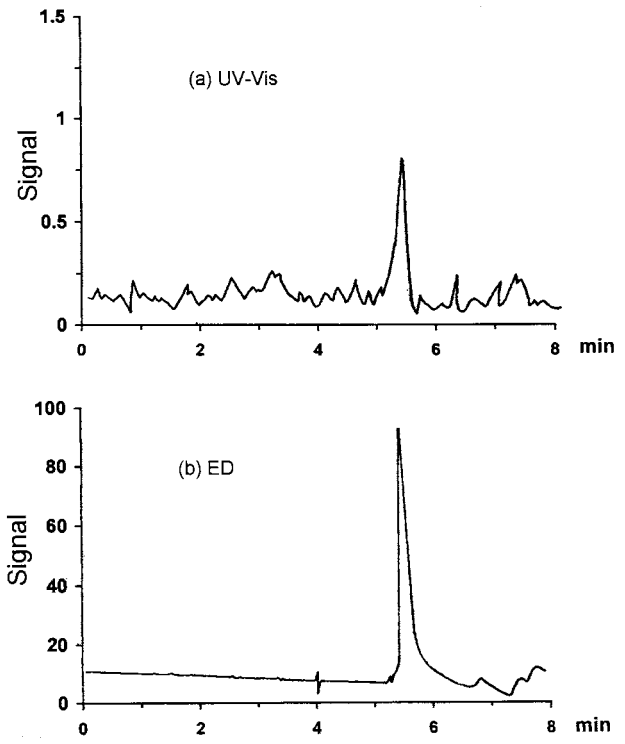


Fig. 5. Comparison between the UV-vis (a) and the ED (b) response of (**8**). Mobile phase: acetonitrile containing $LiClO_4$ (0.01 M), room temperature; flow rate: 0.7 ml min^{-1}.

resentation of (**8**) could be obtained by means of the program for molecular modelling PCMODEL [31]. We employed the structure of estradiol [32] to which we connected the fragment $–C≡C–C_6H_4–NH_2$. The overall molecule has been linked to the organometallic part extracted from the structure of $Co_3(CO)_9C–COOH$ [33]. Then the organometallic moiety was fixed and the remaining bioorganic part of (**8**) freely optimised by the program (Fig. 2).

3.6. HPLC tests

We studied the HPLC behaviour of ethynylestradiol and (**8**) in acetonitrile, acetone, methanol pure and in their mixtures with water.

Since the ED cell employs a Pd pseudo-reference electrode, in order to determine the optimum potential to apply, namely −0.80 V versus Pd, the hydrodynamic voltammogram (HDV) [11] was obtained by repeated injection of solutions of (**8**) at different ED potentials. To increase the S/N ratio, we chose a fixed difference of about 300 mV between the potentials of the guard and the analytical cell, the relative HDV being reported in Fig. 3. The retention times for ethynylestradiol and (**8**) are 3.05 and 2.74 min., respectively. When anodic potentials are applied to ED (usually estrogens are detected by oxidations in HPLC-ED) both species are observed. On the contrary, cathodic potentials allow the evaluation of the tricobalt-marked estradiol (**8**) only. Fig. 4 shows how the applied potential of ED effects the selectivity of the response. The detection limit for (**8**) proved to be about 1 nM, which is fully adequate for pharmacological analyses [34,35]. Fig. 5 shows a comparison between the UV–vis detector ($\lambda = 254$ nm) and ED ($V_{app} = -0.80$ V vs. Pd) responses of (**8**) in acetonitrile, the sensibility of ED is about 100 times higher than that of UV–vis detector.

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