The use of high affinity binding bioligands modified by transition metal carbonyl moieties

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<u>Abstract</u> : The effect of coordinated organometallic fragments such as $Cr(CO)_3$, $Co_2(CO)_6$, $Mo_2Cp_2(CO)_4$ on the binding affinities of certain steroids with their receptors has been examined. In some instances, the biochemical properties of the modified steroids were not significantly affected by the presence of the organometallic labels, which may be detected to the level of a few hundred femtomoles per milligram of protein by Fourier transform infrared techniques (FT-IR).This approach may provide an alternative method to radiochemical procedures for bioassays. In addition, some 17α-modified organometallic steroid derivatives behave as highly effective inactivators of estrogen receptor. This new concept for affinity markers of hormones receptors augurs well for future new developments in bioorganometallic chemistry.

INTRODUCTION

Transition metal reagents offer new avenues in natural product synthesis owing to the remarkable template effects of these species wich have been particularly useful to solve challenging selectivity problems (ref. 1). In this regard, the selective construction of naturally occuring carbocycles, such as steroids, has become a veritable training ground to test the synthetic utility of novel concepts (ref. 2). The concept of selectivity has a direct analogy in biochemistry viz., molecular recognition. In this area, the three major biochemical systems of high specificity of interactions between a ligand and its binding site are : hormone-receptor, antigen-antibody and enzyme-substrate. The idea of targeting a reagent by attaching it to a carrier which will be recognized selectively by the target site may be applied to transition metal complexes provided that the problem of the association of the organometallic-modified bioligand with its binding protein is solved. Parallel to the organic synthesis situation, steroidal hormones also appear to be good candidates for transition-metal labeling with a view to applications in the field of biochemistry, because of the availability of potential sites for complexation. A great deal of work is being done currently on these substrates (ref. 3).

We show in this report how the molecular recognition properties of organometallic labeled hormonal steroids may be exploited as a potential alternative to radiolabeled ligands and as active site directed reagents for steroid protein receptors.
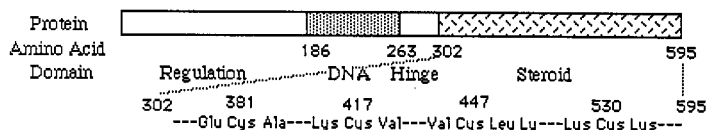
STEROID RECEPTORS

It has been stated that, inside target cells, steroids combine with a cytosolic or nuclear receptor protein which, after activation, binds to nuclear chromatin and elicits a biological response (scheme1)(ref. 4)

Our knowledge of the ligand binding unit has been greatly enriched by recent papers (ref. 5,6).The DNA sequencing data have been used to predict amino acid composition of different receptors (ref. 7-9). At least four domains can be identified in the proteins and there are extensive homologies between the different classes of receptors. The steroid binding domain is at the carboxy terminal end of the protein with the DNA binding region beeing nearer to the amino terminus (Scheme 2).

The sizes of these two domains are relatively similar for the different receptors, the main difference in receptor sizes beeing due to a third amino terminal domain of ill-defined function. The steroid binding domain is hydrophobic in contrast to the hydrophilic nature of the DNA binding region which also contains a high concentration of cysteine residues. The homology of the latter domain between the
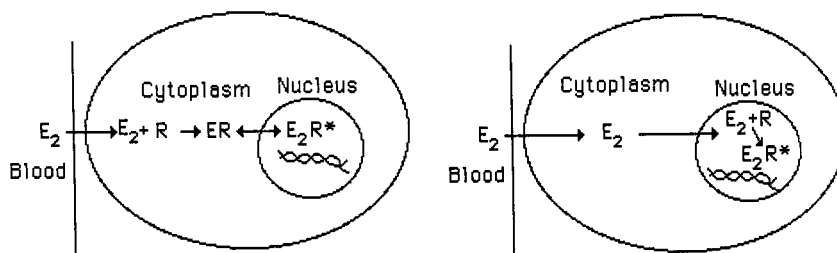
Scheme 1. Intracellular localization of steroid receptors. Steroid (E_2 for example) combines with its receptor (R) as a result of which the "activated receptor" (R*) is able to bind to DNA. In the left-hand model, the initial interaction occurs in the cytoplasm whilst a nuclear locus is indicated in the right-hand model. (ref. 4).

Scheme 2. Prediction of amino-acid composition of estradiol receptor by DNA sequencing data (ref. 5,7).

different receptors is remarkably high while the steroid binding region is moderately conserved. For human estradiol receptor (scheme 2) we note only four cysteins in the hormone binding domain, namely at positions 381,417,447 and 530. It is noticeable that cysteins 417 and 530 are flanked by lysines 416, 529, 531 while cystein 447 is very close to lysine 449. However we should be aware that the tertiary structure of the protein remains unknown.

BINDING AFFINITY OF TRANSITION METAL CARBONYL HORMONES TO ESTRADIOL AND PROGESTERONE RECEPTORS

Current biochemical steroid hormone receptor assays, particularly assays for estradiol and progesterone receptors, are important in therapy (ref. 10,11) but remain expensive, are difficult to perform and require radiolabeled ligands (ref. 12). An alternative receptor assay would ideally involve : a sensitivity comparable to that of radioisotopic detection methods, together with a low cost; a marker with an indefinite shelf-life exempt from any legal or licensing restrictions . We have explored metal carbonyl complexes as markers to fulfill these criteria.

A series of organometal-labeled estradiol derivatives were synthesized and tested to determine their ability to bind estradiol receptor (ref. 13). As a crucial part of this work, the 3-O-(3-hydroxypropyl)-17β-estradiol chromium tricarbonyl complex 1 (scheme 3) was prepared in a tritiated form with a high specific activity (4.58 Ci/mol) (ref. 14).

This allowed us to monitor directly, for the first time, the interaction of such an organometallic hormone with the estradiol receptor. The modification of the 3-hydroxy function greatly increases the stability of the compound; the solid complexes are fairly stable and even in solution in the dark. The stereochemistry of compound 1 was established by a combination of synthetic and spectroscopic correlations based on known X-Ray structures (ref. 15). Compound 1 with a high affinity value (RBA = 28% with respect to estradiol) is an excellent choice for further study on the basis of recognition criteria (ref. 16).

Fig. 1 illustrates the saturation curve obtained after incubation of lamb uterine cytosol in the presence of increasing amonts of [³H]-1 (ref. 17). [³H]-1 binds to the estradiol receptor in a saturable and reversible manner. The level of non-specific binding is low and the hormone is not bound significantly to other non-target tissues.

The saturation curves data are presented in the form of a Scatchard plot in Fig. 2. The organometallic hormone binds exclusively to the high-affinity binding sites of estradiol. The actual binding affinity of[³H]-1 is very similar to that found for estradiol itself (K_d [³H]-E_2 = 0.67 nM; K_d [³H]-1 = 0.85 nM) and the number of binding sites for [³H]-1 is only slightly less (80%) than that for E_2.

The complexation site of the organometallic tripod on the A ring of the steroid strongly discriminates between the α and β-diastereomers with respect to their recognition properties (2, RBA = 1.75%; scheme 3).It should be emphasized that the non-equivalence of the two sides of steroidal A ring would not have been readily

Scheme 3

1, R.B.A. = 28%   2, R.B.A. = 1.75%
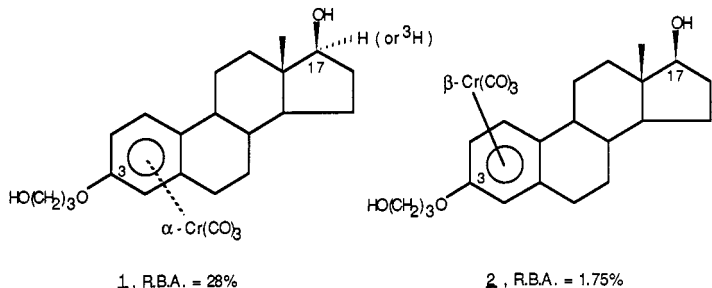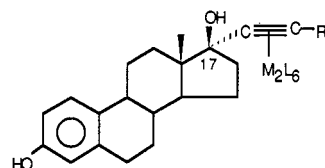
Scheme 4

	R	M_2L_6	RBA (%)
3	H	-	70
4	CH_3	-	44
5	H	$Co_2(CO)_6$	5
6	CH_3	$Co_2(CO)_6$	12
7	H	$Mo_2Cp_2(CO)_4$	13
8	CH_3	$Mo_2Cp_2(CO)_4$	16

Scheme 3. $Cr(CO)_3$ complexation generates a pair of diastereomers readily separated by chromatography 1 shows a high receptor binding affinity (R.B.A. = 28%). [^{3}H]-1 was prepared with a specific activity of 4.58 Ci/mmole.

Scheme 4. Structure and Relative Binding Affinities (RBA) of estradiol hormones modified at the 17α-position by transition metal cluster fragments, such as $Co_2(CO)_6$ and $Mo_2Cp_2(CO)_4$ (Cp = η^5-C_5H_5).

appreciated without the ability of the organometallic label to bond above and below the A ring of the estradiol derivatives.

The photosensitivity of the organochromium steroids might make them difficult to use in routine studies. Therefore, we have synthesized polymetallic steroid complexes, such as those shown in scheme 4 (ref. 18), which are reasonably stable in air, light and solution even for the long period of time necessary for biochemical incubations. We chose Cobalt and Molybdenum carbonyl cluster modifications at the 17α-position because of the known tolerance of the receptor sites to steroidal alteration in this position (ref. 19). The organometallic complexes 5,6,7,8 were incubated in vitro with lamb uterine cytosol and the RBA values for these species were quite satisfactory (scheme 4) with respect to estradiol taken as 100%.
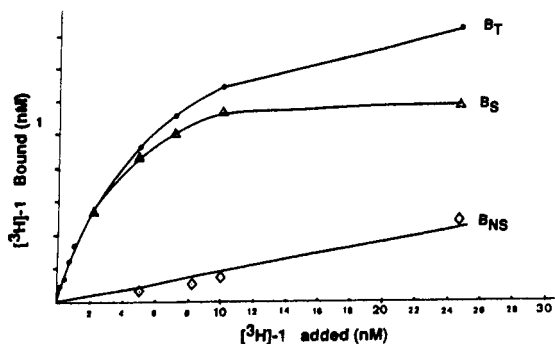

Figure 1 Interaction of [^{3}H]-1 with uterine cytosol estrogen in vitro. Lamb uterine cytosol was incubated with increasing concentrations of [^{3}H]-1 alone or together with a 100 fold excess of unlabeled DES. Binding equilibrium was achieved by incubating the samples approximately 3 h at 4°C. Bound [^{3}H]-1 steroid was measured by protamine sulfate assay (B_T, total binding; B_S, specific binding; B_{NS}, nonspecific binding).
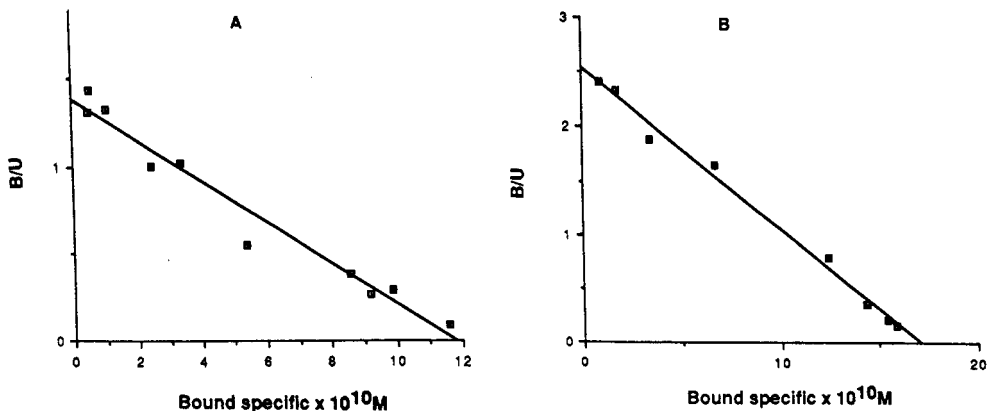

Figure 2 Scatchard analysis of the estrogen receptor from lamb uterus. Binding of [^{3}H]-1 (Panel A) and [^{3}H]-E_2 (Panel B) was measured after incubation of cytosol proteins with increasing concentrations of either [^{3}H]-1 or [^{3}H]-E_2 alone or together with a 100 fold excess of unlabelled DES.
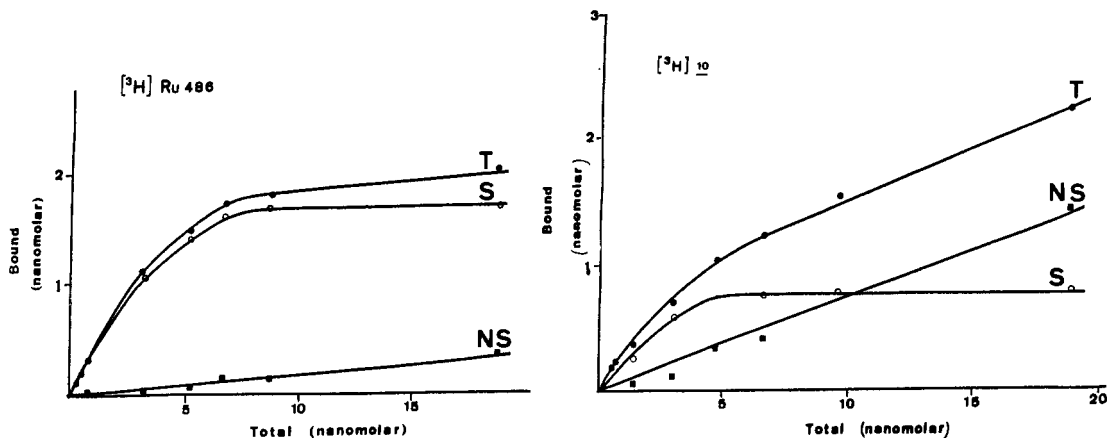
Figure 3. Interaction of [3H]-RU 486 and [3H]-10 with uterine cytosol progestin receptor. Cytosol from estrogen-primed female immature rabbits uteri was incubated with increasing concentrations of [3H]-RU 486 or [3H]-10. Nonspecific binding (NS) was determined in parallel incubations containing a 500-fold excess of cold RU 486. Key (S) specific binding; (T) total binding.

Similar organometallic modifications may be performed with mifepristone (9, RU 486, Scheme 5) a promising new synthetic antiprogesterone (ref. 20). The recognition properties of the receptor for the modified hormone are preserved although complexation brings about a decrease of the RBA values.

	R	M_2L_6	RBA (%) PR	RBA (%) GR
9	-	-	389	251
10	-	$Co_2(CO)_6$	13	17
11	-	$Mo_2Cp_2(Co)_4$	10	8
12	$Cr(CO)_3$	-	20	29
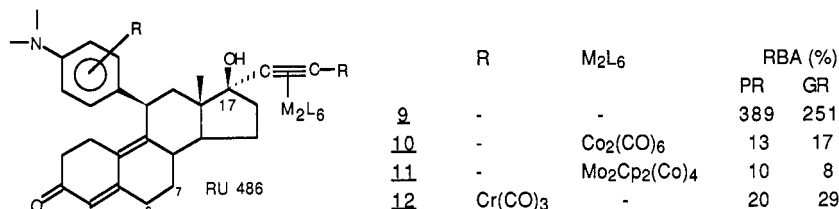
Scheme 5. Relative Binding Affinities (RBA) of RU 486 and RU 486 complexes for the cytosol progestin (PR) and glucocorticoid receptors (GR) (ref. 20).

In order to prove that the transition metal labeled hormone is specifically bound at the progesterone receptor site, we prepared compound 10, the easiest to obtain, tritium labeled at the 6,7 positions (specific activiy : 4.8 Ci/mmole) and performed in vitro biochemical experiments with progesterone uterus receptor.

Figure 3 shows the saturation curves obtained after incubation of rabbit uterus receptor in the presence of increasing amounts of [3H]-10 and [3H]-9. This experiment proves that both [3H]-9 and the organometallic-labeled hormone [3H]-10 bind to the progestin receptor. This binding is saturable and reversible. At saturation concentration (4 X 10-9M), the level of specific binding for [3H]-10 was twice that of non specific binding. Thus complex 10 was found to be suited for progesterone receptor detection.

TRANSITION METAL CARBONYL STEROIDS AS NOVEL INFRARED MARKERS FOR HORMONE RECEPTOR SITE DETECTION

Having identified 1 or 10 as a very suitable probe molecule, the next step was to find a suitably-sensitive analytical technique with which we could monitor the very low concentration (a few femtomoles per milligram of protein) of the organometallic label expected to be complexed to the receptor. There are several well-established procedures to detect Chromium, Molybdenum or Cobalt, e.g., neutron activation and atomic absorption, but these proved to be unsuitable for our purposes. Metal carbonyls, however, exhibit extremely intense, characteristic absorptions in their infrared spectra at about 2000 cm-1 for terminal metal-CO linkages. These absorptions fall into a window between the absorptions of most organic molecules, including those of proteins. Moreover, because of the increased sensitivity and multiscannig capability of Fourier transform infrared (FT-IR) spectrometers, we anticipated that it might be possible to use FT-IR spectroscopy to detect the presence of the $Cr(CO)_3$ labels in the receptor. This has indeed proved to be true.

The FT-IR spectrum of a typical pressed pellet of the uterine cytosol proteins following incubation with 10-8 M of compound 1 is shown in fig.4. The protein absorptions are well off-scale, yet the metal-carbonyl region is devoid to any features except for two small peaks. An expansion of this region is illustrated in Fig. 5. The two

small peaks detected at 1957 and 1880 cm^{-1} correspond reasonably to the v(CO) mode of compound **1**. The reversibility of the binding between compound [3H]-**1** and the receptor was demonstrated by FT-IR spectroscopy. The IR spectrum of a sample obtained subsequent to the competitive binding experiment with excess DES was recorded. The v(CO) region of this spectrum (Fig. 6) reveals the absence of any peaks due to the organometallic-labeled receptor complex confirming the binding reversibility.

Figure 7 depicts the v(CO) region of the difference spectrum obtained by subtraction of the spectrum of the precipitated protein from the in vitro incubation of compound **8** (10^{-8} M) with lamb uterine cytosol from that obtained under identical conditions except in the presence of diethylstilbestrol (DES) as well in order to eliminate the non-specific binding. The necessary subtraction scaling factor was determined from the subtraction of the v(OH) harmonic at 6700 cm^{-1} which was used as the reference peak. These peaks provide for the first time a direct measure of the specific binding of an organometallic steroid (complex **8**) to the estradiol receptor sites, the concentration of which is expected to be 300 fmol/mg protein.
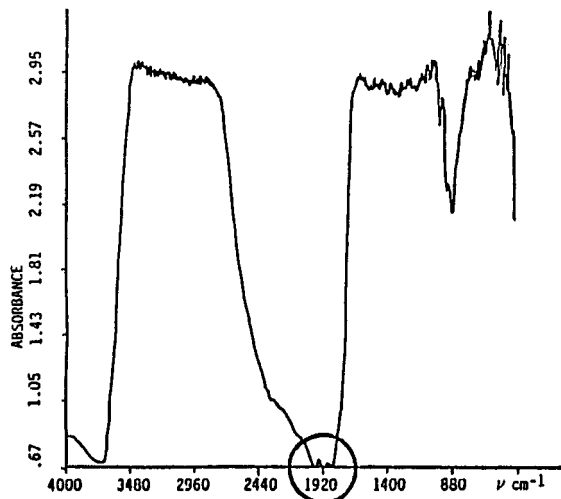

Fig.4. FT-IR spectrum of lamb uterine cytosol following incubation with [3H]-**1** and subsequent precipitation from solution with protamine sulfate (off scale, MCT detector).
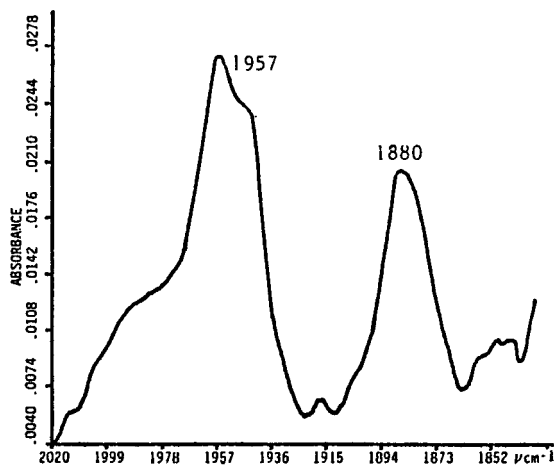

Fig. 5. Expansion of the v(CO) region of the spectrum shown in Fig. 4 and base line correction.
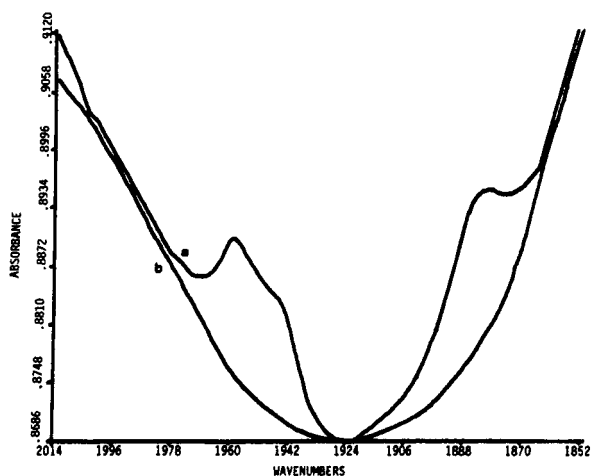

Fig. 6. FT-IR spectrum (26,000 scans, 4 cm^{-1} resolution) of the metal carbonyl area of lamb uterine cytosol incubated with a) 10^{-8}M of **1** and b) 10^{-8}M of **1** plus a 500 fold excess of DES.
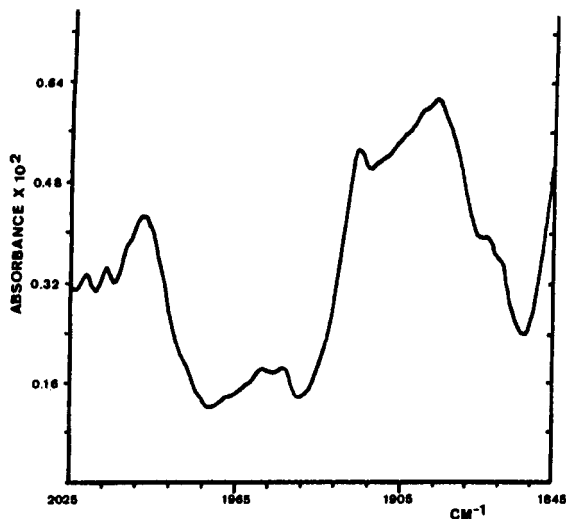

Fig. 7 FT-IR difference spectrum v(CO) region obtained by subtracting the spectrum of precipitated protein, following incubation with **8** (10^{-8}M) in the presence of DES (10^{-3}M) from that obtained after incubation with **8** (10^{-8}M)
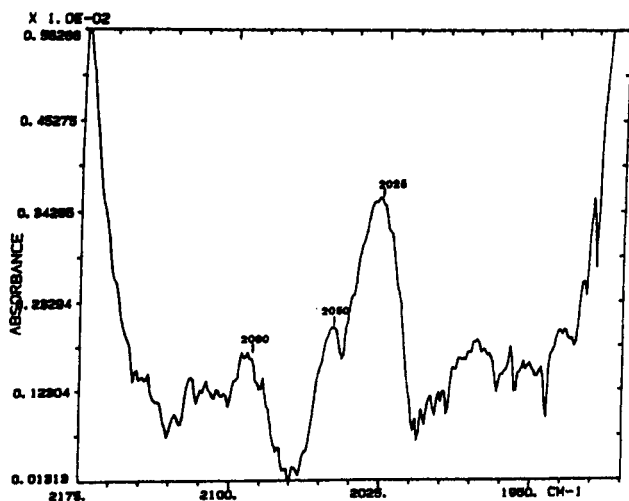
Fig. 8. FT-IR difference spectrum ν(CO) region obtained by subtracting the spectrum of precipitated protein, following incubation with 10 (10⁻⁸M) in the presence of a 500 fold unlabeled 9 from that obtained after incubation with 10 (10⁻⁸M).
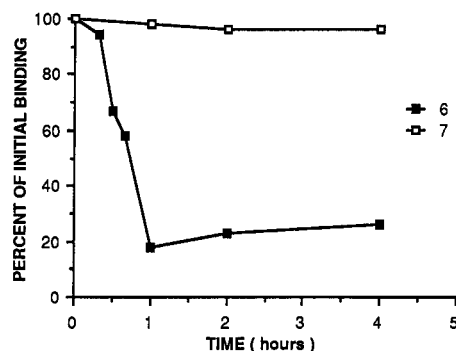

Figure 9. Rate of inactivation of the estrogen receptor in presence of 6 an 7.

Similar difference spectrum technique may be used with compound 10 to detect progesterone receptor. Figure 8 shows clearly two of the characteristic bands of the carbonyl moiety of the cluster at 2025 and 2050 cm⁻¹, while the third and least intense signal at 2090 cm⁻¹ is not clearly discerned above the background noise. The amount of organometallic cluster detected here is in the range of 1 pmol but the results remain qualitative for the present. In order to achieve the necessary sensitivity to observe the relatively weak signals, we employed an InSb detector, which is the most sensitive detector in the 2100-1900 cm⁻¹ region available commercially.

PROJECTIONS FOR SOME FUTURE APPLICATIONS OF BIO-ORGANOMETALLIC CHEMISTRY

Prospectives for this research would be the exploitation of the molecular recognition properties of the organometallic-labeled hormonal steroids, coupled with the chemical specificity associated with certain organometallic fragments (e.g., in the stabilisation of α-carbenium ions), in the design of affinity markers.

The affinty-labeling approach, whereby reversibility binding ligands are elaborated into chemically reactive derivatives capable of covalently labeling binding proteins such as steroid receptors, is a challenging but promising methodology to bring valuable information about the action of hormones at the molecular level (ref. 21-23).

We have seen above that compounds 6, 7 (scheme 4) bind estradiol receptor with apparent relative binding affinities of about 15%. They each possess, upon complexation, a particularly labile 17β-OH function, even in weakly acidic medium, since the resulting carbenium ion in an adjacent position to an organometallic system shows enhanced stability with respect to the free ligand (ref. 24, 25).

The irreversible nature of the interaction of the propynyl estradiol Cobalt hexacarbonyl 6 with receptor was demonstrated by incubating, at pH 7.2 and 25°C, estrogen receptor from lamb uterine cytosol with 10 nM of 6 or 7 and periodically assaying the remaining estrogen binding sites with [3H]-estradiol as shown in Fig.9. One can notice immediately that there is a reversible binding for the Molybdenum complex 7, while for the Cobalt complex 6, the binding is irreversible. In the presence of 6, the labeling is rapid and efficient (about 82% within 1 h. incubation). We have checked that this irreversible action of 6, can be prevented by pre-incubation in presence of 30 nM of estradiol.

These preliminary experiments suggest that the 17β-OH function, modified by complexation in the adjacent position, is selectively activated in the association site of estrogen receptor, presumably by accepting a proton to generate carbenium ion-like reactive species. A nucleophile should be ideally located very close to this protonating group to react instantly with the transient electrophile thus avoiding the formation of the elimination product, as probably occurs with less acidic electrophiles.

As pointed out above, the complete complementary DNA of the estrogen receptor has been cloned and sequenced (ref. 5, 7). From the sequence analysis, several cysteins positions flanked by lysines are good candidates to function with our organometallic since an acidic and nucleophilic group are then neighbours.

The fact that there is a good reason to suspect the presence of the -SH and -NH_2 groups in the steroid binding cavity was established as follows (A. Vessières et al. unpublished results). While the acidic groups usually encountered in natural products such as carboxylic acids and phenols lead, when reacting with 6, to the normal elimination product, Ethane-thiol shows a different behavior. This relatively acidic -SH group forms an electrophilic transient adduct which is not very reactive with alcohols and reasonably reactive with water giving rise to a mixture of products, namely the starting material and the elimination product. It reacts instantly with amines (e.g., butylamine) to form a stable covalent bond in the β-position. It is noticeable that it is possible to prevent in this way a rapid elimination reaction with the tertiary alcohol 6 but not with the hormone 7 which leads to elimination product with the thiol. We should note also the huge difference between the pk_{R+} values of these systems (pk_{R+} = -6.5 for $(Co_2(CO)_6)(H-C \equiv C-C-CH_2^+)$ and pk_{R+} = +3 for $(Mo_2Cp_2(CO)_4)(H-C \equiv C-C-CH_2^+)$ (M. Gruselle et al. unpublished results), in agreement with the observed reactivities.

A tentative sketch of a possible process for this new type of affinity marker is outlined in scheme 6.
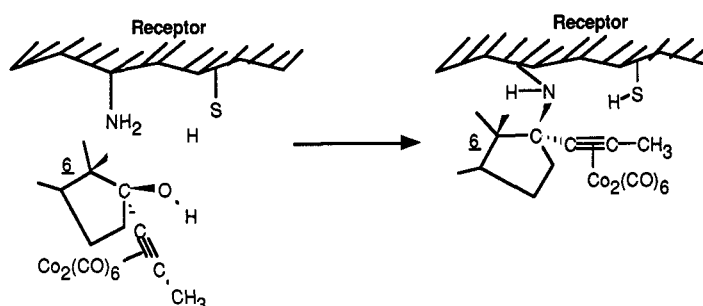
Scheme 6. Tentative mechanism for the organometallic affinity marker.

The question is now to prove this mechanism, to study the generalization of the concept, and to find the exact binding site of the receptor with the 17β position of estradiol. But, even at this early stage, this approach provides a new fascinating area for transition-metal chemistry.

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