Synthesis and Cytotoxicity Studies of Titanocene C Analogues

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From the carbolithiation of 6-\(\mu\)N,\(\mu\)N-dimethylamino fulvene (3) and 2,4[\(\mu\)bis(\(\mu\)N,\(\mu\)N-dimethylamino)\(\mu\)N-methylpyrrolyl)\(\mu\)N-\(\mu\)N-dimethylamino)methyl]benzimidazolyl lithium (2b), \(\mu\)-(\(\mu\)N,\(\mu\)N-dimethylamino)methylphenyl lithium (2c), the corresponding lithium cyclopentadienide intermediate (4a–c) was formed. These three lithiated intermediates underwent a transmetallation reaction with TiCl4, resulting in \(\mu\)N,\(\mu\)N-dimethylamino-functionalised titanocenes 5a–c. When these titanocenes were tested against a pig kidney epithelial cell line (LLC-PK), the IC_{50} values obtained were of 23, and 52 \(\mu\)M for titanocenes 5a and 5b, respectively. The most cytotoxic titanocene in this paper, 5c with an IC_{50} value of 13 \(\mu\)M, was found to be approximately two times less cytotoxic than its analogue Titanocene C (IC_{50} = 5.5 \(\mu\)M) and almost four times less cytotoxic than cisplatin, which showed an IC_{50} value of 3.3 \(\mu\)M when tested on the LLC-PK cell line.

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1. INTRODUCTION

Titanium-based reagents have significant potential against solid tumors. Budotitane ([\(\mu\)cis-diethoxybis(\(\mu\)phenylbutane-1,3-dionate)titanium(IV)]) looked very promising during its preclinical evaluation, but did not go beyond Phase I clinical trials, although a Cremophor EL-based formulation was found for this rapidly hydrolysing molecule [1]. Much more robust in this aspect of hydrolysis is titanocene dichloride (Cp2TiCl2), which shows medium antiproliferative activity in vitro but promising results in vivo [2, 3]. Titanocene dichloride reached clinical trials, but the efficacy of Cp2TiCl2 in Phase II clinical trials in patients with metastatic renal cell carcinoma [4] or metastatic breast cancer [5] was too low to be pursued.

More recently, novel methods starting from fulvenes [6–17] and other precursors [18–20] allow direct access to highly substituted titanocenes via reductive dimerisation, carbolithiation, or hydridolithiation of the fulvene followed by transmetallation in the last two cases.

Titanocene Y was obtained using hydridolithiation of fulvene, and it showed an IC_{50} value of 21 \(\mu\)M [12]. The antiproliferative activity of Titanocene Y has been studied in 36 human tumor cell lines [21] and in explanted human tumors [22]. These in vitro and ex vivo experiments showed that prostate, cervix, and renal cell cancers are prime targets for these novel classes of titanocenes, whereas the IC_{50} values for the breast cancer cell lines were very promising as well. These results were underlined by first mechanistic studies concerning the effect of these titanocenes on apoptosis and the apoptotic pathway in prostate cancer cells [23]. Furthermore, first animal studies have been published recently, reporting the successful treatment of xenografted Ehrlich’s ascites tumor in mice with an ansta-titanocene [24] and xenografted Caki-1 tumors with Titanocene Y [25], showing that the effect of Titanocene Y against xenografted Caki-1 tumors in mice was superior to cisplatin. The structure of Titanocene Y is shown in Figure 1.

So far, our most cytotoxic titanocene, Titanocene C (bis-(\(\mu\)N,\(\mu\)N-dimethylamino-2(\(\mu\)methylpyrrolyl)methylcyclopentadienyl) titanium (IV)) dichloride, was obtained through carbolithiation of fulvenes, which has been published recently [26]. It has an IC_{50} value of 5.5 \(\mu\)M when tested on the LLC-PK cell line. Its structure is shown in Figure 1. This meant significant progress, since Cp2TiCl2 exhibits an IC_{50} value of only 2000 \(\mu\)M against LLC-PK, which explains partly the failed Phase II clinical trials against renal cell carcinoma. The main idea behind the research presented in this paper was to improve the cytotoxicity of Titanocene C by adding extra dimethylamino groups using...
2. EXPERIMENTAL

2.1. General conditions

Titanium tetrachloride (1.0 M solution in toluene) and butyl lithium (2.0 M solution in pentane) were obtained commercially from Aldrich Chemical Co. (Wis, USA). THF was dried over Na and benzophenone, and it was freshly distilled and collected under an atmosphere of argon prior to use. Manipulations of air and moisture sensitive compounds were done using standard Schlenk techniques under an argon atmosphere. NMR spectra were measured on either a Varian 300 or a 400 MHz spectrometer. Chemical shifts are reported in ppm and are referenced to TMS. IR spectra were recorded on a Perkin Elmer Paragon 1000 FT-IR Spectrometer employing a KBr disk. UV/Vis spectra were recorded on a Unicam UV4 Spectrometer, while CHN analysis was done with an Exeter Analytical CE-440 Elemental Analyser.

2.2. Synthesis

6-(N,N-dimethylamino) fulvene (3) was synthesised according to the already published procedure [27].

**Synthesis of bis-[3-[2,4-di(N,N-dimethylamino)methyl]-N-methylpyrrolyl-(N,N-dimethylamino)-methyl-cyclopentadienyl] titanium (IV) dichloride, \( \eta^5-C_5H_5-CH[N(CH_3)\_2]_2[C_6H_5(CH_2-N(CH_3))\_2N(CH_3)_2]_2 TiCl_2 \) (5a)**

To a Schlenk flask with 2,4-bis(N,N-dimethylamino) methyl]-N-methyl pyrrole (1.61 g, 8.25 mmol), 20 ml of THF were added until a transparent solution was formed, while stirring at room temperature. The solution was cooled down to −78°C for 15 minutes and 4.8 mL (8.25 mmol) of butyl lithium were added. The solution was allowed to warm up to 0°C for 20 minutes, resulting in the formation of the yellow lithium intermediate.

In a second Schlenk flask, 6-(N,N-dimethylamino) fulvene (1.00 g, 8.25 mmol) was dissolved in THF, and the resultant orange solution was added via cannula at −78°C to the Schlenk flask containing the lithium intermediate. The reaction mixture was then allowed to warm up to room temperature and left stirring for 40 minutes. Titanium tetrachloride (4.1 ml, 4.13 mmol) was added afterwards in situ at room temperature, and the mixture was refluxed for 20 hours. Subsequently, the solvent was removed under vacuum, resulting in the formation of a dark brown oil that was dissolved in dichloromethane and filtered through Celite to remove the LiCl. The black filtrate was filtered additionally twice by gravity filtration. The solvent was removed under reduced pressure forming a shiny dark red solid, which was washed with 20 ml of pentane and then dried in vacuo (1.44 g, 1.93 mmol, 46.8% yield).

**Synthesis of bis-[((N,N-dimethylaminomethyl) 2-benzimidazolyl)(N’,N’-dimethylamino) methycyclopentadienyl]titanium (IV) dichloride, \( \eta^5-C_5H_5-CH[N(CH_3)_2][C_{10}H_{12}N_2]_2 TiCl_2 \) (5b)**

To a Schlenk flask with N’-(N’,N’-dimethylaminomethyl) benzimidazol (1.45 g, 8.25 mmol), 20 ml of THF were added until a transparent solution was formed, while stirring at room temperature. The solution was cooled down to −78°C for 15 minutes and 14.0 ml (8.25 mmol, 1.7 M) of butyl lithium were added. The solution was allowed to warm up...
to 0°C for 20 minutes, resulting in the formation of the yellow lithium intermediate.

In a second Schlenk flask, 1.00 g (8.25 mmol) of 6-N,N-dimethylamino fulvene was dissolved in THF, and the resultant red solution was added via cannula at −78°C to the Schlenk flask containing the lithiated intermediate. The reaction mixture was then allowed to warm up to room temperature and left stirring for 40 minutes. Titanium tetrachloride (4.1 ml, 4.13 mmol) was added afterwards in situ at 0°C. Subsequently, the solvent was removed under vacuum, resulting in the formation of a dark brown oil that was dissolved in dichloromethane and filtered through Celite to remove the LiCl. The black filtrate was filtered additionally twice by gravity filtration. The solvent was removed under reduced pressure forming a shiny black solid, which was washed with 150 ml of pentane and then dried in vacuo (0.45 g, 0.71 mmol, 82.7% yield).

1H NMR (δ ppm CDCl3, 125 MHz, proton decoupled): 146, 138, 136, 132, 129, 127, 124, 120 [(C6H4 CH2N(CH3)2) and (C6H4)]; 61 [(C6H4–CH–(N(CH3)2(C6H4CH2N(CH3)2))] 60 [(C6H4CH2N(CH3)2)]; 42 [C6H4–CH–(N(CH3)2(C6H4CH2N(CH3)2));] IR absorptions (cm−1 KBr): 3444, 3391, 2958, 2670, 2470, 1621, 1467, 1411, 1261, 1071, 1013, 943, 798. Anal. Calc. for C36H46N4TiCl2: C, 60.93; H, 6.53; N, 15.79; Cl, 9.99 Found: C, 59.99; H, 6.52; N, 15.72; Cl, 9.99.

UV-Vis (CH2Cl2): λ max 263 nm (ε 86 000), λ 275 nm (ε 94 000), λ 298 nm (ε 98 000), λ 320 nm (ε 108 000), λ 340 nm (ε 72 000), λ max 455 nm (weak).

3. RESULTS AND DISCUSSION

3.1. Synthesis

6-(N,N-dimethylamino) fulvene (3) was synthesised according to the already published procedure [27], and its structure is shown in Scheme 1.

The use of aryl lithium in the synthesis of other metalloenes is well known [28–30], and it has recently been used for the synthesis of chiral titanocene dichlorides [26].

This time, the carbolithiation method led to the synthesis of a new group of titanocenes that contain stereo centres (5a–c).

The first step of the reaction consists of the formation of the functionalised lithium intermediates (2a–c) by reacting the corresponding heterocycles (1a–c) with tert-butyl lithium. Side reactions were avoided by cooling the reaction down to −78°C during the addition of tert-butyl lithium, and subsequent warming up to 0°C.

This step was followed by a nucleophilic addition of the lithiated intermediate to the double bond of 6-N,N-dimethylamino fulvene at −78°C. Then the reaction mixture was allowed to warm up to 0°C, resulting in the formation of the appropriately substituted lithium cyclopentadienyl intermediates 4a–c. This reaction occurs with no stereo-selectivity, and the intermediates 4a–c already contain a stereogenic carbon.

After stirring the reaction mixture for 40 minutes, two molar equivalents of 4a, 4b or 4c underwent a transmetallation reaction when reacted with TiCl4 under reflux over 20 h in THF to give titanocenes 5a–c.
The compounds obtained are shiny dark red solids. The synthesis of these compounds is shown in Scheme 1.

All three titanocenes shown in this paper have different isomers as seen in Figure 1. As a result of this, three different signals should be seen for every proton and carbon in the $^1$H and $^{13}$C NMR spectra. The R,R and S,S isomers are enantiomers and thus give identical NMR spectra, whereas for protons or carbons corresponding to the R,S (same as S,R) isomer, two signals can be observed as the environment of the two cyclopentadienyl rings is different. A relation of 2 : 1 : 1 for S,S and R,R, and the two signals for the S,R (or R,S) isomers can be observed in the integration pattern.

### 3.2. Cytotoxicity studies

Preliminary in vitro cell tests were performed on LLC-PK cells in order to compare the cytotoxicity of the compounds presented in this paper. This cell line was chosen based on their long-lasting growth behavior, similar to the one shown in carcinoma cells. It was obtained from the ATCC (American tissue cell culture collection) and maintained in Dulbecco’s modified Eagle medium containing 10% (v/v) FCS (foetal calf serum), 1% (v/v) penicillin streptomycin, and 1% (v/v) L-glutamine. Cells were seeded in 96-well plates containing 200 μL microtiter wells at a density of 5,000-cells/200 μL of medium and were incubated at 37°C for 24 h to allow for exponential growth. Then the compounds used for the testing were dissolved in the minimal amount of DMSO (dimethylsulfoxide) possible and diluted with medium to obtain stock solutions of $5 \times 10^{-4}$ M in concentration and less than 0.7% of DMSO. The cells were then treated with varying concentrations of the compounds and incubated for 48 hours at 37°C. Then the solutions were removed from the wells, the cells were washed with PBS (phosphate buffer solution), and fresh medium was added to the wells. Following a recovery period of 24 h incubation at 37°C, individual wells were treated with a 200 μl of a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in medium. The solution consisted of 30 mg of MTT in 30 ml of medium. The cells were incubated for 3 hours at 37°C. The medium was then removed and the purple formazan crystals were dissolved in 200 μL DMSO per well. Absorbance was then measured at 540 nm by a Wallac Victor (Multilabel HTS Counter) Plate Reader. Cell viability was expressed as a percentage of the absorbance recorded for control wells. The values used for the dose response curves of Figure 2 represent the values obtained from four consistent MTT-based assays for each compound tested.

As seen in Figure 2, Titanocenes 5a–c showed an IC$_{50}$ value of 23, 52, and 13 μM, respectively.

When compared to unsubstituted titanocene dichloride (IC$_{50}$ value = 2000 μM), titanocene 5c shows a major decrease in magnitude in terms of the IC$_{50}$ value, and approximately a fourfold increase in magnitude with respect to cisplatin itself (IC$_{50}$ value = 3.3 μM). However, titanocene 5c shows a decrease in cytotoxicity with respect to Titanocene C. The increased polarity of the new titanocenes together with...
B3LYP level using the 6-31G (DFT) calculations were carried out for titanocene crystal structures were obtained. This might be explained by our efforts to crystallise these three titanocenes, no crystal structures were obtained. This might be explained by the existence of different isomers in the racemic mixture. In order to overcome this problem, density functional theory (DFT) calculations were carried out for titanocene 5c at the B3LYP level using the 6-31G** basis set [31], and compared with that of Titanocene C [26].

Selected bond lengths of the optimised structure of titanocene 5c and C are listed in Table 1 (for atom numbering, see Scheme 2). The calculated structure of 5c is presented in Figure 3.

The length of the bond between the metal centre and the cyclopentadienyl carbons is slightly different for the different Cp rings (251.3 and 248.5 pm, resp.). The same applies for the carbon-carbon bonds of the cyclopentadienyl rings with bond lengths between 140.2 and 143.4 pm.

The bond length between the ethyl carbon centre and the carbon centre of the Cp group is of 152.2 and 152.0 pm, respectively. As well, the length of the bond between the ethyl carbon and the nitrogen of the dimethylamino group, C6 or C6′, and C7 or C7′, respectively, was of 11.42° in both cases, and almost identical to the one formed between each nitrogen of the dimethylamino group, C6 or C6′, and C1 and C1′, respectively.

The DFT calculated structure of 5a was then compared to the calculated structure of its mono-N-methylypyrrolyl-substituted analogue, Titanocene C [26]. In this complex, the length of the bond between the titanium centre and the two Cl atoms appeared to differ in only 1 pm approximately from the one found for 5c, and of 234.9 and 236.1 pm, respectively. The same applies for the bond length between the N1 or N2 and C6 or C6′, respectively, and to the length of the bond between the Cp carbon atoms and the titanium centre.

The Cl–Ti–Cl angle in Titanocene C is very similar to the one calculated for 5c, and of 94.9°, and so is the angle formed between the titanium centre and the centre of the Cp rings (with a difference of 0.3°).

Selected bond lengths from the calculated DFT structure of Titanocene C are listed in Table 1, while atom numbering can be seen in Scheme 2.

**Figure 2:** Cytotoxicity studies of Titanocenes 5a–c against LLC-PK cells.

Despite our efforts to crystallise these three titanocenes, no crystal structures were obtained. This might be explained by the existence of different isomers in the racemic mixture. In order to overcome this problem, density functional theory (DFT) calculations were carried out for titanocene 5c at the B3LYP level using the 6-31G** basis set [31], and compared with that of Titanocene C [26].

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Selected bond lengths from the calculated DFT structure of Titanocene C are listed in Table 1, while atom numbering can be seen in Scheme 2.
4. CONCLUSIONS AND OUTLOOK

The carbolithiation of 6-(N,N-dimethylamino) fulvene with Mannich-functionalised lithiated species followed by transmetallation offers a general way into the synthesis of new chiral N,N-dimethylamino-functionalised metalloccenes. Derivative 5c exhibits an impressive cytotoxicity with an IC\textsubscript{50} value of 13 \(\mu\)M against LLC-PK cells, but is slightly less active with respect to Titanocene C that shows the highest cytotoxicity for a published titanocene so far.

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