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Journal of Organometallic Chemistry 533 (1997) 97–102

Journal of Organometallic Chemistry

Ferrole–estradiol complex as a test for receptor dimerization

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Received 16 August 1996; revised 4 October 1996

Abstract

We describe the synthesis of a structure based on the iron carbonyl mediated head-to-head coupling of two molecules of 17α-ethynylestradiol that leads ultimately to a ferrole. This compound was prepared with the aim of probing the mechanism of action of the estradiol receptor; spectroscopic, molecular modelling and biochemical studies are reported. The estradiol–ferrole cluster retains a weak, but not zero, affinity for the estradiol receptor. There is reversible binding to the receptor, and this is rationalized in terms of the pK_R value of −13.1 found for the bioorganometallic complex.

Keywords: Bioorganometallic chemistry; Iron; Molecular recognition; Estradiol receptor

1. Introduction

The burgeoning role of metal carbonyls in bioorganometallic chemistry is gaining more recognition as their versatility and applicability to a variety of problems become apparent [1]. Their initial use in carbonylmetallo immunoassay (CMIA) procedures has now been extended to the simultaneous quantitative detection of up to three drugs in a single experiment [2]. In a different vein, we have also described the construction of molecules containing both organometallic and steroidal hormone moieties with the aim of probing the current models of the binding of the steroidal hormone estradiol to its specific receptor site [3]. The realization that the active form of estradiol requires receptor dimerization prompted Katzenellenbogen and coworkers [4] to prepare a series of estrogen dimers linked by chains of different length in an attempt to determine the optimal separation between the binding sites in the receptor dimer. However, it remains an open question whether the dimer is of the head-to-head or head-to-tail type.

The reactions of iron carbonyls, notably $Fe(CO)_5$, $Fe_2(CO)_9$ or $Fe_3(CO)_{12}$, with alkynes have been the object of intensive study for many years [5]. Among the organometallic exotica obtained, the major products generally include the ferracyclopentadienyl (ferrole) complexes **1**. Moreover, when the starting material is an unsymmetrical, typically terminal alkyne RC≡CH, three ferrole regioisomers (**1a–1c**) can be isolated (Scheme 1).

This organometallic approach may be used to link with a relatively short spacer two estradiol molecules and the bivalent ligand may be used as a probe for studying the mode of action of the estrogen receptor. In continuation of our studies on organometallic derivatives of steroids, we describe here the synthesis, characterization and biological activity of a ferrole complex possessing two estradiol moieties in a head-to-head fashion.

2. Results and discussion

2.1. Synthesis and characterization of 1a

The reaction of $Fe_3(CO)_{12}$ with 17α-ethynylestradiol in a 1:2 molar ratio in refluxing benzene yields, after separation and work-up, three yellow products whose identical IR and mass spectra unambiguously lead to

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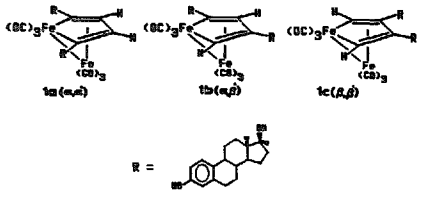
PII S0022-328X(96)06832-5



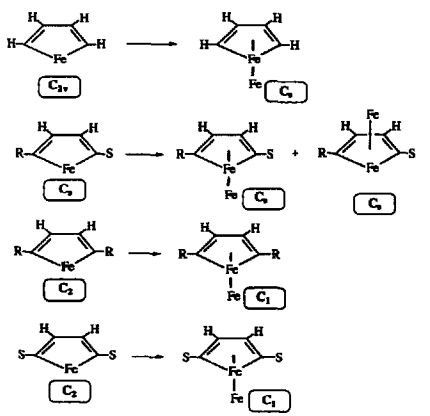
Scheme 1.

their identification as ferroles. Only one of these regioisomers is obtained in reasonable yield (ca. 20%), while the other two are formed merely in trace quantities. The major product exhibits all of the ^{1}H and ^{13}C NMR absorptions characteristic of estradiol itself [6], except that each signal appears as two equally intense resonances. The assignment as the α,α' isomer **1a** (R = estradiol fragment, $C_{18}H_{23}O_2$, in Scheme 1) is readily made by comparison of the ferracyclopentadiene ring ^{1}H and ^{13}C resonances with those of previously reported ferroles [7]. The *C*H resonances are found at 113.9 and 113.3 ppm, with ^{1}J(C–H) values of 172 and 160 Hz respectively; these parameters are characteristic of β-*C*H environments. In contrast, the two steroidal-substituted α-carbons resonate at 189.2 and 186.2 ppm. These assignments are buttressed by the ^{1}H data for the ferracyclopentadiene ring protons which each appear as 3.4 Hz doublets, values entirely consistent with their assignment as hydrogens attached to β-carbons. Typical J(H,H) values in ferrole rings have previously been established as: $J(\alpha,\alpha') = 0.3$ Hz; $J(\alpha,\beta) = 5.3$ Hz; $J(\beta,\beta') = 3.6$ Hz; $J(\alpha,\beta') = 2.1$ Hz [8].

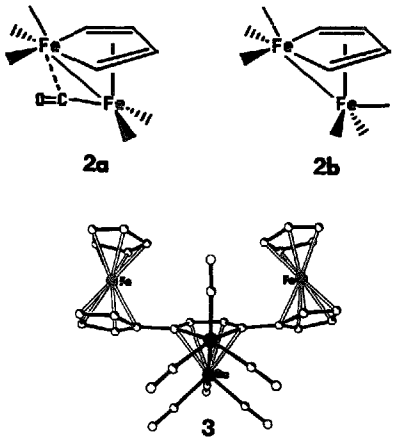
It is useful here to remind ourselves of the consequences of incorporating chiral substituents into the ferrole system [a similar situation, that of the *ortho*-disubstituted (arene)$Cr(CO)_3$ system, has been discussed previously, see Ref. [9]]. The parent system bearing achiral groups can be conveniently viewed as arising from a ferracyclopentadiene ring of C_{2v} symmetry which, upon complexation by a second $Fe(CO)_3$ fragment, yields a molecule possessing a single mirror plane (C_s) (Scheme 2). In contrast, incorporation of two chiral substituents can yield a *meso* ferracyclopentadiene (of C_s symmetry) and a *d,l* pair of ferracyclopentadienes (each of C_2 symmetry). As shown in Scheme 1, attachment of the second $Fe(CO)_3$ moiety to the *meso* ring can occur in two ways; addition to each face yields a different diastereomer, but in each case the symmetry of the resulting ferrole remains at C_s. In contrast, incorporation of the second $Fe(CO)_3$ fragment to one of the C_2 ferracyclopentadienes yields a single ferrole of C_1 symmetry.

In **1a**, which possesses two identical steroidal substituents of known absolute configuration, we would anticipate that the NMR spectra should reflect the asymmetric character of the molecule. The doubling of all the steroidal resonances, together with the non-equivalence of the protons and carbons in the ferracyclopentadiene ring, all bear witness to this phenomenon. We note that the detection of precisely the correct number of ^{13}C resonances for **1a** in the region 90–200 ppm apparently eliminates the possibility of any epimerization at the C17 position of the steroidal units. In particular, the diagnostic region 189–191 ppm unambiguously identifies the number of C(α) sites. Epimerization at even one C17 position would undoubtedly lead to a mixture of SS, SR and RS isomers (each having C_1 symmetry) with consequent peak multiplication. The probability of obtaining only a single isomer with C17 stereochemistry different from that of the estradiol starting material is very low. It is known that metal cluster fragments can stabilize steroidal cations [6,10], but this appears not to have been the case here. Such an epimerization would not, of course, lead to formation of a *meso* compound, since the steroidal skeleton contains four other stereocentres which cannot invert.

The asymmetric nature of the ferrole **1a** is also demonstrated by the observation of three separate carbonyl absorptions for the $Fe(CO)_3$ group within the ferracyclopentadiene ring (207.5, 206.3 and 206.0 ppm). In contrast, the π-bonded $Fe(CO)_3$ fragment yields a singlet at 213.0 ppm, showing that there is rapid rotation of this group on the NMR timescale. The barriers for such localized tripodal rotations are known to be rather small [7], and the low solubility of **1a** in Freon/CD_2Cl_2 precludes the achievement of the low-temperature limiting spectrum in which these three carbonyls should become non-equivalent.



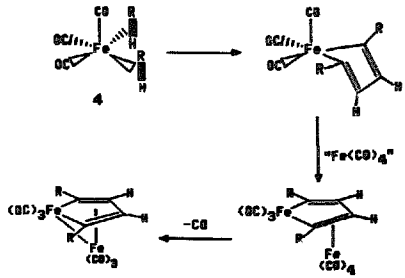
Scheme 2.



Scheme 3.

Ferroles normally adopt one of two conformations [11]: the most common is the 'non-sawhorse' structure **2a**, in which the six CO ligands are staggered with respect to the Fe–Fe vector; moreover, a carbonyl from the capping $Fe(CO)_3$ unit adopts a semi-bridging position so as to alleviate the formal electron deficiency of the iron in the five-membered ring [11]. Less commonly found is the 'sawhorse' **2b**, whereby the six carbonyls are eclipsed (Scheme 3). This latter structure has recently been reported for the ruthenium analogue **3** in which the $Ru(CO)_3$ groups are eclipsed and the two ferrocenyl substituents are bonded to the α-carbons of the ruthenacyclopentadiene ring [12]. These compounds may also be regarded as *nido* clusters.

The preference for the formation of α,α′ isomers **1a** is not unusual in ferrole chemistry; treatment of $Fe_3(CO)_{12}$ with terminal alkynes bearing a bulky substituent such as $^tBuC{\equiv}CH$ or $PhC{\equiv}CH$ gives the corresponding α,α′-ferroles as the predominant products [13]. One possible mechanism for the formation of the ferracyclopentadienyl ring would invoke coupling of the alkynes in a precursor $Fe(CO)_3(RC{\equiv}CH)_2$ complex **4**. Certainly, the mechanism of formation of the corresponding $(C_5H_5)Co(RC{\equiv}CR')_2$ complexes has been intensively studied [14]. If this is indeed the mechanism, it seems not unreasonable to assume that the regiochemistry of the ring closure to the ferracyclopentadiene system is controlled by the favoured conformation of the alkynes in the transition state. As shown in Scheme 4, if **4** can adopt a square-based pyramidal structure, which is usually similar in energy to the trigonal bipyramid, then bond formation between alkynes will be

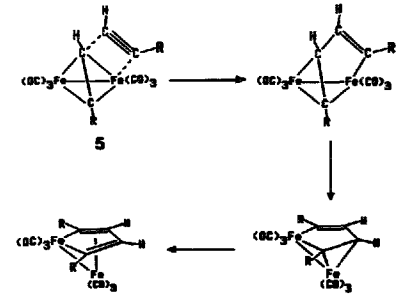


Scheme 4.

easiest for those termini with the less bulky substituents (i.e. hydrogen atoms). Subsequent addition of a second iron fragment would lead directly to the ferrole.

Hoffmann and coworker [15] have offered an alternative suggestion whereby the ferrole system is generated through sideways attack by an alkyne on a preformed (alkyne)$Fe_2(CO)_6$ cluster **5**, as in Scheme 5. Again, coupling between the less sterically encumbered termini would appear to be favoured. We note also that modelling studies indicate that the overall most sterically favoured isomer for the ferrole is the α,β′ isomer **1b**, and so the observed predominance of the α,α′ isomer **1a** appears to be a result of kinetic rather than thermodynamic control.

Although we have not yet obtained X-ray quality crystals of **1a**, a reasonable representation of the structure of $Fe_2(CO)_6(RC{\equiv}CH)_2$ **1a** where $RC{\equiv}CH$ is 17α-ethynylestradiol can be obtained by using the molecular modelling program PC-MODEL [16], which uses the MMX force field (MMX is a version of MM2, modified by J.J. Gajewski and K.E. Gilbert to accommodate transition metal atoms; for more information see Ref. [17]). This representation was built up by taking the X-ray parame-



Scheme 5.

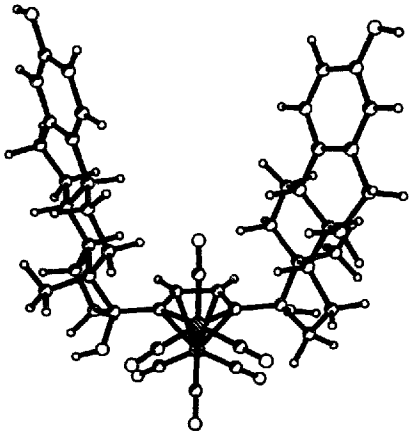


Fig. 1. View of regioisomer **1a**. The structure was obtained by using a molecular modelling program to graft the steroidal fragments onto the organometallic moiety.

ters of $Fe_2(CO)_6[C_4(OH)_2Me_2]$ (non-sawhorse geometry) [18] and those of the estradiol–propanol adduct [19]. The initial structure of **1a** was constructed by replacing the β-methyls in $Fe_2(CO)_6[C_4(OH)_2Me_2]$ by hydrogens, and by detaching the two α-hydroxyl substituents and replacing them with two steroidal fragments whose 17α-hydrogens had been removed. After adjusting the new bond distances to normal values, the resulting structure was energy-minimized by rotating sequentially each steroidal fragment about the C(α)–C(17) bonds. This procedure was iterated several times. Finally, by allowing small oscillations of all atoms (except those in the organometallic skeleton), the overall energy minimum was obtained. While recognizing the approximations inherent in this procedure (e.g. the organometallic segment has been considered as a frozen unit), this method provides a reasonable model of the overall molecular geometry, and the resulting structure appears as in Fig. 1. We note that the orientations of the two estradiol fragments with respect to the capping $Fe(CO)_3$ moiety are not equivalent; indeed, the inherent C_1 symmetry of the system imposes no restrictions on the molecular structure, but the pseudo-C_2 character of the structure is evident. It is noteworthy that the two bulky steroidal substituents are oriented *distal* with respect to the π-bonded $Fe(CO)_3$ group; this effect is also seen in the bis(ferrocenyl)ruthenole **3**. The molecular modelling study suggests that the steroidal moieties have rather limited mobility, and this, to some extent at least, fixes the positions of their hydroxyl substituents. The two phenolic groups, i.e. the C(3)–OH units, are separated by approximately 11.1 Å and, in principle, could be available for binding to a receptor site. In contrast, the C(17)–OH groups are twisted away from each other and are unlikely to be able to interact in any concerted fashion. However, the possibility still exists for each estradiol unit to act independently, since all the hydroxyl groups are sited on the exterior of the molecule. The steroidal fragments are not aligned in a parallel fashion; the interplanar angle between the aromatic A rings of the estradiol units is about 20°.

2.2. *Biological measurements*

The binding affinity of **1a** for the estrogen receptor of lamb uterine cytosol has been measured by a competitive binding method, which is a convenient method for determining the binding affinity of modified hormones [20]. The relative binding affinity (RBA) value found for **1a** is rather low (0.53%; the RBA value of estradiol itself is 100% by definition). There is, however, a fundamental difference between this value, which is low but measurable, and a true RBA of zero. In view of the significant steric crowding in this molecule, the result seems to indicate that the active site of the receptor is not buried in the interior of the protein. One might rationalize the RBA value in terms of the structure proposed for the molecule; the 3-hydroxyl groups in each of the two estradiol fragments are directed towards the exterior of the molecule and still accessible for the receptor. The calculated distance of approximately 11 Å is close to that found in diethylstilbestrol (DES), the well-known hormone mimic [21].

In compound **1a**, one should notice the occurrence of two OH groups located in adjacent positions to the organometallic unit (i.e. $Fe_2(CO)_6$). This is the situation of a potential affinity marker for the estrogen receptor. We have performed the relevant biological experiments and found that the binding between **1a** and the receptor is fully reversible. Therefore there is no covalent link established between **1a** and the receptor. We have previously found that a correlation exists between the carbenium ion stabilization (evaluated by means of its pK_{R^+} value) and the occurrence of an affinity marker property of estradiol coordinated at 17α position by an organometallic moiety [3]. In the case of ferrole, the pK_{R^+} value has not been reported so far. The measurements have been carried out by using the Deno acidity function [22]. We have found a pK_{R^+} value for **1a** of −13.11, which indicates that the cation is quite elusive. This explains why **1a** does not act as an affinity marker for the estradiol receptor: the cation is generated only in a strongly acidic medium ($H_2SO_4 > 60\%$) which is obviously not found in biological systems. By comparison, the bioorganometallic complex $Co_2(CO)_6$(propynylestradiol) works as an affinity marker for the

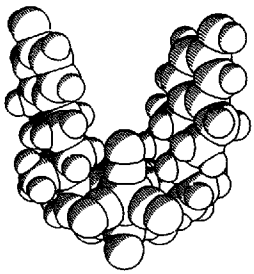


Fig. 2. Space-filling representation of molecule **1a**.

estrogen receptor [23] and the model molecule $Co_2(CO)_6(HC{\equiv}CH_2{-}OH)$ has a pK_{R^+} value of −5.5 [24].

3. Conclusions

Within the hypothesis of a close proximity between the two binding sites of estradiol in the head-to-head estradiol receptor dimer, one should expect a high affinity of this ferrole bivalent ligand **1a**. This is not the case. The reason might be that the linker is too short to fit exactly with the second binding site. However, the hypothesis of a head-to-tail dimerization of the receptor should not be excluded. Recently, Mornon et al. [25] have suggested such a head-to-tail arrangement in their modelling studies of the receptor dimer. Whatever the truth, we have shown that the ferrole strategy provides an interesting way of linking two biomolecules in order to generate bivalent ligands with a rigid organometallic spacer (Fig. 2).

4. Experimental

4.1. Synthesis

The title complex **1a** was synthesized by reacting $Fe_3(CO)_{12}$ (0.50 g, 1 mmol) with 17α-ethynylestradiol (0.59 g, 2 mmol) in 200 ml of anhydrous benzene at reflux for 4 h under Ar. The resulting dark yellow solution was filtered hot on a small SiO_2 column (in order to remove any metallic residue) then concentrated at reduced pressure and chromatographed on TLC plates (adsorbent SiO_2, eluant *n*-hexane/acetone (70:30)). The fast moving green band corresponds to unreacted $Fe_3(CO)_{12}$. Three yellow bands are then eluted; the slowest moving one was isolated in an amount sufficient to be crystallized from *n*-hexane/CH_2Cl_2 (50:50) to give a yellow powder (yield ca. 20%). All the yellow compounds exhibited identical mass spectra and IR spectra. CH_2Cl_2 (ν(CO) cm^{-1}): 2064 m, 2028 vs, 1990 s, 1975 m(sh).

The mass spectra were rather difficult to obtain: both electron impact (EI, 70 eV) and fast atom bombardment (FAB, glycerol matrix) techniques showed extensive fragmentation. Good MS responses could be obtained by the desorption chemical ionization (DCI) technique, employing isobutane as the reagent gas, and collecting negative ions. This procedure afforded an intense $[M-CO]^-$ ion at m/z 844, with concomitant loss of H_2O and CO molecules.

As previously stated, only the regioisomer **1a** was obtained in sufficient quantity to be crystallized and further characterized.

^1H NMR, 400.0 MHz (acetone-d_6): δ/ppm 8.08/8.06 (each s, C_3–OH); 7.18/7.16 (each d, C_1–H); 6.80 and 6.47 (each d, C_β–H, $^3J_{HH}$ = 3.4 Hz); 6.68 (m, C_2–H); 6.65/6.63 (each d, C_4–H); 4.39/4.30 (each s, C_{17}–OH); ca. 3.0–1.3 ppm (all the remaining resonances heavily overlapped); 1.21 (s, Me − 18).

^{13}C NMR, 100.6 MHz ($CDCl_3$): δ/ppm 189.2 and 186.2 (Cα); 153.3/153.2 (C3); 138.1/137.9 (C5); 132.6/132.1 (C10); 126.4/124.3 (C1); 115.1 (C4); 113.9 and 113.3 (Cβ, $^2J_{CH}$ = 172.4 and 160.2 Hz respectively); 112.6/112.5 (C2); 91.5/90.7 (C17); ca. 50.0–20.0 ppm (all the remaining resonances heavily overlapped); 16.4/15.2 (Me − 18).

Anal. Found: C, 63.48; H, 5.33; Fe, 12.89. $C_{46}H_{48}O_{10}Fe_2$ Calc.: C, 63.32; H, 5.54; Fe, 12.80%.

IR and NMR spectra were recorded on Perkin–Elmer 580B and Jeol EX 400 spectrophotometers respectively. DCI mass spectra were recorded on a Finnigan-MAT 95Q instrument with both magnetic and electrostatic analyzers. Isobutane was used as the reagent gas at 0.5 mbar pressure. The ion source temperature was kept at 50 °C, the electron emission current at 0.2 mA, and the electron energy at 200 eV. Both positive and negative ion spectra were recorded.

4.2. Competitive binding assay

Aliquots (200 μl) of lamb uterine cytosol prepared as previously described [21] were incubated for 3 h at 0 °C with 2×10^{-9} M of [^{3}H]-estradiol in the presence or absence of competing unlabelled steroids (non-radioactive estradiol or **1a**; nine concentrations ranging from 1×10^{-10} to 1×10^{-6} M). The percentage reduction in binding of [^{3}H]-estradiol (Y) was calculated using the logit transformation of Y (logit $Y = \ln[Y/(1-Y)]$) versus the log of the mass of the competing steroid. The concentration of unlabelled steroid required to displace 50% of the bound [^{3}H]-estradiol was calculated for each steroid tested, and the results were expressed as RBA.

4.3. Attempted inactivation of the estrogen receptor by *1a*

Fractions of lamb uterine cytosol were first incubated in the presence of 10 nM of **1a**, for 1 h at 25 °C. At the end of the incubation, free tracer was removed by charcoal dextran treatment. The surviving reversible estrogen binding activity was measured after exchange with [^{3}H]-estradiol 1.0×10^{-8} M for 16 h at 25 °C. Protamine sulphate precipitation was used to determine the estradiol receptor concentration [3].

4.4. Determination of the pK_{R^+} value of *1a*

A 10^{-3} M solution of **1a** in acetonitrile was prepared. Aliquots of this organic solution (50 μl) were added to 450 μl of different solutions of H_2SO_4 (final concentration ranging from 66 to 86%). The spectroscopic determination of the carbocation was performed at 458 nm, after 30 min incubation at room temperature. Final calculations were obtained by using the Deno C_0 acidity function [22].

Acknowledgements

We thank the European Union for financial support (Contract ERBCIPA ACT 930027) and Dr. M. Vincenti (Department of Analytical Chemistry, University of Torino) for DCI mass spectra.

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