Novel achiral titanocene anti-cancer drugs synthesised from bis-*N,N*-dimethylamino fulvene and lithiated heterocyclic compounds

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Abstract From the carbolithiation of 6-bis-*N,N*-dimethylamino fulvene (**3a**) and different ortholithiated heterocycles (furan, thiophene and *N*-methylpyrrole), the corresponding lithium cyclopentadienide intermediate (**4a–c**) was formed. These three lithiated intermediates underwent a transmetallation reaction with $TiCl_4$ resulting in bis-*N,N*-dimethylamino-functionalised titanocenes **5a–c**. When these titanocenes were tested against LLC-PK cells, the IC_{50}-values obtained were of 240, and 270 μM for titanocenes **5b** and **5c**, respectively. The most cytotoxic titanocene in this paper, **5a** with an IC_{50}-value of 36 μM was found to be approximately six times less cytotoxic than its mono-*N,N*-dimethylamino substituted analogue Titanocene **C** (IC_{50} = 5.5 μM) and almost ten times less cytotoxic than cisplatin, which showed an IC_{50}-value of 3.3 μM, when tested on the LLC-PK cell line.

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Introduction

Titanium-based reagents have significant potential against solid tumours. Budotitane ([cis-diethoxy-bis(1-phenylbutane-1,3-dionato)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 (Schilling et al. 1996). Much more robust in this aspect of hydrolysis is titanocene dichloride (Cp_2TiCl_2), which shows medium anti-proliferative activity in vitro but promising results in vivo (Melendez 2002; Caruso and Rossi 2004). Titanocene dichloride reached clinical trials, but the efficacy of Cp_2TiCl_2 in Phase II clinical trials in patients with metastatic renal cell carcinoma (Lummen et al. 1998) or metastatic breast cancer (Kröger et al. 2000) was too low to be pursued.

More recently, novel methods starting from fulvenes (Eisch et al. 1998; Fox et al. 2004; Tacke et al. 2004a, b; Rehmann et al. 2005a, b; Sweeney et al. 2005, 2006, 2007; Pampillón et al. 2006a, b, 2007; Strohfeldt et al. 2006) and other precursors (Allen et al. 2004; Causey and Baird 2004; Meyer et al. 2005) 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 μM (Sweeney et al. 2005). The anti-proliferative activity of Titanocene **Y** has been studied in 36 human tumour cell lines (Kelter et al. 2005) and in explanted human tumours (Oberschmidt et al. 2005). These in vitro and ex vivo experiments showed that prostate, cervix and renal cell cancer 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 (O'Connor et al. 2006). Furthermore first animal studies have been published recently reporting the successful treatment of xenografted Ehrlich's ascites tumour in mice with an ansa-titanocene (Valadares et al. 2006) and xenografted Caki-1 tumours with Titanocene **Y** (Fichtner et al. 2006), showing that the effect of Titanocene **Y** against xenografted Caki-1 tumours in mice was superior to cisplatin. The structure of Titanocene **Y** is shown in Fig. 1.

So far our most cytotoxic titanocene, Titanocene **C** (bis-(*N,N*-dimethylamino-2(*N*-methylpyrrolyl)methylcyclopentadienyl) titanium (IV) dichloride, was obtained through carbolithiation of fulvenes which has been published recently (Pampillón et al. 2006a, b). It has an IC_{50}-value of 5.5 μM when tested on the LLC-PK cell line. This meant significant progress, since Cp_2TiCl_2 exhibits an IC_{50}-value of only 2,000 μM against LLC-PK, which explains partly the failed Phase II clinical trials against renal cell carcinoma. However, Titanocene **C** presented various isomers, as it contained a stereocentre. Its structure can be seen in Fig. 1.

The main idea behind the research presented in this paper was to obtain titanocenes with no chiral centre but maintaining the cytotoxicity of Titanocene **C** and its analogues by adding extra dimethylamino groups, as these substituents have proven to be quite useful in increasing the activity of titanocene dichloride derivatives. Within this paper we present a new series of achiral titanocenes, their synthesis from heterocycles and preliminary cytotoxicity studies.

Experimental

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. 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.

Synthesis

6-Bis-(*N,N*-dimethylamino) fulvene was synthesised according to the already published procedure (Hafner et al. 1964).
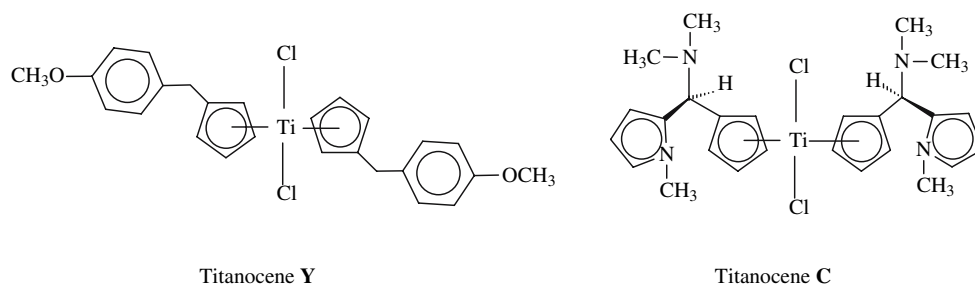

Fig. 1 Structure of titanocenes **Y** and **C**

Bis-(bis- (N,N-dimethylamino)-2-(N'-methylpyrrolyl)methylcyclopentadienyl) titanium (IV) dichloride, {η⁵-C₅H₄-CH[N(CH₃)₂]₂[C₅H₃NCH₃]}₂TiCl₂ (5a)

To a Schlenk flask with 0.49 g (6.08 mmol) of *N*-methylpyrrole, 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 min and 12.0 ml (6.08 mmol) of butyl lithium were added. The solution was allowed to warm up to 0°C for 20 min, resulting in the formation of the yellow lithium intermediate.

In a second Schlenk flask 1.00 g (6.08 mmol) of 6-bis-(*N,N*-dimethylamino) fulvene were dissolved in THF, and the resultant orange 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 min. Titanium tetrachloride (3.04 ml, 3.04 mmol) was added afterwards in situ at room temperature and the mixture was refluxed for 20 h. 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 (1.03 g, 1.70 mmol, 56.0% yield).

^{1}H NMR (δ ppm CDCl₃, 300 MHz): 6.40–6.30 [m, 8H, C₅H_4]; 6.25–6.20 [m, 6H, C₄H_3NCH₃]; 3.04 [s, 30H, C₅H₄-C-(C₄H₃NCH_3)((NCH_3)₂)₂].

^{13}C NMR (δ ppm CDCl₃, 125 MHz, proton decoupled): 135, 122, 110, 108 [C_4H₃NCH₃]; 120, 122 [C_5H₄]; 45 [C₅H₄-C-(C₄H₃NCH₃)((NCH₃)₂)₂]; 29 [C₅H₄-C-(C₄H₃NCH₃)((NCH₃)₂)₂].

IR absorptions (cm^{-1} KBr): 3,100, 2,931, 2,832, 1,606, 1,509, 1,461, 1,425, 1,299, 1,174, 1,108, 1,031, 831.

Anal. Calc. for C₃₀H₄₄N₆TiCl₂: C, 59.31; H, 7.29; N, 13.84; Cl, 11.67 Found: C, 59.30; H, 7.30; N, 13.85; Cl, 11.68.

UV-Vis (CH₂Cl₂): λ 270 nm (ε 33,440), λ 412 nm (ε 3,120), λ 499 nm (ε 216), λ_{max} 520 nm (weak).

Bis-(bis-(N,N-dimethylamino)-2-furylmethylcyclopentadienyl) titanium (IV) dichloride, {η⁵-C₅H₄-CH[N(CH₃)₂]₂[C₅H₃O]}₂TiCl₂ (5b)

To a Schlenk flask with 0.4 ml (6.08 mmol) furan, 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 min and 12.2 ml (6.08 mmol) of butyl lithium were added. The solution was allowed to warm up to 0°C for 20 min, resulting in the formation of the yellow lithium intermediate.

In a second Schlenk flask 1.00 g (6.08 mmol) of 6-bis-*N,N*-dimethylamino fulvene were 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 min. Titanium tetrachloride (3.0 ml, 3.04 mmol) was added afterwards in situ at room temperature and the mixture was refluxed for 20 h. Subsequently the solvent was removed under vacuum, resulting in the formation of a dark green 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 20 ml of pentane and then dried in vacuo (1.02 g, 1.76 mmol, 57.9% yield).

^{1}H NMR (δ ppm CDCl₃, 300 MHz): 7.21–5.90 [m, 8H, C₄H_4O]; 6.40–6.30 [m, 8H, C₅H_4]; 3.06 [s, 30H, C₅H₄-C-(C₄H₄O)((NCH_3)₂)₂].

^{13}C NMR (δ ppm CDCl₃, 125 MHz, proton decoupled): 153, 142, 111, 106 [C_4H₄O]; 120, 121 [C_5H₄]; 45 [C₅H₄-C-(C₄H₄O)((NCH₃)₂)₂]; 29 [C₅H₄-C-(C₄H₄O)((NCH₃)₂)₂].

IR absorptions (cm^{-1} KBr): 3,112, 2,964, 2,780, 1,608, 1,460, 1,405, 1,259, 1,179, 1,086, 1,023, 828, 614.

Anal. Calc. for C₂₈H₃₈N₄O₂TiCl₂: C, 57.84; H, 6.59; N, 9.63; Cl, 12.19 Found: C, 57.80; H, 6.62; N, 9.64, Cl, 12.20.

UV-Vis (CH₂Cl₂): λ 230 nm (ε 22,770), λ 402 nm (ε 2,020), λ 499 nm (ε 210), λ_{max} 523 nm (weak).

Bis-(bis-(N,N-dimethylamino)-2-thiophenylmethylcyclopentadienyl) titanium (IV) dichloride, {η⁵-C₅H₄-CH[N(CH₃)₂]₂[C₅H₃S]}₂TiCl₂ (5c)

To a Schlenk flask with 0.58 ml (6.08 mmol) 2-bromothiophene, 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 and 12.16 ml (6.08 mmol) of butyl lithium were added. The solution was allowed to warm up to 0°C for 20 min, resulting in the formation of the yellow lithium intermediate.

In a second Schlenk flask 1.0 g (6.08 mmol) of 6-bis-(*N,N*-dimethylamino) fulvene were 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 min. Titanium tetrachloride (3.04 ml, 3.04 mmol) was added afterwards in situ at room temperature and the mixture was refluxed for 20 h. Subsequently the solvent was removed under vacuum, resulting in the formation of a dark green 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

20 ml of pentane and then dried in vacuo (1.27 g, 2.06 mmol, 67.8% yield).

^{1}H NMR (δ ppm CDCl₃, 300 MHz): 6.93–6.62 [m, 8H, C₄*H*₄S]; 6.40–6.30 [m, 8H, C₅*H*₄]; 3.14 [s, 30H, C₅H₄-C-(C₄H₄S)((NC*H*₃)₂)₂].

^{13}C NMR (δ ppm CDCl₃, 125 MHz, proton decoupled): 140, 127, 125, 124 [*C₄*H₄S]; 121, 119 [*C₅*H₄]; 48 [C₅H₄-*C*-(C₄H₄S)((N*C*H₃)₂)₂]; 29 [C₅H₄-*C*-(C₄H₄S)((N*C*H₃)₂)₂].

IR absorptions (cm^{-1} KBr): 3,412, 2,931, 2,800, 1,608, 1,460, 1,405, 1,259, 1,179, 1,086, 1,023, 828, 614.

Anal. Calc. for C₂₈H₃₈N₄S₂TiCl₂: C, 54.81; H, 6.24; N, 9.13; S, 10.45; Cl, 11.56 Found: C, 54.82; H, 6.25; N, 9.11; S, 10.45; Cl, 11.55.

UV-Vis (CH₂Cl₂): λ 260 nm (ε 72,770), λ 402 nm (ε 17,020), λ 499 nm (ε 210), λ_{max} 523 nm (weak).

Results and discussion

Synthesis

6-Bis-(*N,N*-dimethylamino) fulvene (**3a**) was synthesised according to the already published procedure (Hafner et al. 1964), and its structure is shown in scheme 1.

The use of aryl lithium in the synthesis of other metallocenes is well known (Qian et al. 1997;
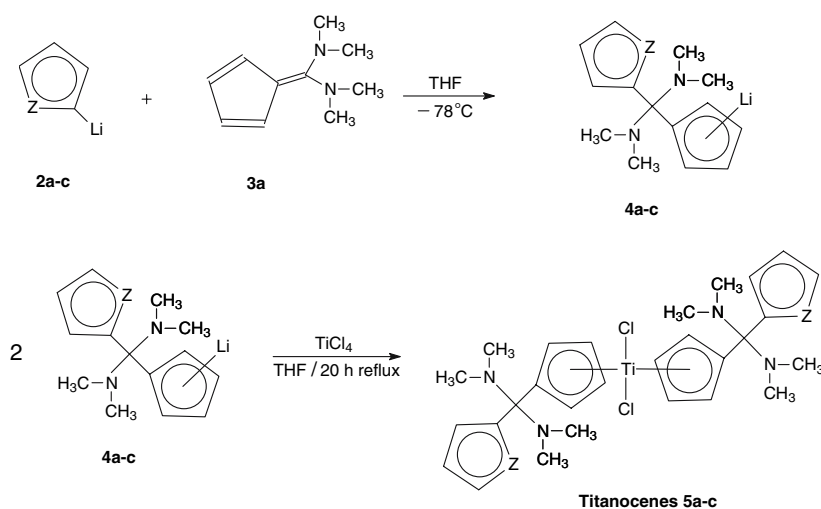
Scheme 1 Synthesis of Titanocenes **5a–c** (Z = N-CH₃, O, S)

Horacek et al. 2002; Knuppel et al. 2005), and it has recently been used for the synthesis of chiral titanocene dichlorides (Pampillón et al. 2006a, b). This time, the carbolithiation method led to the synthesis of a new group of titanocenes that do not contain stereo centres (**5a–c**).

The first step of the reaction consists on 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 stereo selectivity, and the intermediates **4a–c** do not contain any stereogenic carbons.

After stirring the reaction mixture for 40 min, two molar equivalents of **4a**, **4b** or **4c**, underwent a transmetallation reaction when reacted with $TiCl_4$ 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.

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 behaviour, 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 microtitre 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 5×10^{-4} M in concentration and <0.7% of DMSO. The cells were then treated with varying concentrations of the compounds and incubated for 48 h at 37°C. Then, the solutions were removed from the wells and 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.
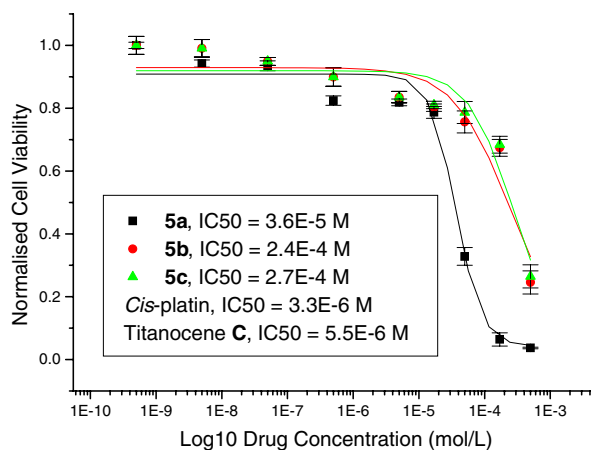

Fig. 2 Cytotoxicity studies of titanocenes **5a–c** against LLC-PK cells

The cells were incubated for 3 h 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 Fig. 2 represent the values obtained from four consistent MTT-based assays for each compound tested.

As seen in Fig. 1, titanocenes **5a–c** showed an IC_{50}-value of 36, 240 and 270 μM, respectively.

When compared to unsubstituted titanocene dichloride, titanocene **5a** has a 55-fold decrease in magnitude in terms of the IC_{50}-value, and approximately, a tenfold increase in magnitude with respect to cisplatin itself (IC_{50}-value = 3.3 μM). However, titanocene **5a** shows a decrease in cytotoxicity with respect to Titanocene **C**. This is likely to be due to the extra *N,N*-dimethylamino groups, as the mono-*N,N*-dimethylamino-substituted analogue of **5a** shows a higher IC_{50}-value of 5.5 μM (Pampillón et al. 2006a, b). The increased polarity of the new titanocenes together with an increase in size might be the cause of the decrease in cytotoxicity shown.

Structural DFT discussion

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 **5a** at the B3LYP level using the 6–31G** basis set (Gaussian '03 2004).

Selected bond lengths of the optimised structure of this titanocene are listed in Table 1 (for atom numbering see scheme 2). The calculated structure of **5a** is presented in Fig. 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 p.m., respectively). The same applies for the carbon–carbon bonds of the cyclopentadienyl rings with bond lengths between 140.2 and 143.4 p.m.

The bond length between the methylic carbon centre and the carbon centre of the Cp group is of 152.2 and 152.0 p.m., respectively. As well, the

Table 1 Selected bond lengths from the DFT- calculated structure of **5a** and DFT- calculated structure of Titanocene **C**

	DFT structure (**5a**) Bond length (p.m.)	DFT structure Titanocene **C** Bond length (p.m.)
$Ti–C_1$	251.3	250.4
$Ti–C_2$	242.9	242.8
$Ti–C_3$	244.2	240.0
$Ti–C_4$	240.4	237.4
$Ti–C_5$	243.2	242.9
$Ti–C_{1'}$	248.5	247.8
$Ti–C_{2'}$	239.1	239.0
$Ti–C_{3'}$	234.2	233.1
$Ti–C_{4'}$	245.6	243.7
$Ti–C_{5'}$	250.2	249.3
$C_1–C_2$	143.4	143.2
$C_2–C_3$	141.6	141.5
$C_3–C_4$	141.2	141.3
$C_4–C_5$	142.3	142.3
$C_5–C_1$	141.4	141.4
$C_{1'}–C_{2'}$	141.4	141.4
$C_{2'}–C_{3'}$	142.2	142.4
$C_{3'}–C_{4'}$	142.3	142.2
$C_{4'}–C_{5'}$	140.2	140.2
$C_{5'}–C_{1'}$	143.0	143.0
$C_1–C_6$	152.2	152.2
$C_{1'}–C_{6'}$	152.2	152.0
$C_6–C_{6'}$	559.5	559.5
$C_6–C_7$	151.5	152.0
$C_{6'}–C_{7'}$	152.0	151.5
$C_6–N_1$	152.0	148.3
$C_{6'}–N_2$	149.7	148.4
$C_6–N_{1'}$	148.2	
$C_{6'}–N_{2'}$	149.7	
$Ti–Cl_1$	240.5	234.9
$Ti–Cl_2$	234.1	236.1

length of the bond between the methylic carbon and the nitrogen of the dimethylamino group is almost identical in all cases, and between 152.0 and 148.2 p.m., respectively. The steric impediment of the extra dimethylamino groups attached to the methylic carbons causes a lengthening of the bond, in order to relieve the resultant steric strain.

The Cl–Ti–Cl angle was calculated to be 95.1°. The angle formed between C_1 and C_1, the respective methylic carbons (C_6 or $C_{6'}$), and C_7 or C_7, respectively, was of 114.2° in both cases, and almost
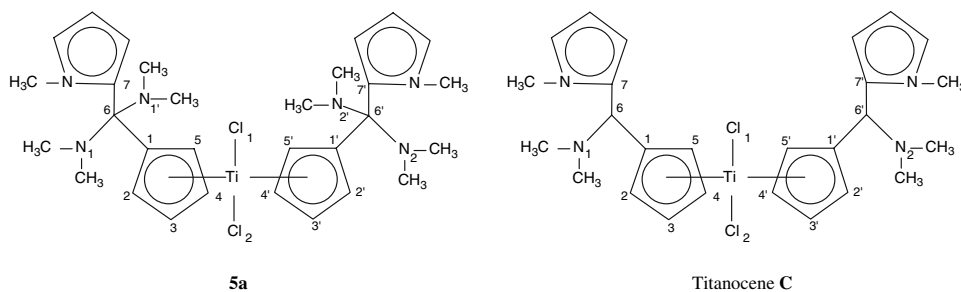
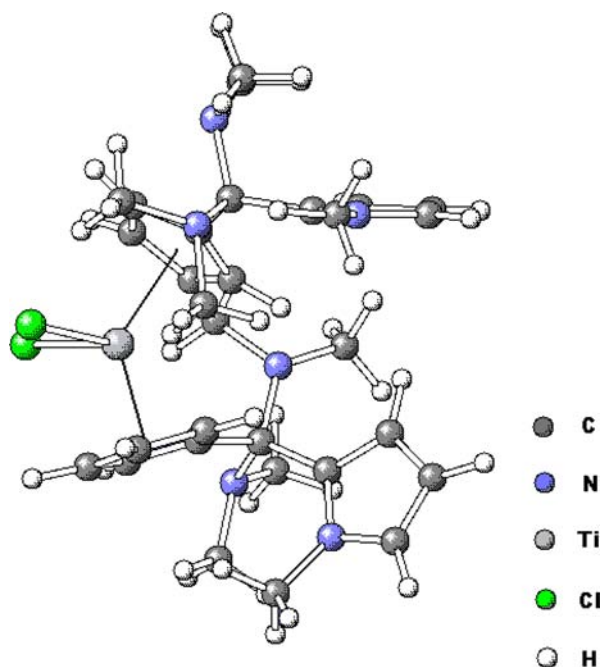**Scheme 2** Numbering scheme of **5a** and Titanocene **C** for the structural DFT discussion of **5a**

Fig. 3 DFT calculated structure of **5a**

identical to the one formed between each nitrogen of the dimethylamino group, C_6 or C_6, and C_1 and C_1, respectively.

The DFT calculated structure of **5a** was then compared to the calculated structure of its mono-*N,N*-dimethylamino- substituted analogue, Titanocene **C** (Pampillón et al. 2006a, b). In this complex, the length of the bond between the titanium centre and the two Cl atoms appeared to differ in only 1 p.m. approximately from the one found for **5a**, and of 234.9 and 236.1 p.m., respectively. The same applies to the bond length between the N_1 or N_2 and C_6 or C_6, 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 **5a**, 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.

Conclusions and outlook

The carbolithiation of 6-bis-(*N,N*-dimethylamino) fulvene with lithiated heteroaryl species followed by transmetallation offers a general way into the

synthesis of new achiral heteroaryl-substituted and bis-*N,N*-dimethylamino-functionalised metallocenes but with a decrease in cytotoxicity with respect to Titanocene **C** that shows the highest cytotoxicity for a titanocene against LLC-PK cells.

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