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Application of Organotransition Metal Carbonyl Complexes as Infrared Markers for Hormonal Steroids in Biological Processes

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Application of Organotransition Metal Carbonyl Complexes as Infrared Markers for Hormonal Steroids in Biological Processes

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INTRODUCTION

The explosive development over the past 25 years in the use of organotransition metal complexes as reagents in organic synthesis is due chiefly to the fact that it is possible to coordinate a metal-containing fragment temporarily to an organic moiety so that the chemical reactivity of the organic moiety is modified sufficiently for reactions to occur which previously were difficult or impossible to achieve by classical routes.[1] There are now several processes based on the intermediacy of organometallic species that have

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become industrially important in such diverse areas as biology, agricultural chemisty, and pharmacology.[2] For example, optically active organorhodium(I) complexes catalyze the selective hydrogenation of prochiral N-acylaminocinnamic acids in the Monsanto production of *l*-DOPA, an important drug widely used in the treatment of Parkinson's disease.[3] Also, useful ceramic materials have recently been produced by the thermal decomposition of organometallic precursors.[4] Organometallic chemistry is therefore expected to provide the chemical industry with many future challenges.

Underlying the use of organometallics in organic synthesis is the concept of selectivity, which is directly analogous to molecular recognition in biochemistry. Any foray of organometallic chemistry into the biochemical field will certainly encounter the problem of association of a modified bioligand with its target binding protein. In biochemical systems, there are essentially three important high-affinity association processes: bioligand–receptor, antigen–antibody, and enzyme–substrate. Several years ago, we decided to undertake a broad study of the application of organotransition metal complexes in hormonal steroid–protein receptor chemistry. This is an extremely active area of research by drug companies throughout the world. Out initial aim was to investigate the effect of coordinating organometallic fragments on the binding affinities of certain hormonal steroids for their protein receptors. If we could demonstrate that the steroid biochemistry was not significantly perturbed by the presence of an organometallic label, we could then proceed to develop mon-radioisotopic methods for the detection of these labels at physiological concentrations. The motive behind developing non-radioisotopic immunoassay methods rather than using existing radiochemical technology was that there has been an increasing effort to find procedures free from legal and licensing constraints, high costs, radiolytic decomposition problems, limited choice of isotopic labels, etc. Among the techniques that have been examined over the past 15 years with this in mind are electron spin resonance spectroscopy,[5] fluorimetry,[6] electrochemistry,[7] and atomic absorption.[8]

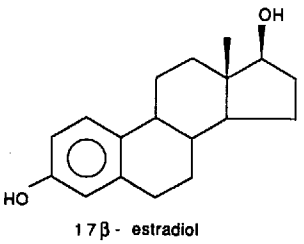
If we were successful in developing new protocols for the detection of organometallic moieties attached to hormonal steroids, we planned next to exploit the molecular recognition properties

of such labels, together with the chemical specificity associated with certain of them (e.g., in the stabilization of α-carbenium ions), in the design of affinity markers which will bind covalently to active sites in protein receptors. The purpose of this Comment is to summarize our research in this new field of bioorganometallic chemistry.

ORGANOMETALLIC HORMONAL STEROIDS

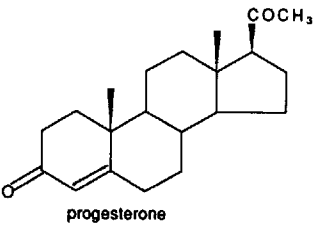
The term "metalloimmunoassay" was originally coined by Cais in 1977,[8] by analogy with the well-established procedure of radioimmunoassay. His research group synthesized a wide range of mercury-, platinum-, and cobalt-labelled estrogens and proteins, prior to doing some metalloimmunoassay work. Apparently, however, they did not do any actual receptor binding studies.

About 10% of all Western women will develop breast cancer sometime during their lives and there is a worldwide search to find new and more reliable methods for early diagnosis of the disease. Breast cancer is a hormone-dependent cancer and the hormones concerned are 17β-estradiol (**1**) and progesterone (**2**).



17β - estradiol

1

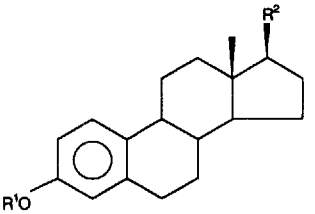


progesterone

2

Determination of the number of hormone receptor sites in the protein-containing tissue from a biopsy is crucial in the prognosis of the disease.[9] For this reason, we selected 17β-estradiol (**1**) and some other arene hormonal steroids for our initial labelling attempts using the metal carbonyl fragments $M(CO)_3$ (M = Cr, Mo, W). This was an area in which we already had considerable experience from both the organic and organometallic chemistry points

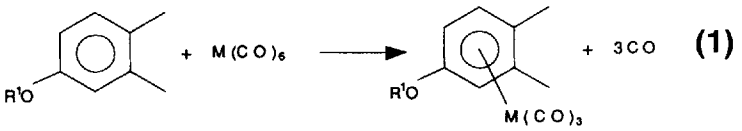
of view.[10,11] In particular, we knew that the arene steroids **1**, **3**, and **4** react with group VIB metal hexacarbonyls, $M(CO)_6$, to produce metal tricarbonyl complexes in which the $M(CO)_3$ moieties are coordinated to the arene A rings of the steroids (Eq. (1))[12]:



1 $R^1 = H$; $R^2 =$ 

3 $R^1 = CH_3$; $R^2 = =O$

4 $R^1 = H$; $R^2 = =O$



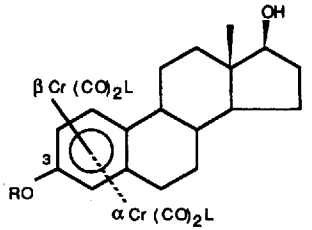
(1)

M = Cr, Mo, W

This first investigation revealed that the chromium complexes were significantly more stable than the analogous molybdenum and tungsten complexes, both in solution and in the solid state. It soon became clear, however, that even the chromium complexes would not be sufficiently stable for the proposed long-term biochemical studies in aqueous-methanol solution unless the 3-OH group was protected. We would therefore have to chemically modify the steroids in some way so as to increase the stability of the resulting organometallic hormones in aqueous-methanol solution while, at the same time, managing to produce organometallic derivatives with reasonable relative binding affinities (RBA)[14] compared to those of the free steroids themselves. A great deal of synthetic research was then undertaken, particularly for 17β-es-

TABLE I

Characteristic data of the estradiol derivatives[15]



Compound	Melting Point °C	$[\alpha]D^{22}$	RBA (%)
1 R = H; 17β-estradiol	173	+76	100
5 R = $Si(CH_3)_2tBu$	158	+58.5	11
6 R = $Si(CH_3)_2tBu/\alpha Cr(CO)_3$	220	+38.7	1.1
7 R = $Si(CH_3)_2tBu/\beta Cr(CO)_3$	179	+62.5	0.4
8 R = $Si(CH_3)_2tBu/\alpha Cr(CO)_2CS$	142	+20.8	1.5
9 R = $HO(CH_2)_3$	168	+70.6	37
10 R = $HO(CH_2)_3/\alpha Cr(CO)_3$	130	+41.7	28
11 R = $HO(CH_2)_3/\beta Cr(CO)_3$	157	+70	1.8

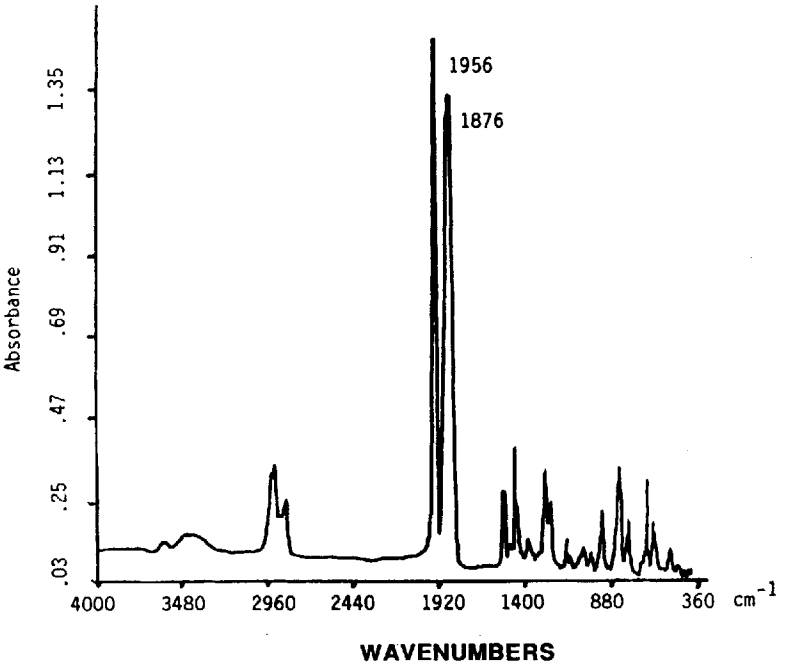
tradiol (**1**), in which the substitutent at the 3-position was changed (Table I).[15]

On the basis of the receptor binding affinity studies, we thought that steroid **9** would be the most suitable for our planned biochemical investigation. This steroid has an RBA value of 37% compared to the 100% value for 17β-estradiol (**1**). Reaction of steroid **9** with $Cr(CO)_6$ yields two diasteromers, **10** (α) and **11**(β), which can be readily separated by thin-layer chromatography and have RBA values of 28% and 1.8%, respectively. Consequently, we selected the α-diastereomer, **10**, as the probe molecule for our hormone receptor investigation.

The next step was to find a sensitive enough analytical technique which would enable us to monitor the very low concentrations (a few femtomoles per milligram of protein, ca. 10^{-15} M) of the organochromium label expected to be complexed to the receptor. Neutron diffraction and atomic absorption are well-established procedures to detect chromium, but in both cases the detection limits were not sufficient for our purposes.

Transition-metal carbonyls exhibit extremely intense absorptions in their infrared (IR) spectra in the 2150–1900 cm^{-1} region due to terminal metal–CO linkages and somewhat lower (ca. 1800 cm^{-1}) for CO groups bridging two or more metals. These IR absorptions fall into a window between the absorptions of most organic molecules, including those of proteins, e.g., ν(NH), ν(OH), and ν(CH): 3600–2800 cm^{-1} and ν(C=O): ~1650 cm^{-1}. In addition, because of the increased sensitivity and multiscanning capability of modern Fourier transform infrared (FT-IR) spectrometers, we anticipated that it might be possible to use FT-IR spectroscopy to detect the presence of the $Cr(CO)_3$ group of compound **10** in the hormone receptor at physiological concentrations.

The FT-IR spectra in the ν(CO) region of compound **10** and that of the precipitated protein (from lamb uterine cytosol) following incubation with **10** are shown in Figs. 1(a) and 1(b), respectively. The two peaks expected for the a_1 and e ν(CO) modes



(a)

of the $Cr(CO)_3$ fragment are clearly visible in Fig. 1(b). In addition, when similar experiments were performed using steroid **8** containing a $Cr(CO)_2(CS)$ moiety rather than a $Cr(CO)_3$ one, the shifts in the ν(CO) peaks expected upon replacement of a CO group by the more electron-withdrawing CS group were observed.[11] Final proof of the origin of the two ν(CO) peaks came from performing the same biochemical experiments using complex **10***, which was tritium-labelled in the D ring at the 17α-position.[16] This tritium-labelled species was prepared by reduction of the 17-keto group in estrone **5** by tritiated sodium borohydride $Na[B(^3H)_4]$[16]; attempts to react the commercially available tritium-labelled estradiol (at position 6 and 7) with $Cr(CO)_6$ only resulted in complete decomposition. Next, we proceeded to measure both the tritium radioactivity and the FT-IR spectrum of the precipitated protein

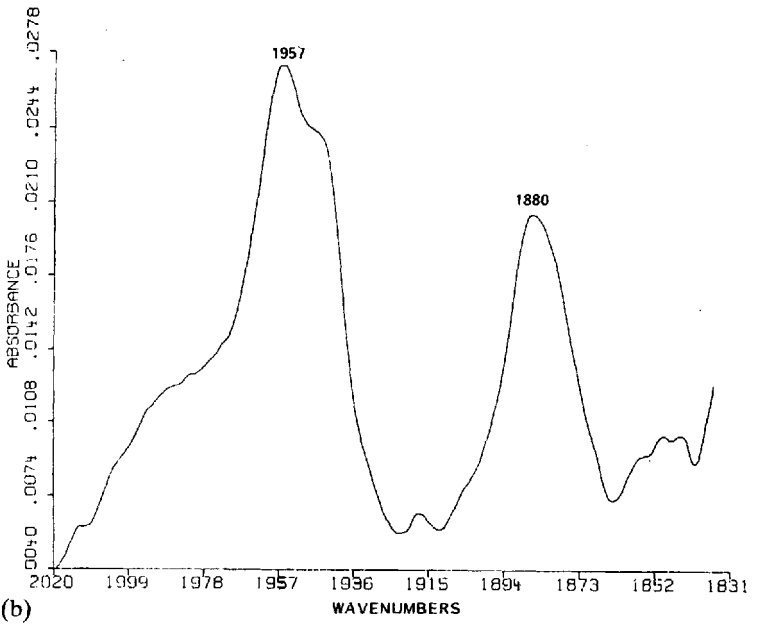


FIGURE 1 (a) FT-IR spectrum of compound **10** in the ν(CO) (CsI 3-mm minipellet). (b) ν(CO) region of the FT-IR spectrum of lamb uterine cytosol following incubation with **10** and subsequent precipitation with protamine sulfate (off-scale, 3-mm minipellet of solid material). Reproduced with permission from Ref. 15(a).

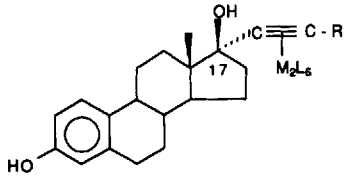
FIGURE 2 FT-IR spectrum (26,000 scans, 4 cm^{-1} resolution; 3-mm minipellet of solid material) of the ν(CO) region of lamb uterine cytosol following incubation with (a) 1×10^{-8} M of **10** and (b) 1×10^{-8} M of **10** plus a 100-fold excess of diethylstilbestrol (DES). Reproduced with permission from Ref. 15(b).

containing the organometallic receptor marker. We were able to show that the same ν(CO) peaks were observed as for **10** (Fig. 2) and that the detection limit of the FT-IR technique is comparable to that for the radiochemical method. Despite this success, however, we discovered shortly afterwards that compound **10** was still not ideally suitable for our purposes because it gradually decomposed in aqueous-methanol in the presence of light, thereby rendering it useless for routine quantitative receptor assay.

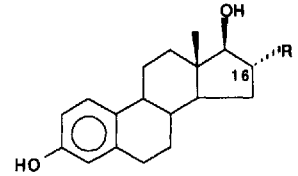
We then turned our attention to synthesizing other organometallic-labelled estradiol derivatives in the hope of finding compounds with more long-term stability in aqueous-methanol solution. In particular, we managed to attach alkyne groups at positions 2, 4, 16, and 17 on the estradiol skeleton.[17] These alkyne groups coordinate readily to cobalt and molybdenum carbonyl fragments to produce small organometallic cluster complexes such as those shown in Table II.

TABLE II

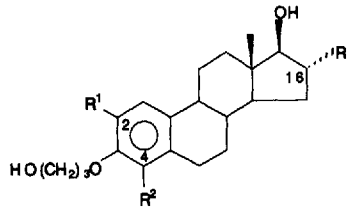
Structure and estrogen receptor binding affinities of estradiol hormone complexes of cobalt and molybdenum (Cp = η^3-C_5H_5)[17]



	R	M_2L_6	RBA (%)
12	H	–	70
13	CH_3	–	44
14	H	$Co_2(CO)_6$	5
15	CH_3	$Co_2(CO)_6$	12
16	H	$Mo_2Cp_2(CO)_4$	16
17	CH_3	$Mo_2Cp_2(CO)_4$	13



	R	RBA (%)
18	$CH_2C{\equiv}CH$	16
19	$CH_2C{\equiv}CH/Co_2(CO)_6$	2



	R^1	R^2	RBA (%)
20	$CH_2C{\equiv}CH$	H	2.5
21	H	$CH_2C{\equiv}CH$	2.5
22	$CH_2C{\equiv}CH/Co_2(CO)_6$	H	0
23	H	$CH_2C{\equiv}CH/Co_2(CO)_6$	2
24	$CH_2C{\equiv}CCH_3/Co_2(CO)_6$	H	0
25	H	$CH_2{\equiv}CCH_3/Co_2(CO)_6$	2

TABLE III

Molar absorptivities of the most intense ν(CO) peaks for representative examples of the three classes of IR markers used in this study (CCl_4 solution)[a]

Compound	ν(CO) (cm^{-1})	Molar Absorptivity ($1\ mol^{-1}\ cm^{-1} \times 10^3$)
10	1960	7.5
15	2047	6.0
17	1973	4.5

[a]Data from ref. 18.

These organocobalt and -molybdenum carbonyl complexes did prove to be appreciably more stable in both solution and the solid state than are the related chromium derivatives. The intensities of the ν(CO) bands in their IR spectra are comparable to those of the $Cr(CO)_3$ complexes. For instance, the molar absorptivities of the most intense ν(CO) peak for examples of the various classes of the compounds used as IR markers in our work are compared in Table III.[18]

The RBA values for the alkyne metal carbonyl clusters were then determined following the same incubation procedure as for the chromium derivatives; some representative values are also listed in Table II. The cobalt compound **15** was selected as an excellent choice for the estradiol receptor studies for the following reactions: (1) it has a good RBA value (12%); (2) it is easily prepared in a single step from a commercially available steroid[19]; (3) its ν(CO) bands in the IR are not overlapped with the bands of water vapor. The molybdenum compounds also have a good RBA value, but a two-step synthesis is required, and the ν(CO) bands are severely overlapped at low concentration with water vapor peaks. The FT-IR spectra in the CO stretching region of the free organocobalt hormone **15**, before and after incubation with lamb uterine cytosol, are compared in Fig. 3. The ν(CO) peaks associated with the cluster compound are just discernible above the background in the IR spectrum of the precipitated protein.

Similar receptor studies have also been performed for progesterone (**2**) receptor.[20] The synthetic antiprogestin mifepristone (**26**) has an extremely high affinity (RBA = 389%) for progesterone receptor (Table IV). It readily forms complexes with the usual

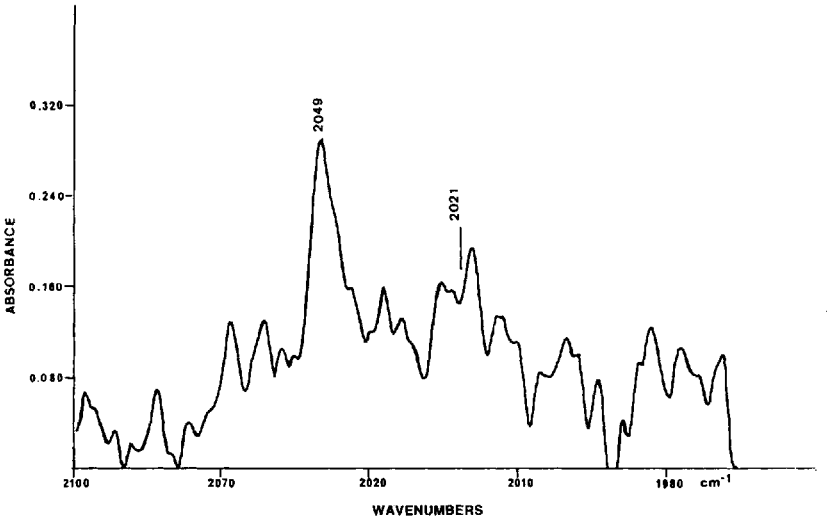
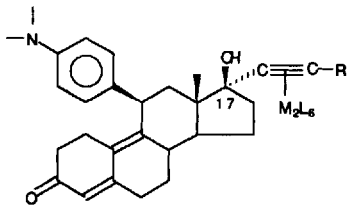


FIGURE 3 FT-IR difference spectrum of the ν(CO) region obtained by subtracting the spectrum of precipitated protein following incubation with **15** (1×10^{-8} M) in the presence of DES (1×10^{-3} M) from that of precipitated protein incubated with 15 (1×10^{-8} M) in the absence of DES (3-mm minipellets of solid materials). Reproduced with permission from Ref. 30.

cobalt and molybdenum carbonyl fragments; these organometallic derivatives have RBA values of around 10%, sufficient for selective labeling of the progesterone receptor site. Subsequent FT-IR analysis of the receptor site using compound **27** as the IR marker

TABLE IV

Relative binding affinities (RBA) of compounds **26–28** for cytosol progestin receptor[a]



	M_2L_6	RBA (%)
26	–	389
27	$Co_2(CO)_6$	13
28	$Mo_2Cp_2(CO)_4$	10

[a]Data taken from Ref. 20.

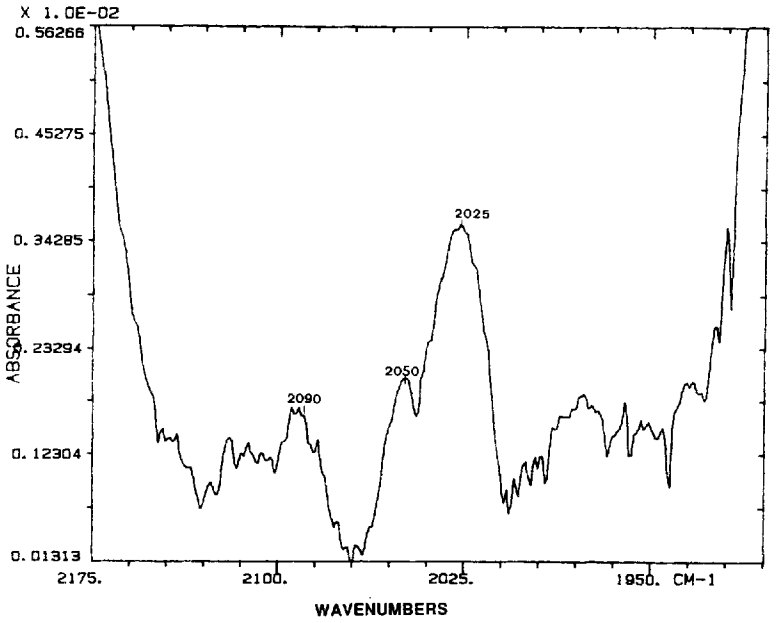


FIGURE 4 Difference spectrum obtained by subtraction of the FT-IR spectrum of precipitated receptor protein subsequent to *in vitro* incubation of uterus cytosol with **27** (3-mm minipellets of solid materials). Reproduced with permission from Ref. 20.

showed that about 1 pmol of the organometallic cluster could be detected (Fig. 4).

The potential utility of organometallic-labelled hormones as IR markers in biological processes has now been demonstrated. However, the procedure remains to be established as a quantitative one. A major step in this direction would be to increase the detection limit of the FT-IR instrument. An increase in the signal/noise of approximately 10^2 can be achieved by using an indium–antimonide (InSb) detector rather than the more commonly available triglycine sulfate (TGS) or mercury–cadmium–telluride (MCT) detectors. A further increase in sensitivity is anticipated if long pathlength, ultra-low volume, solution cells can be constructed to work with the very low concentrations of organometallic labels available from the assaying procedures.

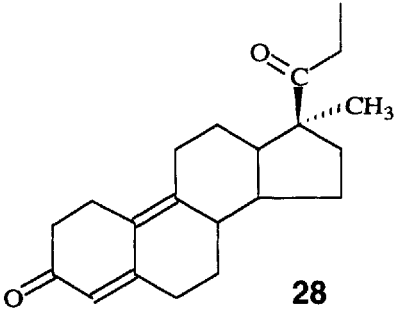
ORGANOMETALLIC AFFINITY MARKERS IN BIOLOGY

In this final section, we will describe our recent efforts in designing a biological affinity marker. This name is given to a molecule capable of forming a covalent bond to a protein receptor, in the present case, a steroidal hormone receptor. The interest in affinity markers is threefold: (1) they may help in the identification, isolation, and purification (under denaturant conditions) of the protein macromolecule; (2) the affinity marker–protein macromolecule complex may still be biologically active and useful comparisons can be made with the activity of the parent macromolecule; (3) it may be possible to locate the receptor binding site and obtain detailed information about the amino acid composition of the labelled active site.

There is an important need for affinity markers which are both efficient and selective. There are essentially two main routes to covalently labelled steroid receptors via either electrophilic functional groups or photoreactive species.[21,22] The latter approach is considered to be superior from the selectivity point of view, but the level of labelling is extremely low. Electrophilic agents, on the other hand, are highly efficient at labelling the receptors, but the selectivity is poor due to subsequent uncontrollable reactivity. Compound **28** is a photochemical affinity marker for progesterone receptor; it is highly selective, but inefficient (15% after 1 h).[22] Two compounds currently used as affinity markers for estradiol receptor are photoactivated hexestrol azide (**29**)[23] and the electrophile tamoxifene aziridine (**30**).[24]

Compound **31** was suspected of being an affinity marker for estradiol receptor, but this has now been shown to be untrue.[25] Consequently, when we began our work there was apparently no convenient affinity marker available for estradiol receptor with a structure based on the estradiol skeleton.

The structural features of compounds **15**, **17**, and **21** are closely similar since the chief differences are related to modifications in the steroid skeleton at the 17α-position. The binding affinities of these three compounds for estradiol (compounds **15** and **17**) and progesterone (compound **27**) are about 12–13%, and all three possess a labile 17β-OH group, even in slightly acidic media. It has been postulated that an acidic active site is implicated in the



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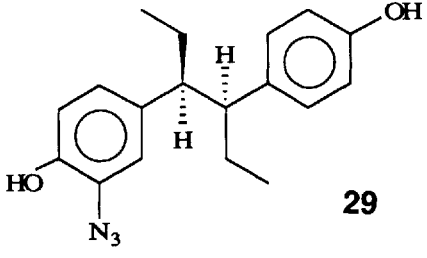

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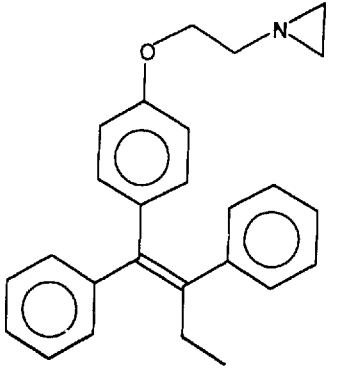

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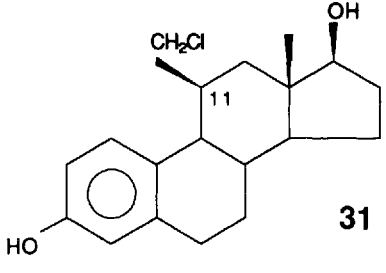


31

association of estradiol and its receptor.[26] The formation of an α-carbenium ion[27] in the 17α-position, adjacent to an organometallic moiety following OH dissociation, results in an enhanced stability for the complex by comparison with that of the free ligand. We felt that compounds such as **15** and **17** might well prove to be useful precursors to the desired affinity markers for estradiol receptor.

With compound **15**, the association with estradiol receptor proved to be irreversible (82% labelling after 1 h), while that for compound **17** was reversible.[28] These binding results are illustrated graphically in Fig. 5. The irreversibility of the interaction with compound **15** can be prevented by pre-incubation in the presence of 17β-estradiol.

These initial results suggest that the 17β-position, modified by complexation in the adjacent 17α-position, is selectively activated in the association site of the estradiol receptor, possibly by proton acceptance leading to formation of a carbenium ion. If there is a nucleophilic group located in the proximity of the protonating group, this group would be expected to react quickly with the transient electrophile to form a strong covalent bond. The difference in reactivity between compounds **15** and **17** lies in the different stabilization of the intermediate electrophilic carbenium ion species.

It is worthwhile to point out that the DNA of the estradiol receptor has been cloned and sequenced; the chicken and human receptor sequences are quite similar.[26] From the sequence analyses, it appears that the domains associated with the estradiol receptor contain four cysteine residues, one of which (#411) is adjacent to a lysine (#410), another (#441) possesses a lysine in position (#443), while a third (#524) is flanked in positions α, α′ by two more lysines (#523, #525). The fourth cysteine is not located near a lysine residue. (Such a situation, namely a cysteine flanked by a lysine, does not exist in the progesterone receptor.) The cysteines might well by ideally positioned to react with an organometallic derivative. However, it should be remembered that the tertiary structure of the protein is still not known. Nevertheless, it does provide us with a good starting point for attempting to elucidate exactly what does occur during association in the active site of the receptor.

In some model studies, we have shown that thiols react with hormone **15** to yield an electrophilic complex which undergoes instantaneous reaction upon treatment with *n*-butylamine to give

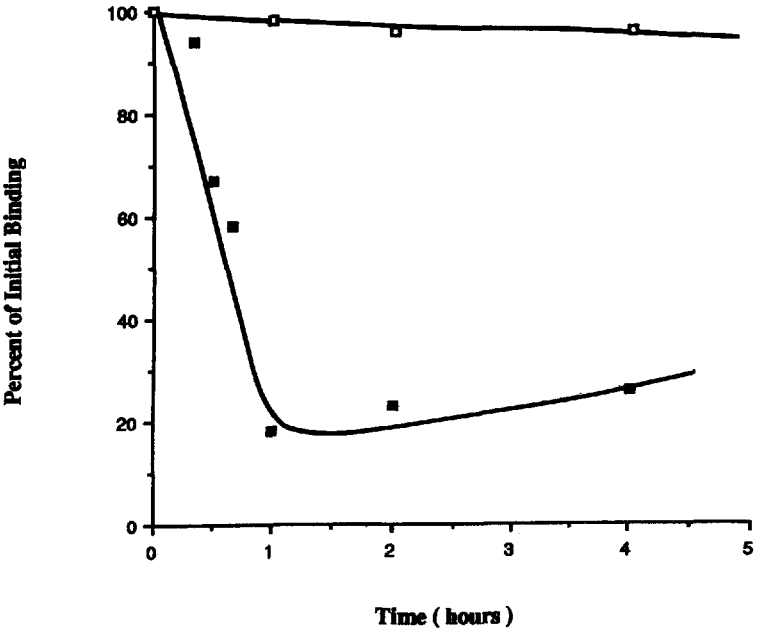


FIGURE 5 Rate of inactivation of the estrogen receptor in the presence of **15** and **17**. Lamb uterine cytosol was incubated at 25° C in the presence of 10 nM of **15** (■) or 10 nM of **17** (□). Free tracer was removed by charcoal dextran treatment (0.5% charcoal, 0.05% dextran). The surviving reversible estrogen binding activity was measured after exchange with 10 nM (^{3}H)-17β-estradiol. Reproduced with permission from Ref. 28.

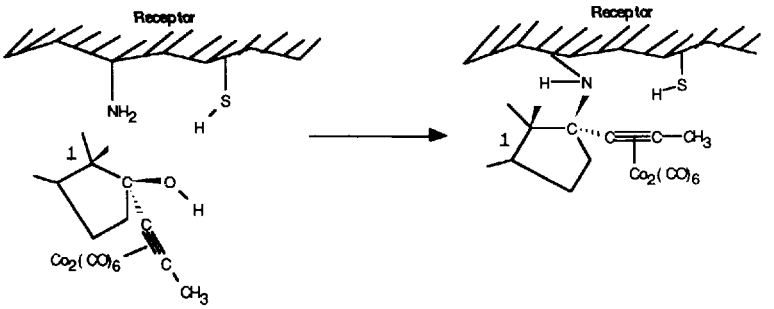
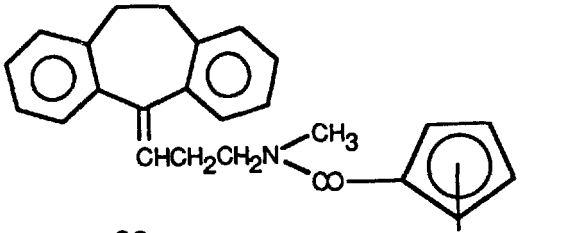


FIGURE 6 Tentative explanation for the function of the organometallic affinity marker. Reproduced with permission from Ref. 28.

a 17α-aminosteroid.[28] On the basis of this result and our knowledge of the cysteine residues, a possible mode of interaction between compound **15** and estradiol receptor has been suggested in Fig. 6. Obviously, this proposal must remain tentative in the absence of more detailed information.

CONCLUSIONS

The utility of organometallics as IR markers shows considerable promise not only in receptor assay and possibly IR metalloimmunoassay in general, but also in affinity labelling. We believe that many other applications of this new field of bioorganometallic chemistry will be forthcoming in the future. Indeed, some labelling work of this type has already been reported for the antidepressant nortryptiline (**32**) using the $Mn(CO)_3$ fragment as the IR marker.[29]



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