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The first titanocenyl dichloride moiety vectorised by a selective estrogen receptor modulator (SERM). Synthesis and preliminary biochemical behaviour

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Abstract

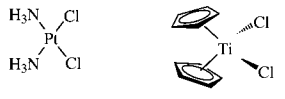
The synthesis of **4′**, a titanocene derivative of the anticancer drug tamoxifen is presented together with its biochemical properties. Compound **4′** is prepared by a McMurry coupling followed by a ligand exchange. Compound **4′** reveals an unexpected proliferative effect on the hormone-dependent cell line MCF7. Surprisingly this effect is also observed with Cp_2TiCl_2 alone.

Keywords: Bioorganometallic; Titanocen; Tamoxifen; Estrogen; SERM; MCF7; Proliferative effect

1. Introduction

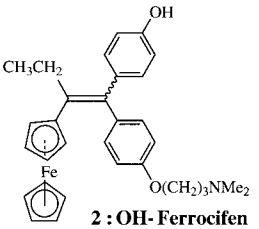
Inorganic platinum complexes, particularly cisplatin **1**, have become established as highly effective antitumoral agents, despite the fact that to date their range of use remains fairly restricted [1]. They have in fact revolutionised the treatment of testicular cancer, which is still one of their most spectacularly successful applications. This breakthrough has inspired much research into the application of metal complexes in oncology [2,3]. In this context, various series of cyclopentadienyl-type organometallic complexes of Ti, Fe, Mo, V, Re, have been evaluated. Titanium in particular is showing promising results, although all stages of clinical trials have not yet been completed [4]. It appears so far however, that these metallocenes act via mechanisms different from those of cisplatin, and thus may lend themselves to treatment of a wider range of cancers [5].

The species mentioned above are most commonly used in their simplest form. We however, have undertaken a program of research focused on attaching them to a well-defined biological vector in an attempt to improve their selectivity and their effectiveness on target cells [6–8]. Our work has led, for example, to OH-Ferrocifens such as **2** capable of producing antiproliferative effects on breast cancer cells whether they are estrogen-dependent ER(+) or -independent ER(−) (ER = estrogen receptor). Resistance to treatment with tamoxifen **3**, encountered in a number of breast cancers, remains a major therapeutic and social problem. This has led to intense activity in research on SERMs (selective estrogen receptor modulators) that would be

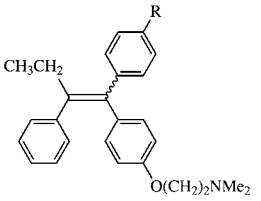


1 : cisplatin

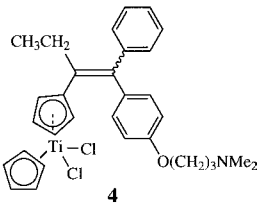
18 : Cp_2TiCl_2



2 : OH- Ferrocifen



3 : Tamoxifen (R = H)
3′ : OH-Tamoxifen (R = OH)



4

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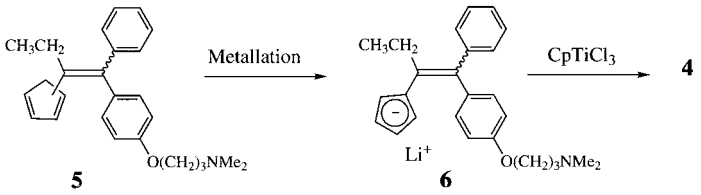


Scheme 1.

more effective than tamoxifen, the primary example of this group [9,10]. Favourable indications concerning the antitumoral potential of dichloro titanocene suggested it as a good candidate for attachment to a tamoxifen-type skeleton, allowing us to evaluate the capability of a complex such as **4** to inhibit cellular proliferation in breast cancers. The difficulties we encountered in synthesising molecule **4** were surprising, and led us to develop a novel preparative approach. Even more surprising was our observation of an unanticipated oestrogenic effect when compound **4** was incubated in presence of MCF7 (ER +) cancer cells. The results of this entire body of work are presented here.

2. Results and discussion

2.1. Synthesis

One potential route to **4** is its preparation starting from 1-phenyl-1-(4-dimethyl aminopropyloxyphenyl)-2-cymantrenyl-1-butene (**5**) (Scheme 1). Metallation of **5** by a lithium or thallium reagent would then give the metal salt **6** which would subsequently be allowed to react with $CpTiCl_3$ [11]. Efforts were therefore made to find synthetic routes to the substituted cyclopentadiene **5**.

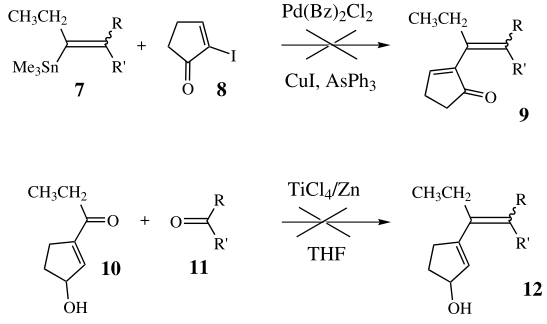
A literature search shows that several possible synthetic routes are possible. The most direct one appears to be the reaction involving formation of a C–C bond catalysed by palladium. This coupling between a stannylated derivative and a halogenide catalysed by palladium, known as a Stille reaction, is very widely used [12]. Johnson et al. have shown the viability of coupling between iodocyclopentenone and a stannylated derivative by using $Pd(Bz)_2Cl_2$–CuI–$AsPh_3$ as the catalyst [13]. In principle the use of this method in the coupling between the tin derivative **7** and iodocyclopentenone **8** should give the ketone **9** which is the precursor of **5** (Scheme 2). In practice, the experiment did not in fact lead to the ketone **9**. This negative result may be attributed to the presence of the ethyl on **7** which could cause a β-elimination reaction.

A second alternative would be a McMurry coupling reaction between the ketone **10** and the ketone **11** [14]. This reaction did not result in the desired product **12** since the ketone **10** did not tolerate the reaction conditions and had completely disappeared by the end of the reaction [15]. Pauson et al. have reported the formation of $(C_5H_4NMe_2)Li$ by a lithium-induced decomposition of dimethylaminoferrocene [16]. However, application of this method to OH-Ferrocifen **2** did not lead to the lithium salt of type **6**, the precursor to the complex **4**. On the other hand, Rosenblum et al. were able to obtain dicyclopentadienylnaphthalene by reacting cyclopentadienyl copper dimethyl sulphide on diiodonaphthalene [17]. In our case, the reaction of cyclopentadienyl copper dimethyl sulfide on a vinylic bromide, an analogue of **7**, also failed to result in **5**.

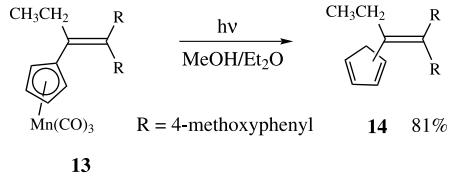
Having tried all these known reactions without success, we were forced to consider a novel synthetic route to compound **5**. The discovery of this new route is based on the observation that cymantrene derivatives decompose slowly through the action of sunlight. By using irradiation from a UV lamp to accelerate decomplexation, and by working in a protic medium, we were able to establish that the cyclopentadienyl ring was generated in very good yield (Scheme 3) [18].

Application of this method should give access to **5** following the synthetic route shown in Scheme 4.

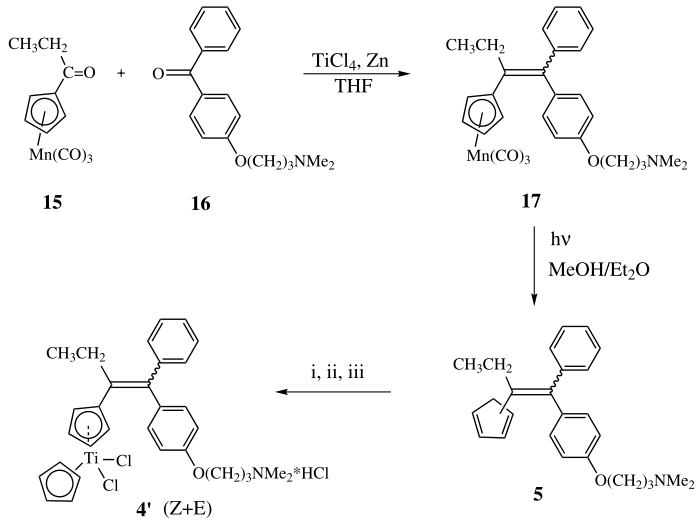
Propionyl cyclopentadienyl manganesetricarbonyl (**15**) and the ketone (**16**) were allowed to react in the presence of McMurry reagent. After heating for 1 h,



Scheme 2.



Scheme 3.



i) n-BuLi, THF, -70°C; ii) $CpTiCl_3$; iii) HCl

Scheme 4.

the complex **17** was obtained in 54% yield. It appeared as a yellow oil containing a mixture of the *cis* and *trans* isomers in 50/50 proportion, as estimated from the NMR spectrum. Next a decomplexation step was performed. UV irradiation of the manganese complex, **17**, in an ethyl ether–methanol mixture gave the cyclopentadienyl derivative, **5**, in excellent yield (98%). This compound, relatively unstable because of a possible Diels–Alder reaction, was characterised only by NMR. Upon preparation, it was immediately converted into a lithium salt by action of *n*-BuLi in THF at -70 °C. Addition of $CpTiCl_3$ to the organolithium caused the immediate formation of the titanium complex **4** which was then transformed into a chlorohydrate salt **4′** by passing over it a flow of HCl gas. A yield of 57% was obtained.

3. Biochemical data

3.1. Determination of RBA values and lipophilicity

The relative binding affinity (RBA) value for **4′** is 8.5% for estrogen receptor alpha (Table 1), a much higher value than expected for a compound with no OH group and more precisely, no phenol ring, which both OH-tamoxifen (**3′**), the active metabolite and **2** (OH-Ferrocifen) do possess. It is in fact well known that this functionality plays a critical role in the binding of the hormone to its receptor. For Cp_2TiCl_2, an RBA value of 0 is found. Lipophilicity of **4′** measured by its octanol–water partition coefficient was found to be 5.83 for the *Z* isomer and 5.95 for the *E* isomer. The addition of the organometallic entity Cp_2TiCl_2 therefore causes an increase in lipophilicity to a level noticeably higher than that observed for the addition of a ferrocene [7] or a $CpRe(CO)_3$ moiety [8].

4. Study of the proliferative/antiproliferative effect on hormone-dependent cell lines (MCF7 and MVLN)

The effect of **4′** on hormone-dependent MCF7 cells, derived from a breast cancer line containing the estrogen receptor ER +, observed after 5 days of incubation**,** is shown in Fig. 1. The results are compared to those found in the presence of estradiol (E_2) which is the standard for the estrogenic effect, and OH-tamoxifen (**3′**), the standard for the antiestrogenic effect. The results obtained are expressed as a percentage of DNA compared to control (cells incubated in the absence of added compound). The result obtained is highly sur-

Table 1
Relative binding affinities (RBA) and partition coefficients (Log Po/w) of the compounds

Compound	RBA (%) (DMSO, 25 °C, ERα) [a]	Log Po/w [b]
4′	8.5	5.83 (*Z*), 5.95 (*E*)
18 (Cp_2TiCl_2)	0	
E_2	100	3.5
3′ (OH-Tamoxifen)	38.5 [c]	3.2 (*Z*) [c]; 3.5 (*E*) [c]
2 (OH-Ferrocifen)	11.5 [c]	4.3 (*Z*) [c]; 4.5 (*E*) [c]

[a] RBA for the estrogen receptor is determined on lamb uterus cytosol as described in Ref. [24].
[b] Octanol–water partition coefficients were determined by reverse phase HPLC using the method described by Pomper [25].
[c] Value from Ref. [7].

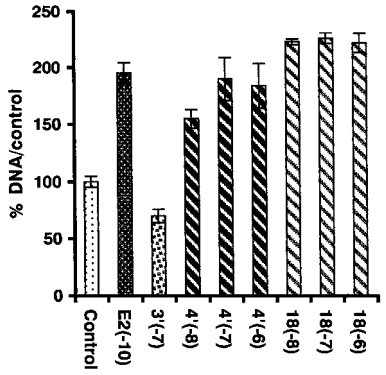


Fig. 1. Effect of E_2 (Estradiol), **3′** (OH-Tamoxifen), **4′** and **18** (Cp_2TiCl_2) on the proliferation of MCF7 cells (estrogen receptor-positive cells). The results are expressed as the percentage of DNA in the sample versus the DNA value of the control. The values are the means of two independent experiments in quadruplicate ± limits of confidence ($P = 0.1$, $t = 1.415$) The values in brackets correspond to the log of molarity of incubation.

prising. For while E_2 and OH-tamoxifen give the expected effects (proliferative for the former, antiproliferative for the latter), **4′** acts as an estrogen almost as active as estradiol itself, at molarities as low as 1×10^{-7} M. This powerful estrogenic effect is strong enough to reverse the antiestrogenic effect produced by the dimethylamino side chain, an effect that has already been observed for tamoxifen derivatives bearing organometallic entities, for example ferrocene [7] or $CpRe(CO)_3$ [8]. This unusual behaviour led us to examine the effect of the Cp_2TiCl_2 entity alone. We observed a proliferative effect greater than that of **4′**. It seems therefore that this effect must essentially be due to the entity itself or to one of its hydrolysates. These results were confirmed on MVLN cells, another hormone dependent cell line, in which expression of luciferase is proportional to the estrogenicity of the tested compounds. After 24 h of incubation, **4′** and Cp_2TiCl_2 showed an estrogenic effect at 1 μM (148, 334%, respectively with E_2 0.1 nM at 261%). Within this short period of incubation the effect observed with **4′** is less pronounced. This is probably due to competition between the antiproliferative effect of the organometallic unit and the tamoxifen moiety.

5. Discussion

In order to try to understand this surprising result, namely that both **4′** and Cp_2TiCl_2 demonstrate strong estrogenic activity, it is necessary first of all to establish the behaviour of these complexes in that most universal of biological solvents, water. In fact, the hydrolysis of Cp_2TiCl_2 has been the subject of a number of studies, and appears to involve a complex series of events that depends largely on the conditions that come into play. In particular, a hydrolysis of the cyclopentadienyl leading to a diene was observed, but also a hydrolysis of the chlorides. Among the species identified were, for example, $Cp_2Ti(H_2O)Cl^+$, $Cp_2Ti(OH)Cl$, $(Cp_2TiCl_2)_2O$ etc. leading ultimately to the precipitation of polymers derived from the various hydrolysis products [19,20]. It is not impossible that one of the products thus formed may exhibit estrogenic behaviour, considering that the estrogen activation site is very receptive to phenol derivatives.

However, an excellent recent study by Sadler [5] has shed new light on the behaviour of Cp_2TiCl_2 in biological media. According to this work, the most rapid hydrolysis occurs at the level of the chlorides. The role of the cyclopentadienes is to slow a too-rapid hydrolysis which would lead to inactive polymers, but the Cp entities are subsequently displaced during the process. Whether or not this is the case, the result is a monomer of Ti^{IV}, a hard metal similar to Fe^{III}, which is quickly captured and stabilised by transferrine, one of the iron-carrying proteins in the blood. It is interesting to note that whatever the initial titanium complex, whether Cp_2TiCl_2 or Budotitane, the result is the capture of the Ti^{IV} ion, preferentially in the case of transferrine where it replaces Fe^{III} and binds to aspartate, histidine, tyrosine and carbonate residues. It has been shown that the level of transferrine receptor is greater at the surface of cancerous cells, perhaps due to their increased demand for iron [5]. Because of the change in pH at this level the Ti^{IV} is then released and is transported to the centre of the cell by binding to ATP. This work attempts to elucidate the nature of the active species, Ti^{IV}, no matter what the precursor may be, or how it is delivered to tumour cells. It is clear from the above that depending on the specific nature of the target cells, differentiated behaviour may be expected. And in fact, although titanium has not yet been studied in this context, the role of certain metal ions has already been the subject of occasional analyses in relation to estrogen receptors [21,22]. It is known that estrogen receptor alpha, which makes up by far the largest proportion of the ER present in MCF7 cells, contains zinc in its natural state. It is these zinc fingers that mediate the association of the DNA binding domain to an estrogen response element (ERE) [23]. Replacing zinc by either nickel (Ni^{2+}) or copper (Cu^{2+}) inhibits this association while Co^+ or Cd^+ have no effect. On the other hand, treatment of the receptor with Cd^+ in very low concentration (1 nM) results in coordination of this metal at the level of the ligand binding domain. The complex formed with this exogenous metal activates ER alpha just as binding to estradiol would. It must be concluded that the effects of Cd^{2+} and Ti^{4+} are similar, in that both lead to proliferation of MCF7 type cancer cells.

A first step in elucidating the role of Cd^{2+} was reported very recently [22]. Cadmium is able to coordinate with cysteins 381 and 447, glutamic acid 523, histidine 524 and aspartic acid 538 of the receptor ligand binding domain. In so doing it inhibits the binding of estradiol. It has been suggested that the hormone binding domain acts somewhat like a mousetrap which is closed by creation of a saline bridge between the two helices H4 and H12 of the receptor. This bridge, after estradiol capture, positions helix 12 such that the transcriptional machinery can be activated. Cysteins 381 and 447 are localised on helices H4 and H8, glutamic acid 523 and histidine 524 are on helix H11 close to the bioligand, and aspartic acid 538 is localised at the interface of helix H12. The idea that this type of coordination may cause the receptor to act in the same way as the natural ligand, by snapping the trap shut, is an appealing one that will be confirmed or denied by further studies. It also remains to be seen whether the explanation advanced for Cd^{2+} holds good for Ti^{4+}.

It should in any case be borne in mind that the estrogen receptor is a critical mediator in the body, and that species that can bind to it and activate it have the potential to increase the risk of breast cancer. The preliminary data presented here suggest that titanium (Ti^{IV}) in the environment could have estrogenic activity and thus be a new risk factor. In addition, if these results were to be confirmed, the future of Cp_2TiCl_2 currently in phase II clinical trials, would have to take into account this previously-unknown parameter.

6. Experimental

6.1. General data

Anhydrous THF and Et_2O were distilled from sodium–benzophenone. Methanol (Prolabo) was used without any further purification. Thin layer chromatography was performed on Silica Gel 60 GF254. UV-photolyses were carried out with a Heraeus TQ150, 150 W high pressure Hg lamp. FT-IR spectra were recorded on a BOMEM Michelson-100 spectrometer. ^{1}H- and ^{13}C-NMR spectra were acquired on Bruker 200, 250 or 400 MHz spectrometers. Mass spectrometry was performed by laboratoire de spectrométrie de masse (ENSCP) and by CRMPO (Université de Rennes 1). Melting points were measured with a Kofler device. Elemental analyses were performed by the Regional Microanalysis Department of Université Pierre et Marie Curie.

6.2. Synthesis of the compounds

6.2.1. Propionylcyclopentadienyltricarbonylmanganese (15)

In a Schlenk tube purged with Ar, cymantrene (3.00 g, 14.70 mmol) and propionyl chloride (1.35 g, 14.70 mmol) were dissolved in CS_2 (30 ml). $AlCl_3$ (0.977 g, 7.35 mmol) was added in small portions to the solution of cymantrene, and the solution became red. After 1.5 h, the CS_2 was removed under vacuum and the oil obtained was hydrolysed with water at 0 °C. After extraction with Et_2O (3×20 ml), and drying over $MgSO_4$, the solution was concentrated, whereupon addition of pentane yielded (propionylcyclopentadienyl)tricarbonylmanganese (3.540 g, 92%) as a yellow powder. Recrystallization from ether/pentane furnished yellow crystals, m.p. 250 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 5.44 (t, 2H, $J = 2.1$ Hz, η^5-C_5H_4), 4.85 (t, 2H, $J = 2.1$ Hz, η^5-C_5H_4), 2.64 (q, 2H, CH_2), 1.17 (t, 3H, CH_3). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 197.9 (CO), 91.5 (C_1 of η^5-C_5H_4), 86.4 (2C, C_5H_4), 83.4 (2C, C_5H_4), 32.2 (CH_2), 7.9 (CH_3). IR (CH_2Cl_2): ν_{CO} at 2030 (s), 1948 (s), 1686 cm^{-1} (m). Anal. Calc. for $C_{11}H_9MnO_4$: C, 50.79; H, 3.49. Found: C, 50.83; H, 3.41%.

6.2.2. 4-Dimethylaminopropyloxy benzophenone (16)

In a Schlenk tube purged with Ar, 4-hydroxybenzophenone (3.96 g, 0.02 mol) was dissolved in CH_3COCH_3 (80 ml). Powdered NaOH (1.60 g, 0.04 mol) was added into the solution, and the mixture heated at reflux for 15 min. After that time, dimethylaminopropionylchloride hydrochloride (3.16 g, 0.02 mol) was added into the mixture in one portion. The heating was maintained for 15 h. After cooling to room temperature (r.t.), the solution was filtrated and evaporated. The crude product obtained was chromatographed on a silica gel column. The column was first eluted with CH_3COCH_3 to remove the unreacted hydroxybenzophenone, and then with CH_3COCH_3–Et_3N (10:1). Dimethylaminopropyloxy benzophenone was isolated as an colourless oil which became a solid upon addition of pentane (4.40 g, 77%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.84 and 6.96 (d, d, 2H, 2H, $J = 8.9$ Hz, C_6H_4), 7.75 and 7.50 (m, m, 2H, 3H, C_6H_5), 4.11 (t, 2H, $J = 6.4$ Hz, OCH_2), 2.49 (t, 2H, $J = 7.4$ Hz, NCH_2), 2.28 (s, 6H, NMe_2), 2.00 (m, 2H, CH_2). MS (EI, 70 eV) m/z, 283 [M^+]. Anal. Calc. for $C_{18}H_{21}NO_2$: C, 76.30; H, 7.47; N, 4.94. Found: C, 76.30; H, 7.58; N, 4.92%.

6.2.3. 1-Phenyl,-1-(4-dimethylaminopropyloxyphenyl)-2-cymantrenyl-1-butene (17)

$TiCl_4$ (1.71 g, 9 mmol) was added dropwise to a suspension of Zn powder (1.17 g, 12 mmol) in THF (30 ml) at 0 °C. The blue mixture obtained was heated at reflux for 2 h, the solution became black, and the oil bath was removed. A second solution was prepared by dissolving 4-dimethylaminopropyloxy benzophenone, **16**, (0.849 g, 3 mmol) and propionylcymantrene, **15**, (0.780 g, 3 mmol) in THF (15 ml). The latter solution was added dropwise to the first solution, and the resulting mixture was again heated for 1 h. After cooling to

r.t., the mixture was hydrolysed with 100 ml of a 10% Na_2CO_3 solution. After $CHCl_3$ extraction and solvent removal, the crude product was chromatographed on silica gel plates with THF–Et_3N 10:1 as eluent to give **17** ($Z+E$) as an orange oil (0.828 g, 54% yield). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.32–6.78 (m, 9H, aromatic rings), 4.50 (m, 4H, C_5H_4), 3.98 (t, 2H, OCH_2), 2.45 (t, 2H, CH_2–N), 2.30 (q, 2H, CH_2CH_3, hindered by NMe_2 signal), 2.25 (s, 6H, NMe_2), 1.94 (t, 2H, CH_2), 1.05 (t, 3H, CH_2CH_3). MS (EI, 70 eV) m/z, 511 [M^+], 427 [$(M-3CO)^+$].

6.2.4. Titanium complex 4′

Compound **17** (0.200 g, 0.39 mmol) was dissolved in a mixture of technical grade ethyl ether (20 ml) and MeOH (10 ml). The pyrex tube containing the solution was placed under a UV lamp (TQ150) for 30 min irradiation. Significant gas evolution was observed and a brown powder precipitated. The solution was filtered and evaporated. TLC on silica gel with 20:1 THF–Et_3N as eluent gave **5** (0.144 g) as a beige oil. Compound **5** was immediately used to prepare titanium complex. Compound **5** was dissolved in THF (7 ml) and cooled to -70 °C. *n*-BuLi (0.34 ml of a 1.2 M solution in hexane, 0.41 mmol,) was added dropwise. The lithium salt solution was stirred for 1 h 15 min. while the temperature was allowed to rise slowly to -20 °C, and the solution became orange. The solution was re-cooled to -70 °C, then $CpTiCl_3$ (0.085 g, 0.39 mmol) was added as a solid in one portion, and the solution immediately turned dark red. After stirring for 1.5 h, a flow of HCl gas was passed into the mixture for 5 min. The THF was removed under vacuum and the dark brown solid obtained was washed twice by Et_2O. The solid was redissolved in CH_2Cl_2 saturated with HCl (15 ml). The solution was filtered and concentrated to 2 ml. Et_2O was added yielding a dark red precipitate of **4′** (0.130 g, 57%). ^{1}H-NMR (200 MHz, CD_3OD): δ 7.32–6.83 (m, 9H, aromatic rings), 6.53 and 6.52 (s, s, 5H, C_5H_5), 6.32 (m, 2H, C_5H_4), 6.06 and 6.00 (t, t, 2H, C_5H_4), 4.10 (q, 2H, OCH_2), 2.93 (s, 6H, NMe_2), 2.41 (m, 2H, CH_2CH_3), 2.21 (m, 2H, CH_2), 0.98 and 0.99 (t, t, 3H, CH_2CH_3). MS (FAB) 556 [$M+H]^+$, 520 [$M-Cl]^+$, 372 [$M-CpTiCl_2]^+$. High resolution MS: Calc. [$M+H]^+$ 556.1657, Found: [$M+H]^+$ 556.1651. Anal. Calc. for $C_{31}H_{35}Cl_2NOTi\cdot HCl$: C, 62.80; H, 6.12; N, 2.36. Found: C, 61.60; H, 6.66; N, 2.41%.

7. Biochemical studies

In order to obtain reproducible results new stock solutions (1×10^{-3} M) have to be prepared for each experiment with freshly purified organometallic complexes.

7.1. Determination of the relative binding affinity (RBA) of the compounds for the estrogen receptor alpha (ERα)

Aliquots (200 μl) of sheep uterine cytosol prepared as described in Ref. [24] were incubated for 3 h at 0 °C with [6,7-^{3}H]-estradiol (2×10^{-9} M, specific activity 1.96 TBq mmol^{-1}) in the presence of nine concentrations of the hormones to be tested. Stock solutions (1×10^{-3} M) of the compounds were prepared in DMSO. At the end of the incubation period, the free and bound fractions of the tracer were separated by protamine sulphate precipitation. 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 unlabeled steroid required to displace 50% of the bound [^{3}H]-estradiol was calculated for each steroid tested, and the results expressed as RBA. The RBA value of estradiol is by definition equal to 100%.

7.2. Measurement of the lipophilicity of 4′

Log Po/w were measured by reverse phase HPLC using the method described by Pomper et al. [25].

7.3. Test on MCF7 cells

7.3.1. Culture materials

Earle's based minimal essential medium (MEM), fetal bovine serum (FBS), L-glutamine, penicillin, gentamicin, streptomycin were obtained from Gibco (Ghent, Belgium), plastic culture materials from Falcon (Ghent, Belgium).

7.3.2. Culture conditions

MCF7 cells were from the Michigan Cancer Foundations (Detroit). Cells are maintained in monolayer culture in Dulbecco–MEM added with 10% thermally inactivated FBS, L-glutamine (0.6 mg ml^{-1}) and a cocktail of antibiotics (gentamicin 40 μg ml^{-1}, penicillin 100 U ml^{-1}, streptomycin 100 μg ml^{-1}). Growth of the cells was assessed by measuring the DNA content of treated and untreated (control) cells after 5 days of culture [26].

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