

# Stabilization of Carbenium Ions Derived from Ethynylestradiol by Different Adjacent Organometallic Moieties. Implication in the Inactivation of the Estrogen Receptor

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The stabilization of carbenium ions by an adjacent organotransition metal moiety has been evaluated using the classical Deno's method as well as a new procedure based on DCI-MS spectrometry. The ability of transition metal clusters to enhance the stability of a proximate carbocationic center

in 17 $\alpha$ -alkynylestradiol-[OM] derivatives {[OM] = Co<sub>2</sub>(CO)<sub>6</sub>; Co<sub>2</sub>(CO)<sub>4</sub>(dppm); Mo<sub>2</sub>Cp<sub>2</sub>(CO)<sub>4</sub>; M<sub>3</sub>(CO)<sub>10</sub>, M = Ru, Os; HRu<sub>3</sub>(CO)<sub>9</sub>} has been related to their capacity to inactivate the estrogen receptor (affinity marker properties).

The stabilization of carbenium ions by an adjacent organotransition metal moiety is a well documented phenomenon.<sup>[1,2]</sup> The study of the hexacarbonyldicobalt unit as a protecting group for the C $\equiv$ C triple bond<sup>[3]</sup> led Nicholas et al. to discover the fast acid-promoted hydration/dehydration equilibrium, (Figure 1) which generates the [(propargyl)Co<sub>2</sub>(CO)<sub>6</sub>]<sup>+</sup> cation as an intermediate.<sup>[4]</sup>

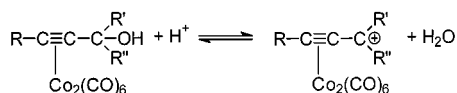


Figure 1. Hydration/dehydration equilibrium involving a [(propargyl)Co<sub>2</sub>(CO)<sub>6</sub>]-like compound

A large variety of propargyl cations coordinated to many different organometallic fragments have been investigated to date.<sup>[5–8]</sup> The major interest in these compounds is based on the possibility of employing them as electrophilic synthons. In this context, 17 $\alpha$ -alkynylestradiol organometallic derivatives (Figure 2) may be regarded as analogous to the propargylic alcohol complexes.

Several examples of transition-metal carbonyl fragments, used as labeling agents for 17 $\alpha$ -alkynylestradiol, have been reported in the literature {[OM] = Co<sub>2</sub>(CO)<sub>6</sub>; Mo<sub>2</sub>Cp<sub>2</sub>(CO)<sub>4</sub>; M<sub>3</sub>(CO)<sub>10</sub>, M = Ru, Os}.<sup>[9]</sup> An appreciable

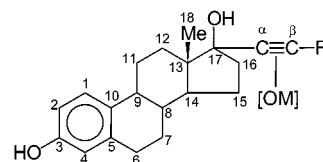


Figure 2. Conventional representation of the [OM]-steroidal ring system, showing the numbers used to identify the carbon atoms

recognition level for the receptor, evaluated in terms of the *relative binding affinity (RBA)*, is maintained even when relatively bulky groups are linked to a 17 $\alpha$ -alkynylestradiol.<sup>[10]</sup> This particular labeling of steroids has been used in a new non-isotopic immunoassay (*carbonylmetalloimmunoassay, CMTA*) for assaying circulating hormones such as cortisol<sup>[9]</sup> or in receptorology for studying the interaction between the hormone and its receptor.

The synthesis of a large number of organometallic labeled hormones led to the discovery of a new family of *affinity markers* for the estrogen receptor, i.e. molecules able to bind irreversibly to the receptor, probably through a direct covalent bond. This unusual behavior may be attributed to the stability of the associated organometallic carbenium ion, as previously discussed. The 17 $\beta$ -OH group is essential for inactivation of the receptor.<sup>[11]</sup> The presence of cysteine acid residues near the association site of the receptor can account for the formation of the carbocation through an acid-promoted dehydration reaction on the OH group at the 17 $\beta$  position (Figure 3). Unfortunately, the recently published X-ray structure for the Ligand Binding Domain (LBD) of the  $\alpha$ -estradiol receptor was obtained after chemical modification of the cysteines.<sup>[12]</sup> Therefore, this hypothesis cannot be proven for the moment, although these organometallic markers may help in the elucidation of the structure of the receptor binding site.<sup>[13,14,15]</sup>

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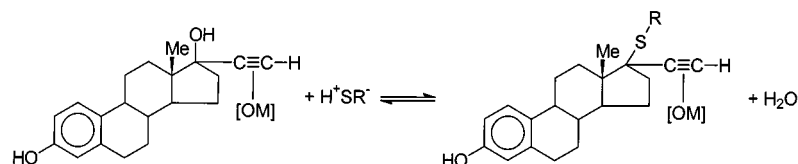


Figure 3. Proposed mechanism for the formation of the carbocation through an acid-promoted dehydration reaction on the 17 $\beta$ -OH group

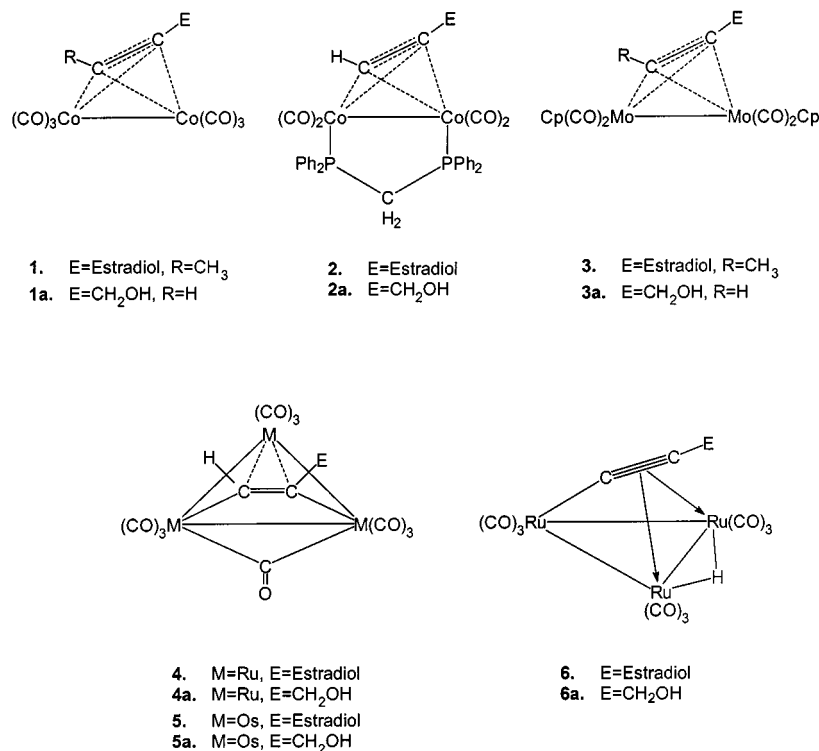


Figure 4. Sketch of structures of complexes 1–6 and 1a–6a

The aim of this work is to investigate both the relationship between the ability of transition metal clusters to enhance the stability of the neighboring carbocationic center in 17 $\alpha$ -alkynylestradiol derivatives, and also to investigate their affinity marker properties. Breast cancers that retain estrogen receptor (ER) can usually be treated effectively by endocrine therapy, such as by the use of the antiestrogen tamoxifen. Moreover, by labeling estradiol with cytotoxic metal derivatives one could expect to synthesize a new family of anticancer drugs able to use the receptor system as a vector to reach the target cells and remain attached for a period sufficient to damage the cancerous tissue.

## Results and Discussion

Several 17 $\alpha$ -alkynylestradiol organometallic derivatives 1–6 and the corresponding propargylic model complexes 1a–6a have been synthesized (Figure 4).

Compound **6a** [HRu<sub>3</sub>(CO)<sub>9</sub>(–C $\equiv$ CCH<sub>2</sub>OH)] was obtained in good yield by thermolysis of Ru<sub>3</sub>(–CO)<sub>10</sub>(HC $\equiv$ CCH<sub>2</sub>OH) (**4a**). In contrast, the direct reaction of Ru<sub>3</sub>(CO)<sub>12</sub> and HC $\equiv$ CCH<sub>2</sub>OH gave compound **6a** only

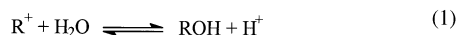
in trace amounts; the major product was metallacyclopentadiene Ru<sub>2</sub>(CO)<sub>6</sub>(alkyne)<sub>2</sub>.<sup>[16]</sup>

The relative binding affinities of these complexes for the ER were determined in a competition binding assay using lamb uterine cytosol as a source of ER, and [<sup>3</sup>H]estradiol as the tracer. The values found range between 2.5 and 33% (estradiol itself being taken as 100%), indicating moderate recognition. Roughly, RBA values are inversely proportional to the steric bulk of the organometallic graft.

The possible existence of a covalent bond between the hormone derivatives and the receptor was investigated by receptor inactivation (RI) studies. The RI procedure involves a preliminary incubation of the organometallic tracer with the cytosol receptor after which the free organometallic tracer is removed by means of charcoal-dextran treatment. The remaining reversible estrogen binding activity of the tracer-receptor adduct is then measured by an exchange reaction of [<sup>3</sup>H]estradiol with the organometallic tracer not covalently bound to the estrogen binding site. The quantification of the [<sup>3</sup>H]estradiol specifically bound to the ER was then performed by protamine sulfate precipitation as previously described.<sup>[17]</sup> The residual radioactivity

on the precipitate is related to the remaining estrogen binding activity: the higher the radioactivity, the lower the receptor inhibition ( $RI\% = 0$  for estradiol itself).<sup>[18]</sup>

Carbenium ions can be generated in presence of strong acids. The  $pK_{R+}$  values for ionization of alcohols in aqueous sulfuric acid were determined spectrophotometrically. The position of the alcohol–carbenium ion equilibrium (Equation 1):



was studied as a function of sulfuric acid concentration, using the empirical acidity function ( $C_0$ ) proposed by Deno.<sup>[19]</sup> The Deno's method is a facile way of obtaining the thermodynamic stability of the carbenium ions, provided that a sharp change in the UV/Vis spectrum is produced upon acid addition. However, the strongly acidic conditions required by this method may induce rearrangements of the steroid skeleton to give several enyne complexes (Figure 5), a possibility proposed in a previous study.<sup>[6]</sup>

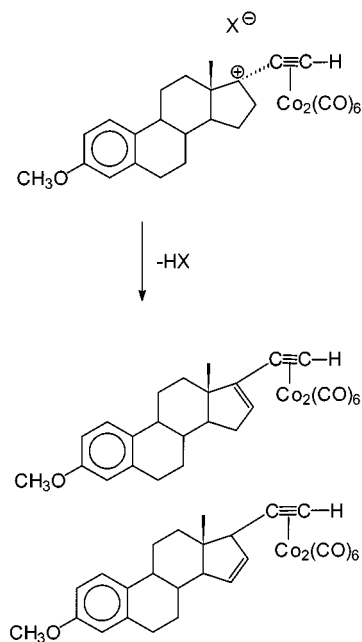


Figure 5. Proposed mechanism for the formation of enyne complexes through rearrangements of the steroid skeleton

Since the  $[R^+]/[ROH]$  ratio is evaluated from the UV/Vis absorptions of both species, any chemical transformation of either is likely to alter the overall spectrum profile and, consequently, the  $pK_{R+}$  value. To prevent misinterpretations, several measurements have been carried out on the corresponding propargylic alcohol as models. This simplification is reasonable, as the  $pK_{R+}$  values measured for the propynylestradiol complexes **1** and **3** proved to be similar to those of the model propargyl complexes.<sup>[6]</sup>

To overcome the limitations inherent in the Deno's method, we propose an alternative gas phase procedure for evaluating carbocation stability with respect to the parent

alcohol. This procedure utilizes chemical ionization mass spectrometry in combination with the fast thermal vaporization of the organometallic complex (DCI-MS). A relatively high pressure of reagent gas (i.e. isobutane) and a low pressure of analyte ( $M$ ) are simultaneously present in the ionization chamber. The reagent gas is ionized by electron impact, and fragments in a conventional manner. Several collisions occur between ions and neutral isobutane molecules yielding the *tert*-butylcarbonium ion  $[(CH_3)_3C]^+$  as a stable species. This may subsequently react with the analyte ( $M$ ) by proton transfer to generate the quasi-molecular ion  $[M + H]^+$ . The facility of such a process, and thus the intensity of the quasi-molecular ion peak  $[M + H]^+$ , depends on the acid-base properties of the species involved in the equilibrium (Equation 2):



In the present case, the protonated ion  $[M + H]^+$  can eliminate  $H_2O$ , provided the corresponding carbocation is sufficiently stable (Figure 6). Thus, the ratio,  $[M - OH]^+ / [M + H]^+$ , between ion peak intensity represents a good estimate of the corresponding carbocation stability in the gas phase.

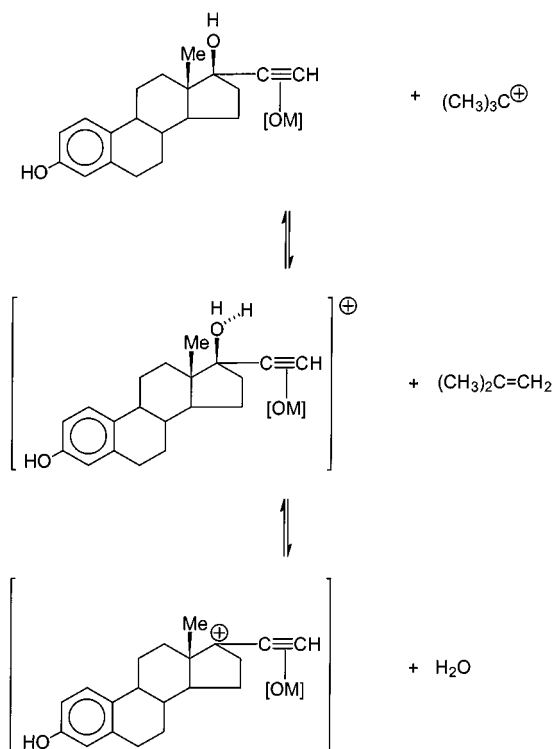


Figure 6. Water elimination reaction on the organometallic estradiol

The DCI method is experimentally simpler than the (chemical) Deno's method. Furthermore, the ionization process and ion detection is so rapid that elimination reactions on the carbenium ion do not occur, allowing a meaningful measure on real estradiol–organometallic derivatives.

The presence of the 17 $\beta$ -OH group is essential for carbocation formation; in fact, the DCI-MS spectrum of estrone (C=O group in lieu of the OH group) does not exhibit the  $[M - OH]^+$  peak. The contribution of the phenolic OH in position 3 to the protonation-dehydration process is negligible:  $[M - OH]^+/[M + H]^+$  ratios of 17 $\alpha$ -ethynylestradiol and 3-methoxy-17 $\alpha$ -ethynylestradiol are identical, within error.

Compounds **4** and **5** show a fragmentation pathway different to all the other complexes. Since the major process is the loss of a CO ligand, a reaction previously found in solution,<sup>[20]</sup> the  $[M + H]^+$  and  $[M - OH]^+$  peaks are too small to be evaluated properly. For this reason, the mass ratio has been evaluated by the relative abundance of the daughter ions  $[(M - CO) - OH]^+ / [(M - CO) + H]^+$ .

The DCI-MS data for the propargyl models **1a–6a** are similar to those of the estradiol homologues: the  $[M - OH]^+/[M + H]^+$  ratios of the former are always higher than those of the latter. This result can be explained by postulating that the  $[M + H]^+$  internal energy for estradiol derivatives is distributed over a much larger number of chemical bonds than for propargyl derivatives. Less energy is therefore available to induce the loss-of-water fragmentation process.

Complexes **1–6** may be separated into three categories:

(i) Complex **2** has a  $pK_{R+}$  value of  $-11$  and a DCI-MS ratio of 0.87, indicating a highly unstable carbenium ion, and gives no alkylation of receptor at all (RI% = 0);

(ii) Compounds **1** and **4–6** have  $pK_{R+}$  values in the  $-6.0$  to  $-1.4$  range and DCI-MS ratios from 7.2 to 1.4, indicating the formation of moderately stable but still reactive carbocations; they efficiently inactivate the estradiol receptor ( $80 < \text{RI\%} < 60$ );

(iii) Complex **3** shows the highest  $pK_{R+}$  and DCI-MS values ( $+2.7$  and  $12.0$ , respectively) indicating a very stable but almost unreactive carbocation, associated with a low inactivation of the receptor (RI% = 22).

The best affinity marker was found to be compound **1**, which has a  $pK_{R+}$  around  $-6$ . With smaller or higher  $pK_{R+}$  values this phenomenon of receptor inactivation is not so apparent. Although 17 $\alpha$ -propynylestradiol shows the same  $pK_{R+}$  value as **1**, it does not reveal any detectable RI effect. This implies that the C $\equiv$ C triple bond in the  $\alpha$  position is itself able to stabilize the adjacent carbocation, although the metal core should play a further active role in establishing a covalent bond with the receptor, for instance with the thiol groups of cysteine residues present in the LBD. In this context, it is known that cobalt carbonyls are able to

Table 1. Relative binding affinity (RBA%) of the estradiol receptor, inactivation of the receptor (RI%),  $pK_{R+}$  and DCI-MS ( $[M - OH]^+ / [M + H]^+$ ) ratio

	Compound	RBA	RI%	$pK_{R+}$	$[M - OH]^+ / [M + H]^+$
	estradiol (E-H)	100% <sup>[a]</sup>	0% <sup>[a]</sup>	$-13.0$ <sup>[b]</sup>	0.3 <sup>[b]</sup>
	17 $\alpha$ -ethynylestradiol (E-C $\equiv$ CH)	70% <sup>[b]</sup>	0% <sup>[b]</sup>	<sup>[d]</sup>	1.2 <sup>[b]</sup>
	17 $\alpha$ -propynylestradiol (E-C $\equiv$ C-CH <sub>3</sub> )	44% <sup>[b]</sup>	0% <sup>[b]</sup>	$-6.4$ <sup>[b]</sup>	1.6 <sup>[b]</sup>
<b>1</b>	Co <sub>2</sub> (CO) <sub>6</sub> (EC $\equiv$ C-CH <sub>3</sub> )	18% <sup>[c]</sup>	80% <sup>[c]</sup>	$-6.0$ <sup>[b]</sup>	7.2 <sup>[b]</sup>
<b>1a</b>	Co <sub>2</sub> (CO) <sub>6</sub> (HC $\equiv$ CCH <sub>2</sub> OH)	—	—	$-5.5$ <sup>[e]</sup>	16.0 <sup>[b]</sup>
<b>2</b>	Co <sub>2</sub> (CO) <sub>4</sub> (dppm)(EC $\equiv$ CH)	4.2% <sup>[b]</sup>	0% <sup>[b]</sup>	$-11.0$ <sup>[b]</sup>	0.9 <sup>[b]</sup>
<b>2a</b>	Co <sub>2</sub> (CO) <sub>4</sub> (dppm)(HC $\equiv$ CCH <sub>2</sub> OH)	—	—	<sup>[d]</sup>	1.3 <sup>[b]</sup>
<b>3</b>	Mo <sub>2</sub> Cp <sub>2</sub> (CO) <sub>4</sub> (EC $\equiv$ C-CH <sub>3</sub> )	33% <sup>[c]</sup>	22% <sup>[c]</sup>	$+2.7$ <sup>[e]</sup>	12.0 <sup>[b]</sup>
<b>3a</b>	Mo <sub>2</sub> Cp <sub>2</sub> (CO) <sub>4</sub> (HC $\equiv$ CCH <sub>2</sub> OH)	—	—	$+3.0$ <sup>[e]</sup>	12.3 <sup>[b]</sup>
<b>4</b>	Ru <sub>3</sub> (CO) <sub>10</sub> (EC $\equiv$ CH)	8% <sup>[c]</sup>	68% <sup>[c]</sup>	<sup>[d]</sup>	2.0 <sup>[b,f]</sup>
<b>4a</b>	Ru <sub>3</sub> (CO) <sub>10</sub> (HC $\equiv$ CCH <sub>2</sub> OH)	—	—	$-1.4$ <sup>[c]</sup>	2.0 <sup>[b,f]</sup>
<b>5</b>	Os <sub>3</sub> (CO) <sub>10</sub> (EC $\equiv$ CH)	2.5% <sup>[c]</sup>	65% <sup>[c]</sup>	<sup>[d]</sup>	1.4 <sup>[b,f]</sup>
<b>5a</b>	Os <sub>3</sub> (CO) <sub>10</sub> (HC $\equiv$ CCH <sub>2</sub> OH)	—	—	$-3.4$ <sup>[c]</sup>	1.6 <sup>[b,f]</sup>
<b>6</b>	HRu <sub>3</sub> (CO) <sub>9</sub> (-C $\equiv$ CE)	2.6% <sup>[c]</sup>	60% <sup>[c]</sup>	<sup>[d]</sup>	1.5 <sup>[b]</sup>
<b>6a</b>	HRu <sub>3</sub> (CO) <sub>9</sub> (-C $\equiv$ CCH <sub>2</sub> OH)	—	—	<sup>[d]</sup>	2.8 <sup>[b]</sup>

<sup>[a]</sup> Values by definition. — <sup>[b]</sup> This work. — <sup>[c]</sup> ref. [10]. — <sup>[d]</sup> No shape change in the UV/Vis spectrum occurred upon acid addition. This prevented the correct evaluation of  $pK_{R+}$  values. — <sup>[e]</sup> ref. [6] — <sup>[f]</sup> Calculated as the ratio  $[(M - Co) - OH]^+ / [(M - Co) + H]^+$ .

The experimental results are summarized in Table 1.

There is a rough correlation between  $pK_{R+}$  and DCI-MS values, although the metallic grafts are very different throughout the series. Higher (less negative)  $pK_{R+}$  values correspond to higher DCI-MS ratios; both results point to a higher stabilization of the corresponding carbocations.

As previously mentioned, an approximate correlation exists between the affinity marker properties of estradiol organometallic derivatives (evaluated by their RI% value) and the carbenium ions stabilization (evaluated from both  $pK_{R+}$  and DCI-MS values).<sup>[9]</sup> However, the interactions in a sophisticated biological system are always more complex than in our simple model experiment.

react with thiol functionalities, giving rise to a plethora of compounds.<sup>[21]</sup>

As mentioned above, compound **1** shows the highest RI value (80%). Replacement of one carbonyl group on hexacarbonyldicobalt-coordinated propargyl cations by phosphane is expected to increase the stability, and hence the  $pK_{R+}$  values of these compounds. This would occur by increasing the electron density on the Co<sub>2</sub> moiety, in the manner qualitatively described by Nicholas.<sup>[22]</sup> Kuhn et al. quantified this effect in a kinetic study on the electrophilic reactivity of  $[\text{Co}_2(\text{CO})_5(\text{PPh}_3)(\text{HC}\equiv\text{CPh})]^+$  cation.<sup>[23]</sup> Thus, the replacement of two carbonyls by diphosphanes in compound **1** (to give **2**) is expected to increase the carbocation



stability. Surprisingly, compound **2** gave a  $pK_{R+}$  value of  $-11$  and a mass ratio of  $0.87$ , both indicating a highly unstable cation. This unexpected behavior can be explained in terms of steric hindrance.

The build-up of a positive charge results in a shift of the cationic center towards one of the Co atoms. It has to be recognized that the mechanism of carbocation stabilization involves rearrangements on the overall skeleton which facilitate the metal–C(17)<sup>+</sup> interaction. Melikyan et al. resolved the structure of a Co<sub>2</sub>(CO)<sub>6</sub>-complexed propargyl cation showing that the C<sup>+</sup> atom is no longer equidistant from Co atoms.<sup>[24]</sup> The same behavior was observed in **3**<sup>+</sup>, where the organometallic moiety shifts below the estradiol D ring, enabling a Mo–C(17)<sup>+</sup> interaction to be established.<sup>[6]</sup> In the case of **3**<sup>+</sup> this interaction is weaker than that reported for **3a**<sup>+</sup>, and interestingly, this difference has been attributed to the conformational constraints imposed by the bioligand.

In **2** the steric repulsion should be enhanced by the presence of two bulky diphosphane and the large steroidal skeleton. In order to investigate this hypothesis, we compared the size of **2** with respect to **1**, by representing them using the molecular modeling program PC Model.<sup>[25]</sup> The organometallic fragments were derived from the X-ray structure of Co<sub>2</sub>(CO)<sub>6</sub>(PhC≡CPh)<sup>[26]</sup> and [Co<sub>2</sub>(CO)<sub>4</sub>(dppm)]<sub>2</sub>[PhC≡C–C≡CPh].<sup>[27]</sup> The steroidal moiety structure was taken from the crystallographic data of the estradiol-propanol adduct.<sup>[28]</sup> The fragments were combined according to **1** and **2**. After adjusting the new bond distances to normal values, the resulting structures were energy-minimized by rotating the steroidal fragment around the C(17)–C $\alpha$  bond (fixed at  $1.50 \text{ \AA}$ ). This procedure was repeated several times. Lastly, by allowing small oscillations of all atoms (except those in the organometallic moiety), the overall energy minimum was obtained. Although the approximation inherent to this procedure should be recognized (e.g. the organometallic fragment is considered as a frozen unit), this method provides a reasonable model of the overall molecular geometries of **1** and **2**. The molecular surfaces were obtained by simply considering the atomic radii of their

constituents (CPK surface). The resulting space-filling models of **1** and **2** (Figure 7) suggest that the large size of the diphosphane ligand buries the cobalt metals and therefore hinders any C(17)<sup>+</sup>–Co interaction, in the corresponding carbocation **2**<sup>+</sup>, that is responsible for carbocation stabilization.

## Experimental Section

**General Chemical Procedures and Materials:** All reactions were carried out under an atmosphere of dry nitrogen with standard Schlenk techniques. Solvents were distilled under nitrogen from appropriate drying agents. Published procedures were employed for the synthesis of 17 $\alpha$ -ethynylestradiol, **1** and **3**,<sup>[29]</sup> **4**–**6**,<sup>[10]</sup> and model complex **3a**.<sup>[30]</sup> Model complexes **1a**, **2a**, **4a**, and **5a** were synthesized by reaction of Co<sub>2</sub>(CO)<sub>8</sub>, Co<sub>2</sub>(CO)<sub>6</sub>(dppm),<sup>[31]</sup> or M<sub>3</sub>(CO)<sub>10</sub>(NCCH<sub>3</sub>)<sub>2</sub>,<sup>[32]</sup> (M = Ru for **7a** and Os for **8a**) with propargyl alcohol. Their purity was checked by IR, MS, and NMR spectroscopy. All elemental analyses are within  $\pm 0.5\%$  of the theoretical values. Solution infrared spectra were recorded on a Bruker Equinox 55 in matched air-free cells with NaCl windows. <sup>1</sup>H-, <sup>13</sup>C-, and <sup>31</sup>P-NMR spectra were acquired on a JEOL EX-400 spectrometer operating at 400 MHz for <sup>1</sup>H, 100.6 MHz for <sup>13</sup>C, and 161.9 MHz for <sup>31</sup>P. Chemical shifts are reported downfield, positive with respect to the reference standard SiMe<sub>4</sub> ( $\delta = 0$ ) using the residual H signals as internal standard (<sup>1</sup>H) or the characteristic resonances of the solvent nuclei (<sup>13</sup>C); for <sup>31</sup>P, 85% H<sub>3</sub>PO<sub>4</sub> was used as reference. <sup>31</sup>P and <sup>13</sup>C data are proton decoupled. DCI-MS spectra were recorded on a Finnigan-MAT 95Q instrument with 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. Positive ion spectra were collected. A computer program provides a digital readout of peak intensity and their comparison with the simulated isotopic pattern. The fit was excellent for all of the compounds under investigation.

**Synthesis of Co<sub>2</sub>(CO)<sub>4</sub>(dppm)(EC≡CH) (**2**):** Stoichiometric amounts of 17 $\alpha$ -ethynylestradiol and Co<sub>2</sub>(CO)<sub>6</sub>(dppm), prepared according to the literature procedure,<sup>[33]</sup> were heated at reflux in benzene for 2 h, under nitrogen. The hot reaction mixture was filtered over a short silica column to remove any insoluble impurities and eluted with ethyl acetate/cyclohexane (40:60). The filtrate was dried under vacuum, and the complex was then purified by recrystallization from hexane/CH<sub>2</sub>Cl<sub>2</sub> at  $-20 \text{ }^{\circ}\text{C}$ . Yields 60%. – IR (CH<sub>2</sub>Cl<sub>2</sub>):  $\tilde{\nu}_{\text{CO}} = 2022 \text{ s, } 1991 \text{ sh, } 1989 \text{ vs, } 1966 \text{ s cm}^{-1}$ . – <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta = 8.12 \text{ (s, 1 H, C3-OH), } 7.50\text{--}7.20 \text{ (m, 20 H, Ph), } 7.10 \text{ (s, 1 H, C1-H), } 6.62 \text{ (dd, 1 H, C2-H), } 6.56 \text{ (d, 1 H, C4-H), } 5.65 \text{ (t, } \equiv\text{C21-H, } ^3J_{\text{HP}} = 7.7 \text{ Hz), } 4.69 \text{ (s, C17-OH), } 3.62 \text{ and } 3.22 \text{ (each m, 1 H, P-CH}_2\text{-P)}^{[35]}, \text{ ca. } 3.0\text{--}1.3 \text{ (all the remaining resonances heavily overlapped), } 1.11 \text{ (s, 3 H, Me-18)}$ . – <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta = 204.42 \text{ (br, CO), } 153.25 \text{ (C3), } 138.34 \text{ (C5), } 136\text{--}128 \text{ (Ph), } 132.90 \text{ (C10), } 126.42 \text{ (C1), } 115.14 \text{ (C4), } 112.51 \text{ (C2); } 110.73 \text{ (C20), } 85.04 \text{ (C17), } 75.12 \text{ (C21), } 48.21 \text{ (C13), } 41.62 \text{ (t, P-CH}_2\text{-P, } J_{\text{CP}} = 20.2 \text{ Hz), } 42\text{--}20 \text{ (all the remaining resonances heavily overlapped); } 15.73 \text{ (Me-18)}$ . – <sup>31</sup>P NMR (CDCl<sub>3</sub>):  $\delta = 42.5 \text{ (s)}$ .

**Synthesis of HRu<sub>3</sub>(CO)<sub>9</sub>–(C≡C–CH<sub>2</sub>OH) (**6a**):** A solution of **6** in cyclohexane was heated at 75 °C under nitrogen for 45 min. The mixture was allowed to reach room temperature and purified by chromatography on a silica column. Cyclohexane first eluted some

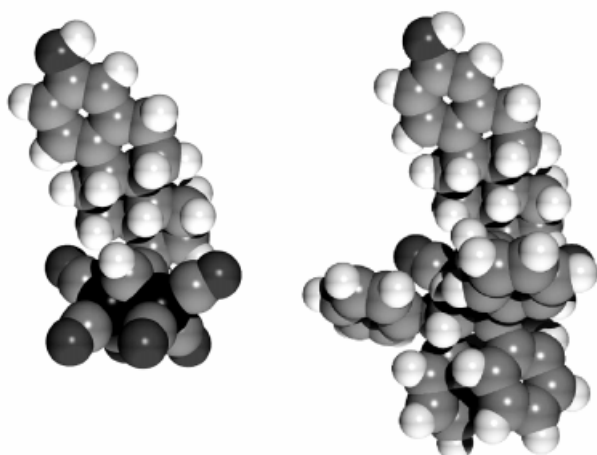


Figure 7. Space-filling models of **1** (left) and **2** (right). The picture was obtained by using molecular modeling program to graft the steroidal fragment onto the organometallic moiety

adventitious  $\text{Ru}_3(\text{CO})_{12}$ ; pure  $\text{CH}_2\text{Cl}_2$  then eluted the desired compound. Yield 50%. – IR ( $\text{CH}_2\text{Cl}_2$ ):  $\tilde{\nu}_{\text{CO}}$  = 2100 w, 2074 vs, 2054 vs, 2022 vs, 1989 m  $\text{cm}^{-1}$ . –  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  = 4.89 (d, 2 H,  $-\text{CH}_2-$ ,  $J_{\text{HH}}$  = 6.1 Hz), 2.07 (t, 1 H,  $-\text{OH}$ ),  $-21.03$  (s, 1 H, hydride). –  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  = 197.15 and 188.19 ( $\text{Ru}-\text{CO}$ ),<sup>[36]</sup> 160.58 ( $=\text{C}-\text{Ru}$ ), 102.64 ( $=\text{C}-\text{CH}_2$ ), 61.05 ( $\text{CH}_2$ ).

**pK<sub>R+</sub>**: Aliquots of a  $10^{-3}$  M solution of the compound under investigation in acetonitrile (50  $\mu\text{L}$ ) were added to 450  $\mu\text{L}$  of  $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$  solutions of increasing acid concentration. The wavelength of maximum absorption was sought by scanning the 190–600 nm range and the approximate concentration of  $\text{H}_2\text{SO}_4$  required to achieve 50% ionization of organometallic hormone determined by comparing the maximum UV/Vis absorbance for the different  $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$  solutions.

A minimum of four aqueous  $\text{H}_2\text{SO}_4$  solutions, whose concentrations were around the roughly estimated value, were prepared. Thirty minutes after the initial injection of acetonitrile solution into the acid solutions, a spectroscopic determination of the ratio  $[\text{R}^+]/[\text{ROH}]$  was performed. The final pK<sub>R+</sub> value was calculated by use the following Equation (Equation 3):

$$\text{pK}_{\text{R}^+} = \text{C}_0 + \log \frac{[\text{R}^+]}{[\text{ROH}]} \quad (3)$$

where  $\text{C}_0$  is the Deno acidity function for  $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$  solutions.<sup>[34]</sup>

**Biochemical Materials and Methods:** Estradiol, 17 $\alpha$ -ethynylestradiol, charcoal (Norit A), and protamine sulfate (from salmon, grade X) were from Sigma. [6,7- $^3\text{H}$ ]Estradiol (specific activity 70 Ci/mmol) was purchased from C.E.A. (Saclay, France).

**Preparation of Lamb Uterine Cytosol and Binding Assays:** Lamb uteri, used as a source of estrogen receptor, were obtained from the slaughterhouse of Mantes-la-Jolie, France and kept frozen in liquid nitrogen until used. The lamb uteri were thawed, and the minced tissues homogenized with an Ultra-Turrax in buffer A (0.05 M Tris-HCl, sucrose 0.25 M, 0.1% 3 $\beta$ -mercaptoethanol, pH 7.4). The homogenate was centrifuged at 40,000 r.p.m. for 35 min in the TFT 65-13 rotor of a Kontron T-1160 ultracentrifuge. Aliquots (200  $\mu\text{L}$ ) of the supernatant (usually called cytosol) were incubated for 3 h at 0 °C with [ $^3\text{H}$ ]estradiol ( $6 \cdot 10^{-9}$  M) in presence of nine different concentrations (ranging from  $1 \cdot 10^{-8}$  M to  $1 \cdot 10^{-6}$  M) of the compound under investigation, acting as the competitor. An equivalent incubation, containing the same aliquots of cytosol and the same concentration of [ $^3\text{H}$ ]estradiol, was prepared with nine different concentrations (ranging from  $1 \cdot 10^{-8}$  M to  $1 \cdot 10^{-6}$  M) of non-radioactive estradiol as the competitor. A blank (without competitors) was added to the incubation set to evaluate the maximum receptor binding capacity. At the end of the incubation, separation of the bound fractions of the steroids was achieved by using protamine sulfate precipitation.<sup>[17]</sup> The bound fractions were collected on glass fiber paper (Whatman GF/C) under a light vacuum and washed extensively with phosphate buffer (pH = 7.2). The filters were then transferred into a vial containing 10 mL of scintillation fluid (A.C.S., Amersham) and the radioactivity was measured in an LKB-1211 Rack-Beta scintillation counter. The percentage reduction in binding of [ $^3\text{H}$ ]estradiol (Y) was calculated using the logit transformation of Y, where  $\text{logit } Y = \ln(Y/1 - Y)$ , vs. the log of the mass of competing steroid. The concentration of competitor required to displace 50% of the bound [ $^3\text{H}$ ]estradiol was calculated for the compound under investigation ( $\text{C}_{50}$ ) and for non-radioactive estradiol ( $\text{C}_{50\text{e}}$ ); the result was expressed as the relative binding affinity [ $\text{RBA} = (\text{C}_{50\text{e}}/\text{C}_{50}) \times 100$ ].

**Receptor Inactivation Assays:** Aliquots ( $3 \times 200$   $\mu\text{L}$ ) of lamb uterine cytosol were first incubated for 2 h at 0 °C in the presence of  $1 \cdot 10^{-8}$  M of compound under investigation (estradiol, 17 $\alpha$ -ethynylestradiol, 17 $\alpha$ -propynylestradiol, or **1–6**), then [ $^3\text{H}$ ]estradiol (final concentration  $2.65 \cdot 10^{-9}$  M) was added to each fraction, which was again incubated for 1 h at 25 °C. At the end of this second incubation, the fraction of [ $^3\text{H}$ ]estradiol specifically bound to its receptor was measured by protamine sulfate precipitation (vide supra). This value corresponds to the fraction of the estradiol receptor reversibly bound to the organometallic hormone in the first incubation. This value is compared to that of a control, i.e. aliquots of uterine cytosol ( $3 \times 200$   $\mu\text{L}$ ) incubated under the same conditions of time and temperature, but in the presence of tritiated estradiol only.

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