A series of ferrocenyl ester complexes, varying the lipophilic character of the pendant groups, was prepared and characterized by spectroscopic and analytical methods. The syntheses of Fe(C5H4CO2CH3)2, Fe(CpCOOCH3) (CpCOOCH2CH3), and Fe(CpCOOCH2CH3)2 are reported. The solid-state structure of Fe(C5H4CO2CH3)2 has been determined by X-ray crystallography. Fe(C5H4CO2CH3)2 has the cyclopentadienyl rings virtually in an eclipsed conformation with the pendant groups not completely opposite to each other. Cyclic voltammetry characterization showed that the functionalized ferrocenes oxidize at potentials, Epa, higher than ferrocene as a result of the electro withdrawing effect of the pendant groups on the cyclopentadienyl ligand. The cytotoxicities of Fe(C5H4CO2CH2CH2OH), Fe(C5H4CO2CH2CH=CH2), Fe(C5H4CO2CH3), Fe(CpCOOCH3), and Fe(CpCOOCH2CH3) in colon cancer HT-29 and breast cancer MCF-7 cell lines were measured by the MTT biological viability assay and compared to ferrocene and ferrocenium. Fe(C5H4CO2CH2CH=CH2) showed the best IC50 values, 180(10) μM for HT-29 and 190(30) μM for MCF-7 cell lines, with cytotoxicities similar to ferrocenium. The cytotoxic data suggest that as we increase the lipophilic character of the functionalized ferrocene, the cytotoxicity improves approaching to the cytotoxic activity of ferrocenium.

1. Introduction

Modern organometallic chemistry has been greatly influenced by the accidental discovery of bis(cyclopentadienyl)iron(II) (ferrocene) in 1951 [1, 2]. In fact, ferrocene (Cp2Fe) has been the most extensively studied organometallic species. Its applications in catalysis, organic synthesis, and industrial processes are numerous [3–6]. The thermal stability, inert character in concentrated acid and base solutions as well as the redox properties of ferrocene makes this species a versatile compound in many research areas.

Until 1979, the biological properties of metallocenes were unexplored. The report of titanocene dichloride (Cp2TiCl2) as the first metalocene to possess antitumor activity opened a new area of research, bioorganometallics, which has been developing rapidly in the last twenty eight years [7, 8].

In 1984, Köpf-Maier et al. reported the anticancer activity of ferrocene complex in Ehrlich ascites tumor [9]. Since then, ferrocene has been studied for potential biological and medicinal applications [8, 10, 11]. Originally, the oxidized species of ferrocene, ferrocenium (Cp2Fe+) was reported to be responsible for the cytotoxic properties on DNA mediated through its capacity to generate oxygen-free radical species [12, 13]. Ferrocene has been recently reported to have antitumor properties due to the metabolic formation of ferrocenium ions which induces oxidative damage to DNA [14, 15]. As a result, many functionalized ferrocenes have been prepared and tested in cancer cells [11].

We have initiated a project on functionalized ferrocene chemistry, with pendant groups (functional groups, R) on the Cp rings varying their polar and lipophilic characters, see Scheme 1. The objective of this study is to understand how the polar and lipophilic characters of the pendant groups change the anticancer activity of the corresponding ferrocenyl derivatives. We have investigated the synthesis, structure, electrochemistry, and cytotoxic properties of five ferrocenyl esters in HT-29 colon cancer and MCF-7 breast cancer cell lines. The objective of this study is to report these novel findings.
2. Results and Discussion

2.1. Synthesis and Structure. The syntheses of Fe(C₅H₄–CO₂CH₂CH₃)₂, Fe(C₅H₄CO₂CH₂CH₃)₂, and Fe(C₅H₄–CO₂CH₂CH₂OH)₂ have been reported previously [16, 17]. We applied the methodology developed by Busetto et al. to synthesize Fe(C₅H₄CO₂CH₂CH₃)₂ and Fe(C₅H₄CO₂–CH₂CH₃)(C₅H₄CO₂CH₃) (mixed ferrocene) [17]. Interestingly, using this synthetic methodology, the reaction of FeCl₃ and one equivalent of NaC₅H₄CO₂CH₂CH₃ and NaC₅H₄CO₂CH₃, even at room temperature, allowed the selective incorporation of two distinct functionalized Cp ligands, affording the mixed ferrocene complex in good yield without the formation of Fe(C₅H₄CO₂CH₂CH₃)₂ and Fe(C₅H₄CO₂CH₂CH₃)(C₅H₄CO₂CH₃).

The NMR, IR, and elemental analysis corroborate the identities of the ferrocenyl ester complexes. The IR spectral data showed ν(C=O) broadbands at 1708 cm⁻¹ (Fe(C₅H₄CO₂CH₂CH₃)₂) and 1712 cm⁻¹ (Fe(C₅H₄CO₂–CH₂CH₃)(C₅H₄CO₂CH₃) corresponding to the carbonyl groups of the esters. In the ¹H NMR spectrum, Fe(C₅H₄–CO₂CH₂CH₃)₂ exhibits two sets of resonances at 4.42 and 4.85 ppm. These Cp protons exhibit coupling belonging to an AA’BB’ spin system. For the Fe(C₅H₄CO₂CH₂–CH₃)(C₅H₄CO₂CH₃), the ¹H NMR spectrum shows two sets of resonances at 4.42 and 4.85 ppm corresponding to the Cp protons of C₅H₄CO₂CH₂CH₃ and C₅H₄CO₂CH₃ rings. While we might expect four sets of resonances in the vinyl region corresponding to two different substituted Cp rings, the fact that we observed only two sets suggests overlapping between these Cp proton signals. Particularly interesting in the ¹³C NMR spectrum, at room temperature, two sets of resonances for each carbon atom are observed in a ratio of 1:1. This suggests that two possible conformational diastereoisomers, syn and anti, coexist in solution as discussed below (Scheme 2) [18].

In solution, rapid ring oscillation about Cp–Fe–Cp axis allows H₂ and H₃ (H₁' and H₃') and H₂ and H₄ (H₃ and H₄') to become equivalent. This situation applies for both substituted Cp rings. Since both Cp rings have ester groups, overlapping of the H₂/H₃ and H₂'/H₃' and H₃/H₄ and H₃'/H₄' signals in the ¹H NMR spectrum is expected. However, the position of the ester groups yields to conformational diastereoisomers, syn and anti, which differ in the orientation of the carbonyl groups [18]. This condition applies when the C(Cp)–C(CO) bond is slow in the NMR scale time.

When the rotation is fast, having enough energy to overcome the rotational barrier of the C(Cp)–C(CO) bond, such stereolabile chirality disappears. In the ¹³C NMR spectrum, the carbonyl orientation (syn and anti) makes significantly different magnetic environment on the Cp carbons as well as on the carbonyl groups such that two sets of resonances corresponding to syn and anti conformers can be observed. Variable temperature NMR studies, in toluene-d₈, showed that at 80°C in the ¹H spectrum, the multiplet signal at 4.85 ppm became a single line and the multiplet at 4.43 ppm broadened. These signals belong to the Cp rings. In the ¹³C NMR spectrum at 80°C, the carbon signals of one conformer begin to increase in intensity at expense of the other conformer, reaching a ratio of 1:2. This suggests that conformational diastereoisomers, syn and anti, at room temperature, have very similar stability but at high temperature, one diastereoisomer (presumably) anti becomes more populated.

In general, the present synthetic route developed by Busetto et al. [17] for the ferrocenyl ester complexes (Cp functionalization and formation of the corresponding functionalized ferrocene) represents a convenient alternative procedure for the functionalization of metalloenes and has been used by our group to synthesize functionalized titanocenes [19].

The solid-state structure of Fe(C₅H₄CO₂CH₂CH₃)₂ was investigated by single-crystal X-ray diffraction, see Figure 1. Crystal data and structure refinement are summarized in the Supplementary Material available online at doi: 10.1155/2009/420784 (Cambridge Crystallographic Data Centre and the deposition number is CCDC 705387). Bonding parameters are included in Supplementary Table 1.

As shown by X-ray, this ferrocene is a sandwich complex with the cyclopentadienyl ligands adopting an eclipsed conformation and with the pendant groups not completely opposite to each other. The average Fe–C(Cp) distances for Fe(C₅H₄CO₂CH₂CH₃)₂ are 2.049 and 2.048 Å which is very similar to those reported for ferrocene, 2.045 Å [20]. The shortest Fe–C(Cp) bond distances are on C(5) and C(7). The shorter bond distances could be attributed to the inductive (electro-withdrawing) effect of the ester groups.
on these carbon atoms. The ester groups are not coplanar with the Cp ring, one of the OCH$_3$ is bent toward the iron atom (below the Cp plane) with a torsion angle of 15.2° while the other is bent away from the iron atom by 6.9°. The C(Cp)–C(CO) bonds (C(5)–C(13) and C(7)–C(11)) are somewhat shorter than a typical C–C single bond, 1.477 Å versus 1.54 Å (for a single bond), suggesting these bonds have partial double bond character as a result of conjugation with the Cp π system [21]. The later might explain the high C(Cp)–C(CO) rotational barrier encountered in the Fe(C$_5$H$_4$CO$_2$CH$_2$CH$_3$)(C$_5$H$_4$CO$_2$CH$_3$) complex. In addition, the two CO$_2$Me groups are anti to each other.

While the solid structure of Fe(C$_5$H$_4$CO$_2$CH$_3$)$_2$ has been reported previously, our solid-state structure has a fundamental difference to that published by Cetina et al. [22]. That is, in the lattice, they determined a hydrogen bonding network between the carbonyl group and the CH$_3$ of the adjacent molecule. We could not determine such hydrogen bonding network between the carbonyl group and the CH$_3$ of the adjacent molecule even though our data was collected at lower temperature (173 K versus 293 K) and we obtained better refinement parameter (R (I$_{2sigma}$ (I)) 0.0397 versus 0.046).

2.2. Cyclic Voltammetry. Electrochemical characterization of the subject complexes was performed by mean of cyclic voltammetry (CV). It is well known that ferrocene easily undergoes one electron oxidation to form ferrocenium (Cp$_2$Fe$^+$) in a reversible manner. Thus, we investigated the functionalized ferrocenes electrochemical behaviors and compared to ferrocene in organic solvent. Table 1 presents the CV results. As it can be seen in the cyclic voltammograms (Figure 2), ferrocene and Fe(C$_5$H$_4$CO$_2$CH$_3$)$_2$, as well as all the remaining functionalized ferrocenes undergo a reversible redox process with an $i_{pa}/i_{pc}$ ratio close to one. All the functionalized ferrocene demonstrated oxidation potentials, $E_{pa}$, higher than ferrocene. This is the result of the electron withdrawing (inductive) effect of the ester groups on the Cp rings. Since ferrocene is known to undergo one-electron redox process, it can be deduced that the subject complexes also undergo one-electron redox change. Thus, this major redox process on the functionalized ferrocenes is associated to the ferrocene/ferrocenium couple.

2.3. Cytotoxic Studies—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT): Colorimetric Assay for Cytotoxicity Analysis on the Colon Cancer HT-29 and Breast MCF-7 Cell Lines. The cytotoxicities of the ferrocenyl ester complexes on the HT-29 colon cancer and MCF-7 breast cancer cell lines were measured using a slightly modified MTT assay [23, 24]. Ferrocene was initially evaluated at time intervals of 72, 96, and 120 hours at concentrations that ranged from 10–1200 μM, in HT-29, to determine its optimal

### Table 1: Redox potential of ferrocenyl esters in CH$_3$CN 1M CH$_3$CN 1M [NBu$^+$$_4$]PF$_6$ at a scan rate of 100 mV/s, referenced to ferrocene/ferrocenium redox couple. Ferrocene concentration of $2 \times 10^{-3}$ M. $E_{1/2}$ is an average of the anodic and cathodic peaks potentials.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$E_{1/2}$ (mV)</th>
<th>$\Delta E$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(C$_5$H$_4$CO$_2$CH$_3$)$_2$</td>
<td>454</td>
<td>75</td>
</tr>
<tr>
<td>Fe(CpCOOCH$_3$CH$_3$)$_2$</td>
<td>448</td>
<td>74</td>
</tr>
<tr>
<td>Fe(CpCOOCH$_3$)(CpCOOCH$_2$CH$_3$)$_2$</td>
<td>452</td>
<td>78</td>
</tr>
<tr>
<td>Fe(C$_5$H$_4$CO$_2$CH$_2$CH=CH$_2$)$_2$</td>
<td>453</td>
<td>85</td>
</tr>
<tr>
<td>Fe(C$_5$H$_4$CO$_2$CH$_2$CH$_2$OH)$_2$</td>
<td>456</td>
<td>89</td>
</tr>
</tbody>
</table>
activity (Supplementary Figure 1S). Ferrocene displayed comparable activity at all three time intervals, with an IC$_{50}$ value of $3.6 \times 10^{-4}$ M. Since exposing the cells to ferrocene at longer periods of time did not increase its cytotoxic activity, all subsequent experiments were performed at a drug exposure time of 72 hours. Ferrocene, ferrocenium, and the functionalized complexes ($\text{CO}_2R$, $R$=Me, Et) were tested in concentrations which ranged from 10–1200 $\mu$M. In addition, two control experiments were run 100% Medium and 5% DMSO/95% Medium. Both control experiments behaved identical demonstrating that 5% DMSO in the Medium does not have any cytotoxic effect on these cells. Table 2 summarizes the results of the cytotoxicity experiments and Figure 3 depicts the cytotoxic curves from MTT assays showing the effect of the ferrocene complexes on the viability of HT-29 colon cancer cell line. The IC$_{50}$ value represents the concentration of the ferrocene at which the cell growth is inhibited by 50%. It can be noted that bis(carboethoxycyclopentadienyl)ferrocene showed activity comparable to ferrocene, with values of 370 and 360 $\mu$M, respectively but it has less cytotoxicity than ferrocenium (180 $\mu$M). In contrast, (carboethoxycyclopentadienyl)-(carboxethoxycyclopentadienyl)-ferrocene and the bis(carboxethoxycyclopentadienyl)-ferrocene exhibited less cytotoxic activity than ferrocene and ferrocenium. The carboxethoxy functionalization has been shown previously to inactivate or lower the cytotoxic activity of resulting titanocene complexes [19]. The present results clearly show how the presence of the carboxethoxy substituent in the cyclopentadienyl ring lowers the activity of ferrocene in a stepwise manner, being the bis(carboxethoxy)ferrocene less active than (carboxethoxy)(carboxethoxy)ferrocene. The carboxethoxy functionalization does not improve the activity of the ferrocene complexes as compared to ferrocene.

Other ferrocenyl complexes, varying the lipophilic character of the carboalkoxy groups, were also evaluated for cytotoxic activity against HT-29 cells. Ferrocene complexes with either terminal alcohol or allyl groups were tested in concentrations that ranged from 13 to 1300 $\mu$M at a 72-hour time interval. IC$_{50}$ values for these complexes are also summarized in Table 2, and Figure 4 shows the cytotoxic curves for these complexes along with ferrocene and ferrocenium for comparison. The ferrocene alcohol complex shows an IC$_{50}$ value of 370 $\mu$M which is comparable to ferrocene, while (allyl) complex shows higher cytotoxic activity than ferrocene and equal to the ferrocenium at the time interval studied, with an IC$_{50}$ value of 180 $\mu$M.

The initial work performed by Kopf-Maier et al. demonstrated that ferrocenium possesses in vivo anticancer activity in breast cancer [9]. Based on this precedent, we investigated our functionalized ferrocenes in MCF-7 breast cancer cell line. Surprisingly, ferrocene showed to be less cytotoxic in breast cancer than in colon cancer. With regard to the functionalized ferrocenes and similar to the results on HT-29 cell line, a pattern in the cytotoxicity as we change the functional group (pendant group) is evident. First, in the ferroceny with carboalkoxy groups, the incorporation of methyl ester ($\text{CO}_2\text{CH}_3$) groups on the Cp rings decreases the cytotoxic activity of the resulting complexes. Second, see Figure 5, the increase in the lipophilic character on the carboalkoxy substituents such as in Fe($\text{C}_5\text{H}_4\text{CO}_2\text{CH}_2\text{CH}=\text{CH}_2$)$_2$, where the ethyl group is substituted by an olefin, increases the cytotoxic activity. Such behavior has been reported previously by two-independent research groups, as described below [12, 25].
A wide variety of ferrocenyl derivatives have been synthesized by several research groups aimed to improve their anticancer activity as well as to understand the structure-activity relationship. Very active ferrocenyl derivatives have been synthesized containing estrogen receptor modulators as pendant groups [26]. Among them, 1,1′-bis-(4′-hydroxyphenyl)-2-ferrocenyl-but-1-ene showed strong antiproliferative activity in both hormone-dependent (MCF-7) and hormone-independent (MDA-MDA231) breast cancer cells with IC\textsubscript{50} = 0.7 and 0.6 \mu M, respectively [26]. On the other hand, ferrocenyl carbohydrides conjugates prepared by Orvig et al. showed IC\textsubscript{50} values between 87–468 \mu M on HTB-129 human breast cancer cell line [27]. Our ferrocenyl esters showed IC\textsubscript{50} values similar to those of the ferrocenyl carbohydrides [27] and ferrocenium derivatives tested on MCF-7 cell line: ferroceniumcarboxylic acid tetrafluoroborate (IC\textsubscript{50} = 340 \mu M), decamethylferrocene tetrafluoroborate (IC\textsubscript{50} = 37 \mu M), 1,1′-dimethylferrocenium tetrafluoroborate (IC\textsubscript{50} = 320 \mu M), and ferrocenium boronic acid tetrafluoroborate (IC\textsubscript{50} = 317 \mu M) [12]. It should be clearly noticed that, as reported previously, the increase in the lipophilic character on the ferrocene or ferrocenium complex increases the cytotoxic activity [12, 25].

For colon cancer, the cytotoxic data is more limited for a more meaningful comparison and assessment. On a recent review on the medicinal properties of ferrocenes, it was described a series of ferrocene conjugates (functionalized) anchored to polyspartimides (water soluble carrier polymers) and evaluated on Colo and Hela colon cancer cell lines [11, 28]. The IC\textsubscript{50} values determined are in the 10\textsuperscript{−4}–10\textsuperscript{−3} M range, analogous to some of our most active species. From these studies, Neuse et al. found that increasing the hydrophilicity of the polyspartamide side chains does not necessarily improve the cytotoxicity of the resulting ferrocenes, similar to our findings [28]. On the other hand, opposite to our findings, Kenny et al. studied the antiproliferative effect of a series of N-(ferrocenyl)benzoyl dipeptide esters on H1229 lung cancer cells and found that increasing the alky chain length of the amino acid lower the cytotoxic activity [29]. However, since the data come from different cancer cell lines, the comparison of the IC\textsubscript{50} values becomes less accurate.

Finally, an important conclusion derived from this study is that minor changes in the pendant group on the Cp ring may have notable impact in the cytotoxic activity of the resulting ferrocenes. This is in good agreement with previous reports [12, 25]. Increasing the lipophilic character of the ferrocenyl esters apparently improves their biological activity, as a result of better transport of these species into the target place inside the cell [12, 13, 25]. This increased lipophilic character on the ferrocenes increases the cellular uptake (improving cell membrane permeability of the ferrocene), and more ferrocene molecules could become available inside the cell to express their activity. It has been proposed by Lander et al. that not only ferrocenium cation but also ferrocene is able to express oxidative stress [15]. Ferrocene is able to generate \text{H}_2\text{O}_2 by autoxidation, forming ferrocenium ions. The immune-stimulatory properties of ferrocene are postulated to be mediated by redox-sensitive signaling proteins [15].

3. Experimental

3.1. General Procedure. All reactions were performed under an atmosphere of dry nitrogen using Schlenk glassware or a glovebox, unless otherwise stated. Reaction vessels were flame dried under a stream of nitrogen, and anhydrous solvents were transferred by oven-dried syringes or cannula. Tetrahydrofuran was dried and deoxygenated by distillation over K-benzophenone under nitrogen. Infrared spectra were recorded on a Bruker Vector-22 spectrometer with the samples as compressed Kbr discs. The NMR spectra were obtained on a 500 MHz Bruker spectrometer. Elemental analyses were obtained from Atlantic Microlab, Inc., Ga, USA. Electrochemical characterization was carried out on a BAS CV050W voltammetric analyzer of Bioanalytical Systems, Inc. with a three-stand electrode cell. Cyclic voltammetric experiments were performed in deoxygenated CH\textsubscript{3}CN solution of ferrocene complexes with 1M of [NBu\textsubscript{n}4PF\textsubscript{6} as supporting electrolyte and ferrocene complex concentration of 2 \times 10\textsuperscript{−3} M. The three electrodes used were platinum disk as the working electrode, Ag/AgCl as a reference electrode, and Pt wire as an auxiliary electrode. The working electrode was polished with 0.05 \mu m alumina slurry for 1–2 minutes, and then rinsed with double-distilled and deionized water. This cleaning process is done before each CV experiment and a sweep between 0 and 2000 mV is performed on the electrolyte solution to detect any possible deposition of ferrocene on the electrode surface.

Dimethyl carbonate, diethyl carbonate, diallyl carbonate, ethylene carbonate, diethyl ether (anhydrous \geq 99.7\%), anhydrous FeCl\textsubscript{3}, [Cp\textsubscript{2}Fe]BF\textsubscript{4}, and CDCl\textsubscript{3} were purchased from Sigma-Aldrich. NaCp was prepared in situ by reacting freshly distilled cyclopentadiene and NaH in THF. Silica gel was heated at about 200°C while a slow stream of dry nitrogen was passed through it.

The syntheses of sodium 1-carboethoxycyclopentadienide (NaCpCOOCH\textsubscript{3}) and sodium 1-carboethoxycyclopentadienide (NaCpCOOCH\textsubscript{2}CH\textsubscript{3}) have been reported previously by our group [18].
The colon cancer cell line HT-29 and the breast adenocarcinoma cell line MCF7 were purchased from American Type Culture Collection, Va, USA, and were at 37°C and 95% Air/5%CO₂. Growth medium for HT-29 was McCoy’s 5A complete medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antimycotic. Growth medium for MCF7 was Eagle’s Minimum Essential Media supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotic/antimycotic, nonessential amino acids, and 0.01 mg/mL bovine insulin. MTT and Triton X-100 used for the cytotoxic assay were obtained from Sigma. All MTT manipulations were performed in a dark room.

3.2. Synthesis

3.2.1. Synthesis of 1,1′-bis(carbomethoxy)-ferrocene (Fe(Cp–COOCH₂CH₃)).

To a solution of NaCpCOOCH₂CH₃ (0.47 g, 3.1 mmol) in THF 20 mL, was added solid CH₂Cl₂ (0.25 g, 1.58 mmol). The solution was stirred for 24 hours at room temperature. The solvent was removed under vacuum, and CH₂Cl₂ was added; the red suspension was first filtered on a Celite pad and then chromatographed on silica gel eluting with Et₂O to give 0.45 g (95%) of orange viscous oil. The product was dissolved in chloroform/hexane (1:9) at −20°C and orange solid could be obtained.¹¹HNMR (500 MHz, CDCl₃): δ (ppm) 3.85 (s, 6H; –OCH₂CH₃), 4.43 (t, 4H, 3J (H, H) = 1.5 Hz, AA’BB’; Cp), 4.85 (t, 4H, 3J (H, H) = 1.5 Hz; AA’BB’; Cp), 13CNMR (125 MHz, CDCl₃): δ (ppm) 51.71 (–OCH₂CH₃), 71.61 (CH; Cp), 72.62 (CH; Cp), 72.89 (ipso-C; Cp), 170.82 (C=O). IR (KBr, cm⁻¹): 2957, 1703, 1470, 1379, 1284, 1143, 1030, 774, 514. Anal. Calcd for C₁₆H₁₈O₄Fe: C, 58.22; H, 5.11. Found: C, 56.01; H, 4.74.

3.2.2. Synthesis of 1,1′-bis(carboethoxy)-ferrocene (Fe(Cp–COOCH₃)(CH₂CH=CH₂)).

To a solution of NaCpCOOCH₃ (0.47 g, 3.1 mmol) in THF 20 mL, was added solid FeCl₂ (0.50 g, 3.16 mmol). The solution was stirred for 24 hours at room temperature. The solvent was removed under vacuum, and CH₂Cl₂ was added; the red suspension was first filtered on a Celite pad and then chromatographed on silica gel eluting with Et₂O to give 0.48 g (92%) of orange viscous oil. The product was dissolved in chloroform/hexane (1:9) at −20°C and orange solid could be obtained.¹¹HNMR (500 MHz, CDCl₃): δ (ppm) 1.39 (t, 3H, 3J (H, H) = 7.0 Hz; –OCH₂CH₃), 4.31 (q, 4H, 3J (H, H) = 7.0 Hz; –OCH₂CH₃), 4.42 (t, 4H, 3J (H, H) = 1.5 Hz; AA’BB’; Cp), 4.84 (t, 4H, 3J (H, H) = 1.5 Hz; AA’BB’; Cp), 13CNMR (125 MHz, CDCl₃): δ (ppm) 14.53 (–OCH₂CH₃), 60.46 (–OCH₂CH₃), 71.57, 71.61 (CH; Cp), 72.73, 72.77 (CH; Cp), 72.86, 72.86, 73.21 (ipso-C; Cp), 170.43, 170.43 (C=O). IR (KBr, cm⁻¹): 2957, 1703, 1470, 1379, 1284, 1143, 1030, 774, 514. Anal. Calcd for C₁₅H₁₆O₄Fe: C, 57.00; H, 5.07. Found: C, 57.19; H, 5.08.

The two complexes [Fe(C₅H₄CO₂CH₂CH=CH₂)]₂ and [Fe(C₅H₄CO₂(CH₂)₁OH₁₂]₂ were prepared as described by Busetto et al. from sodium cyclopentadienide and diallyl carbonate or solid ethylene carbonate [17].

3.3. Cytotoxic Assay. Biological activity was determined using the MTT assay originally described by Mossmann [19a] but using 10% Triton in isopropanol as a solvent for the MTB formazan crystals [19b]. HT-29 and MCF7 cells were maintained at 37°C and 95% Air/5%CO₂ in McCoy’s 5A (ATCC) complete medium, which had been supplemented with 10% (v/v) fetal bovine serum (ATCC) and 1% (v/v) antibiotic/antimycotic (Sigma). Asynchronously, growing cells were seeded at 1.5 × 10⁴ cells per well in 96-well plates containing 100 μL of complete growth medium, and allowed to recover overnight. Various concentrations of the complexes (10–1300 μM) dissolved in 5% DMSO/95% Medium were added to the wells (eight wells per concentration, experiments performed in quadruplicate plates). The complexes solutions were prepared first by dissolving the corresponding ferrocene in DMSO and then Medium was added to a final composition of 5% DMSO/95% Medium. In addition to the cells treated with the ferrocenes, two controls experiments were run one without any addition of solvent mixture (5% DMSO/95% Medium) and one adding 5% DMSO/95% Medium to the cells. Both control experiments behaved identical, showing that 5% of DMSO in the Medium did not render toxic to these types of cells. The cells were incubated for an additional 70 hours. After this time, MTT dissolved in complete growth medium was added to each well to a final concentration of 1.0 mg/mL and incubated for an additional 70 hours. After this period of time, MTTs dissolved in corresponding ferrocene in DMSO and then Medium were added to the wells (eight wells per concentration, experiments performed in quadruplicate plates). The complexes solutions (10–1300 μM) dissolved in 5% DMSO/95% Medium were added to the wells (eight wells per concentration, experiments performed in quadruplicate plates). The complexes solutions were prepared first by dissolving the corresponding ferrocene in DMSO and then Medium was added to a final composition of 5% DMSO/95% Medium. In addition to the cells treated with the ferrocenes, two controls experiments were run one without any addition of solvent mixture (5% DMSO/95% Medium) and one adding 5% DMSO/95% Medium to the cells. Both control experiments behaved identical, showing that 5% of DMSO in the Medium did not render toxic to these types of cells. The cells were incubated for an additional 70 hours. After this time, MTT dissolved in complete growth medium was added to each well to a final concentration of 1.0 mg/mL and incubated for two additional hours. After this period of time, all MTTs containing medium were removed, cells were washed with cold PBS and dissolved with 200 μL of a 10% (v/v) Triton X-100 solution in isopropanol. After complete dissolution of the formazan crystals, well absorbances were recorded in triplicates on a 340 ATTC Microplate Reader (SLT Lab Instruments) at 570 nm with background subtraction at 630 nm. Concentrations of compounds required to inhibit cell proliferation by 50% (IC₅₀) were calculated by fitting data to a four-parameter logistic plot by means of SigmaPlot software from SPSS, Ill, USA.
3.4. X-Ray Crystallographic Analysis. A light orange needle crystal with $0.15 \times 0.04 \times 0.01$ mm in size was mounted on a cryoloop with Paratone oil. Data was collected in a nitrogen gas stream at $-173°C$ on a Bruker Smart system. Data collection was 99.6% complete to 25° in $\theta$. The data was integrated using the Bruker SAINT software program. The structure was solved by direct methods and all nonhydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-97). The crystal structure has been deposited at the Cambridge Crystallographic Data Centre and the deposition number is CCDC 705387.

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