Available online at www.sciencedirect.com
ScienceDirect
Journal of Organometallic Chemistry 693 (2008) 675–684
Journal of Organometallic Chemistry
www.elsevier.com/locate/jorganchem

Synthesis, characterization and antitumor activity of 1,2-disubstituted ferrocenes and cyclodextrin inclusion complexes

Željko Petrovski [a], Marta R.P. Norton de Matos [a,b], Susana S. Braga [c], Cláudia C.L. Pereira [a,c], Márcia L. Matos [b], Isabel S. Gonçalves [c,*], Martyn Pillinger [c], Paula M. Alves [a], Carlos C. Romão [a,b,*]

[a] *Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa, Av. da Republica, Estação Agronómica Nacional, 2780-157 Oeiras, Portugal*
[b] *Alfama Ltd., Taguspark, Núcleo Central 267, 2740-122, Porto Salvo, Portugal*
[c] *Department of Chemistry, CICECO, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal*

Received 26 October 2007; received in revised form 27 November 2007; accepted 27 November 2007
Available online 4 December 2007

Abstract

Seven different ferrocene derivatives have been tested in vitro against Ehrlich ascites tumor cells. Neither ferrocene nor the monosubstituted derivative *N*,*N*-dimethylaminomethylferrocene showed cytotoxic activity ($IC_{50} > 1000$ μM for 3 h treatments). Better results were obtained with 1,2-disubstituted derivatives. The IC_{50} values ranged from 376.6 μM for 1,2-diformylferrocene to 71.2 μM for racemic 2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxamide. The latter derivative was also encapsulated in native β-cyclodextrin (CD), heptakis-2,3,6-tri-*O*-methyl-β-CD (TRIMEB) and 2-hydroxypropyl-β-CD (HPβCD) to give 1:1 (host:guest) inclusion compounds. The existence of true inclusion complexes in the solid state was confirmed by a combination of powder X-ray diffraction, thermogravimetric analysis, FTIR and ^{13}C CP MAS NMR spectroscopy. The IC_{50} value for the β-CD inclusion compound was identical to that obtained for the nonincluded ferrocene derivative. By contrast, the inclusion compounds comprising TRIMEB and HPβCD yielded IC_{50} values of 25.2 and 20.0 μM, respectively. No obvious relationship could be established between the redox behavior of the compounds determined by cyclic voltammetry and the biochemical data.

Keywords: Iron compounds; Ferrocene derivatives; Antitumor agents; Ehrlich ascites tumor; Cyclodextrins; Cyclic voltammetry

1. Introduction

The successful development of metal-based anticancer drugs starts with *cis*-$[PtCl_2(NH_3)_2]$, often referred to as cisplatin [1–3]. Cisplatin is currently used in the treatment of testicular, ovarian, bladder and neck cancers. The drug does, however, present several drawbacks, such as solubility problems and severe side effects. Also, many human cancers either have a natural resistance to cisplatin or acquire resistance during treatment. For these reasons, modified versions of cisplatin have been studied (the so-called 2nd and 3rd generation drugs), of which carboplatin and oxaliplatin stand out. There has also been a huge effort to develop other transition metal compounds as antitumor agents [4–11]. Ruthenium(II) and ruthenium(III) complexes [12–17], metallocene dihalides Cp_2MX_2 (M = Ti, V, Nb, Mo) and their derivatives [18–20], ferrocenium salts [21–25] and organotin compounds [26] are just some of the examples reported to exhibit promising in vitro or in vivo antitumor activities against a range of tumor cell lines. Recently, a ruthenium-dimethyl sulfoxide complex

* Corresponding authors. Address: Department of Chemistry, CICECO, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal (I.S. Gonçalves). Tel.: +351 234378190; fax: +351 234370084.
E-mail address: igoncalves@ua.pt (I.S. Gonçalves).

0022-328X/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jorganchem.2007.11.053

(NAMI-A) has successfully completed Phase I Clinical Trials [27]. These different classes of metal-containing antitumor agents do not all function by the same mechanism. For example, Osella and co-workers reported that the cytotoxic behavior of ferrocenium derivatives is based on their ability to generate oxygen active species which can damage DNA [24,25]. However, as illustrated by work with ferrocenyl derivatives of tamoxifen (the so-called ferrocifens) [10,28–31], the ferrocene(II) derivatives can have excellent cancerostatic properties provided that the Cp-ring functionalization allows a bio-interaction and in situ oxidation to ferrocenium cation [32]. The bioorganometallic and medicinal chemistry of ferrocene has been extensively reviewed recently [33–35].

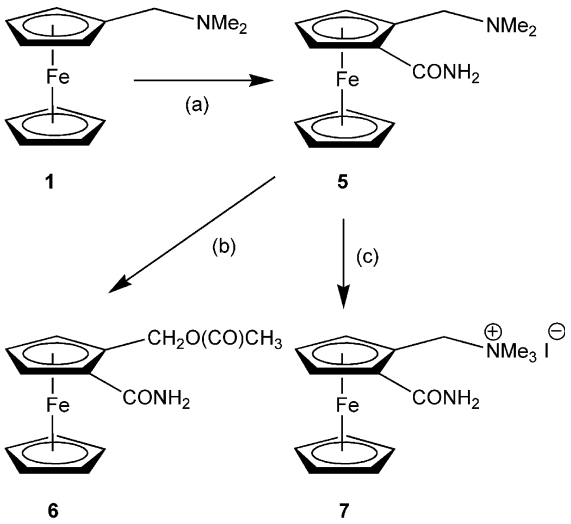
Considering the conflicting results in the literature about the ferrocene/ferrocenium cancerostatic properties, we decided to carry out a preliminary examination of the in vitro cytotoxicity of the ferrocenes we had available against the Ehrlich ascites tumor (EAT) cell line. We also report the synthesis, characterization and antitumor activities of three different cyclodextrin (CD) inclusion compounds containing one of the ferrocene derivatives. CD inclusion complexes are interesting for pharmaceutical use due to enhanced solubility, stability and bioavailability of the drug molecules [36,37]. For example, complexation of the promising antitumor agent rhodium(II) citrate with hydroxypropyl-β-cyclodextrin (HPβCD) improved encapsulation and release kinetics from biodegradable polymer microspheres [38]. Other potential benefits of using CD drug delivery systems in chemotherapy include improved activities and reduction of toxic side effects. CD inclusion compounds containing the anticancer agents carboplatin [39–41] and Cp_2MX_2 (M = Ti, V, Nb, Mo) [42–45] have already been described. Utsuki et al. reported that interstitial delivery of a carboplatin-HPαCD complex from a microencapsulated formulation was effective against an experimental rat glioma model [46]. Cytotoxicity experiments carried out on human adenocarcinoma cells indicated that the anti-tumor activity of Cp_2MoCl_2 can be enhanced by association with cyclodextrins, in particular the modified cyclodextrin heptakis-2,3,6-tri-*O*-methyl-β-CD (TRIMEB) [47]. CDs are known to form stable inclusion complexes with a large number of ferrocene derivatives [48]. In the present work, native β-CD hydrate, TRIMEB and HPβCD have been studied as the host compounds. The chemically modified HPβCD has attracted particular interest for drug formulations due to an improved aqueous solubility and safety profile as compared with unmodified β-CD [49].

2. Results and discussion

2.1. Synthesis and characterization of ferrocene derivatives

As part of an ongoing project in our laboratories involving the synthesis of disubstituted ferrocenes, we had available *N*,*N*-dimethylaminomethylferrocene (**1**) [50], 1,2-bis(hydroxymethyl)ferrocene (**2**) [51], 1,2-diformylferrocene (**3**) [51], and 2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxaldehyde (**4**) [52]. This group of compounds was expanded to include three additional 1,2-disubstituted ferrocene derivatives. The racemic 2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxamide (**5**) was prepared from **1** by *ortho*-lithiation and subsequent reaction with carbon dioxide (Scheme 1). Without isolation, the unstable carboxylation product was further reacted with oxalyl chloride and ammonia to give the amide as a stable orange crystalline solid in good yield. The ^{1}H NMR spectrum of **5** shows two multiplets at δ 4.73 and 4.29–4.25 ppm attributed to the 1,2-disubstituted cyclopentadienyl ring, a singlet at δ 4.11 ppm due to the Cp ring, a pair of doublets (AX pattern, $J_{A,X} = 12.6$ Hz) at δ 2.98 and 4.06 ppm for the methylene protons, and a singlet at δ 2.11 ppm for the methyl groups. The presence of the amide functional group in **5** was confirmed by the IR spectrum which showed bands at 3400 (ν_{NH}) and 1643 cm^{-1} ($\nu_{C=O}$).

Transformation of the tertiary amine substituent in compound **5** gave the compounds 2-carbamoylferrocenylmethyl acetate (**6**) and *N*,*N*,*N*-trimethyl-*N*-(2-carbamoylferrocenylmethyl)ammonium iodide (**7**) (Scheme 1). To the best of our knowledge, only the methyl ester of 2-dimethylaminomethylferrocene carboxylic acid has been reported [53], prepared by stoichiometric *ortho*-palladation of Ugi's amine (dimethylaminomethylferrocene), followed by carbonylation in methanol. Compound **6** was identified by a ^{1}H NMR singlet at δ 1.96 ppm, attributed to the methyl group in $CH_3(CO)OCH_2$–, and two ^{13}C NMR resonances at δ 176.0 and 172.7 for the carbonyl groups in $NH_2(CO)$– and $CH_3(CO)$–, respectively. The formation of compound **7** was confirmed by a ^{1}H NMR signal at δ 2.91 ppm for the methyl groups of the ammonium ion.



Scheme 1. (a) (i) *t*-BuLi, Et_2O, 15 min, (ii) CO_2, CF_3COOH, (iii) ClCOCOCl, 2 h, (iv) NH_3 (conc.). (b) Ac_2O. (c) CH_3I, Et_2O–CH_3CN.

2.2. Synthesis and characterization of β-CD inclusion compounds

The inclusion compounds were prepared from mixed water/ethanol solutions containing equimolar amounts of the CD host and compound **5**. For native β-CD, the inclusion compound precipitated as an orange microcrystalline powder, designated as β-CD · Fc(5) (**8**). Elemental analysis indicated that the host:guest ratio in the product was 1:1. Due to the high solubility of the modified β-CDs TRIMEB and HPβCD, precipitation at room temperature was not possible and the solvents were removed by lyophilization. The products are designated as TRIMEB · Fc(5) (**9**) and HPβCD · Fc(5) (**10**). The powder X-ray diffraction (XRD) pattern of compound **8** showed the formation of a new, moderately crystalline phase, different from either native β-CD hydrate or compound **5**, and therefore consistent with the presence of a true inclusion complex (Fig. 1) [54]. Compounds **9** and **10** were poorly crystalline, showing only two or three very broad XRD features. Since the low crystallinity could be due to lyophilization, the samples were rehydrated and the XRD patterns measured again (Fig. 1). No significant change was noted, although a few weak sharp lines are present for the TRIMEB adduct. The lack of crystallinity for compound **10** is not surprising since the host itself, HPβCD, is amorphous (Fig. 1f). On the other hand, when considering TRIMEB, a crystalline host, the formation of an amorphous inclusion compound with the ferrocene derivative indicates a profound structural change.

Thermogravimetric analysis (TGA) curves are shown in Fig. 2 for β-CD hydrate, the 1,2-disubstituted ferrocene **5**, a 1:1 physical mixture of β-CD and **5**, and the inclusion compound **8**. Compound **5** features a small mass loss of 3% from 80 to 100 °C and decomposition from 150 to 250 °C, followed by a second abrupt mass loss in the range 350–400 °C. The residual mass is around 20%. Native β-CD hydrate shows loss of water up to 80 °C (15%, 11 water molecules per β-CD molecule), and starts to melt and decompose above 250 °C. In an equimolar physical mixture of β-CD and **5**, each component behaves independently. For example, a step is present around 180 °C corresponding to the first decomposition event of nonincluded **5**. This feature is not present in the TGA curve of the inclusion compound **8**, indicating that the organometallic molecules are complexed by β-CD molecules. The host–guest interaction promotes simultaneous decomposition of the two components, which begins just above 200 °C, significantly lower than that observed for β-CD hydrate. The loss of water up to 140 °C was 10.0%, in agreement with elemental analysis which indicated 9 water molecules per β-CD molecule.

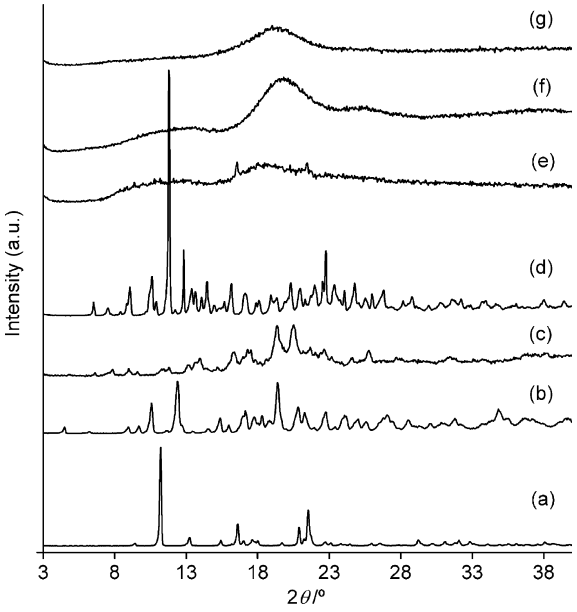


Fig. 1. Powder XRD patterns of (a) the 1,2-disubstituted ferrocene **5**, (b) native β-CD hydrate, (c) the β-CD inclusion compound **8**, (d) TRIMEB, (e) the TRIMEB inclusion compound **9**, (f) HPβCD, and (g) the HPβCD inclusion compound **10**. Intensities for (c), (e), (f) and (g) are scaled by a factor of 4 relative to those for the other diffraction patterns.

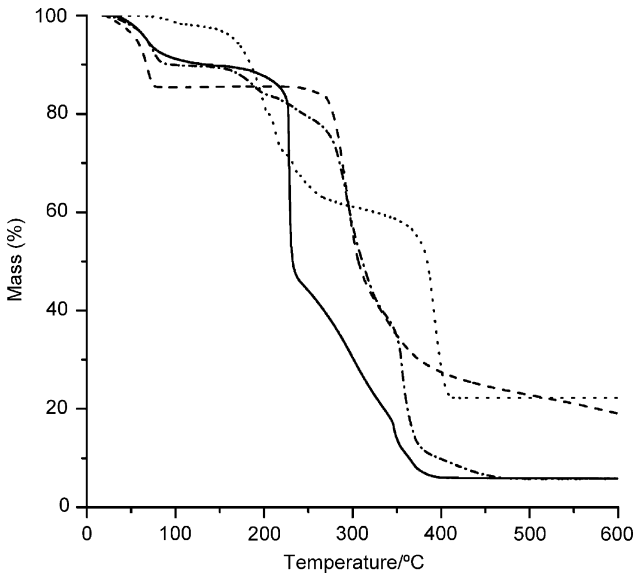


Fig. 2. Thermogravimetric analysis profiles of native β-CD hydrate (– – –), the 1,2-disubstituted ferrocene **5** (· · · · · ·), a 1:1 physical mixture of β-CD and **5** (– · – ·), and the β-CD inclusion compound **8** (——).

TGA was also useful for the recognition of complex formation in the adducts **9** and **10** (Fig. 3). Decomposition of compound **9** occurs in a single step. The behavior is not very different from that exhibited by TRIMEB except that the mass loss for **9** is more abrupt, centered around 305 °C. Contrastingly, for compound **10**, three main steps are observed in the temperature range 190–440 °C, none of which coincide exactly with the decomposition steps observed for the nonincluded organometallic **5** or the host HPβCD. The initial thermal degradation of the guest occurs at a higher temperature than that for the bulk compound **5**.

Evidence for inclusion complexation in the adducts **8**–**10** was also obtained by comparing their KBr IR spectra with

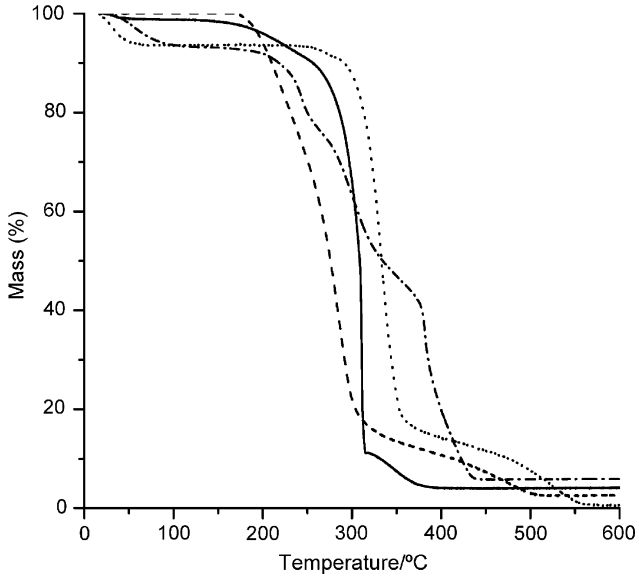


Fig. 3. Thermogravimetric analysis profiles of TRIMEB (– – –), the TRIMEB inclusion compound **9** (——), HPβCD (·····), and the HPβCD inclusion compound **10** (– · – ·).

that of the nonincluded 1,2-disubstituted ferrocene **5**. The carbonyl stretching absorption (amide I band) shifted from 1643 cm^{-1} for **5** to 1658 cm^{-1} for the inclusion compounds **8** and **10**, possibly due to changes in hydrogen bonding interactions induced by encapsulation. Accordingly, the shift in this band was even higher for the TRIMEB adduct **9** (to 1670 cm^{-1}), which was shown by TGA to have a low water content. The permethylated host molecule is known for its nonpolar cavity. For the organometallic **5** the expected amide II band (mainly N–H bending) was obscured by the strong amide I band at 1643 cm^{-1}. However, for the inclusion compounds, the shift in the amide I band allowed the amide II band to be resolved as a weaker peak at about 1600 cm^{-1} for **8** and **10**, and 1629 cm^{-1} for **9**.

Fig. 4 shows the ^{13}C CP MAS NMR spectra of the three β-CD precursors, the ferrocene derivative **5** and the inclusion complexes **8**–**10**. The spectrum of β-CD hydrate is similar to that previously reported and exhibits multiple resonances for each type of carbon atom [55–57]. This has been mainly correlated with different torsion angles about the (1 → 4) linkages for C-1 and C-4 [55,56], and with torsion angles describing the orientation of the hydroxyl groups (see Fig. 5 for carbon atom numbering scheme) [57]. The different carbon resonances are assigned to C-1 (101–104 ppm), C-4 (78–84 ppm), C-2,3,5 (71–76 ppm) and C-6 (57–65 ppm). Like the parent compound β-CD, TRIMEB gives rise to several resonances for each type of carbon atom. By reference to reported solution spectra [58,59], the different carbon resonances are assigned to C-1 (93–102 ppm), C-2,3,4 (74–88 ppm), C-5,6 (68–73 ppm) and O–CH_3 (54–64 ppm). When the guest **5** is included in the two β-CD hosts to form compounds **8** and **9**, the multiplicity of host resonances is reduced. In fact, some of the signals for the same type of carbon, C-1 for example,

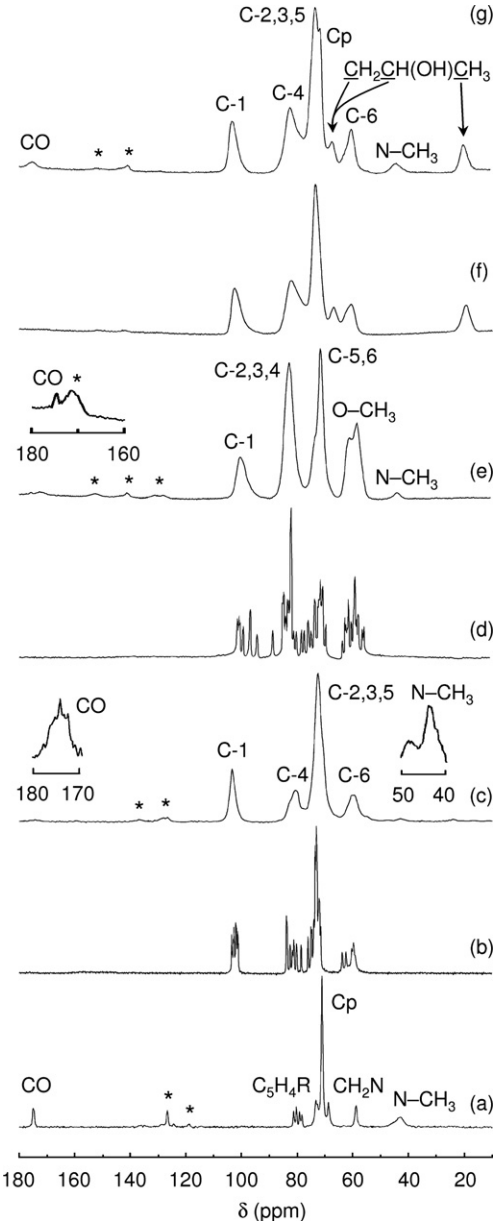


Fig. 4. Solid-state ^{13}C CP MAS NMR spectra of (a) the 1,2-disubstituted ferrocene **5**, (b) native β-CD hydrate, (c) the β-CD inclusion compound **8**, (d) TRIMEB, (e) the TRIMEB inclusion compound **9**, (f) HPβCD, and (g) the HPβCD inclusion compound **10**. Asterisks denote spinning sidebands.

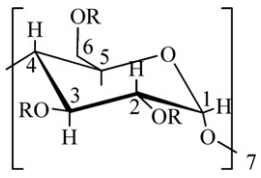


Fig. 5. Carbon atom numbering scheme for β-CD (R = H), TRIMEB (R = CH_3) and HPβCD [R = $CH_2CH(OH)CH_3$ or H].

coalesce into one broad signal. For the parent β-CD this feature has been associated with the effect of the included guest upon the symmetry of the macrocycle and a similar effect may be occurring in the TRIMEB inclusion

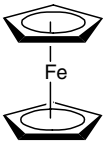
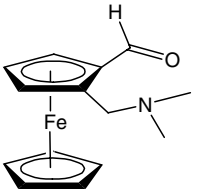
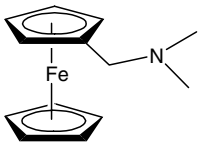
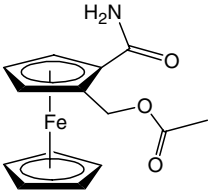
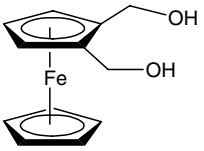
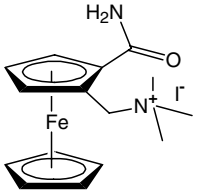
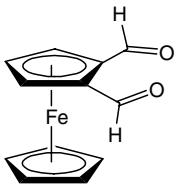
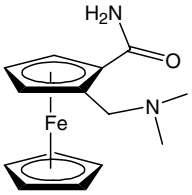
compound. Inclusion of guest molecules in the β-CD cavities will result in the host molecule adopting a more symmetrical conformation, with each glucose unit in a similar environment [45,60]. The changes in the spectra may also be associated with changes in crystallinity, particularly for the TRIMEB adduct. In addition to the resonances for the β-CD carbons, the spectra contain weak, broad lines that can be assigned to the carbonyl and methyl carbon atoms of the guest molecule. These are weakly shifted compared with the corresponding lines for the nonincluded compound. Other peaks due to the guest overlap with those of the hosts and are not resolved.

For hydroxypropyl-β-CD, the precursor compound is amorphous and only broad lines are observed in the ^{13}C CP MAS NMR spectrum. No significant changes occurred upon inclusion of the ferrocene derivative, other than the appearance of guest resonances at 43.2, 70.9 and 175.0 ppm.

2.3. Cytotoxicity assays

The cytotoxicity of the seven ferrocene derivatives and the inclusion compounds **8–10** was evaluated on the EAT line. Ferrocene was also tested. Full details are given in the experimental part and Table 1 shows the IC_{50} values. Compounds **1–6** and ferrocene are insoluble in water and so DMSO was used as the solvent (1.5% DMSO aqueous solutions were prepared). Neither ferrocene nor the monosubstituted derivative **1** exhibited activity. These two compounds were also found to be inactive against P-388 mouse leukaemia tumor [61]. On the other hand, Fiorina et al. reported that some ferrocenyl polyamine compounds were active against P-388 lymphocytic leukaemia in mice [62]. The other ferrocene derivatives tested in this work have two adjacent substituents on one of the cyclopentadienyl rings. Compounds **2** and **3**, containing either two CH_2OH or two CHO groups, did not show any significant cytotoxic

Table 1
Cytotoxicity of ferrocenyl derivatives and inclusion compounds against Ehrlich ascites tumor

Compound	Structure	IC_{50} (μM)[a]	Compound	Structure	IC_{50} (μM)
Fc		>1000	**5**		71.2 ± 1.1
1		>1000	**6**		91.6 ± 1.2
2		>1000	**7**		76.2 ± 1.2
3		376.6 ± 1.3	β-CD · Fc(5) (**8**)		71.0 ± 1.0
4		131.1 ± 1.1	TRIMEB · Fc(5) (**9**)		25.2 ± 1.0
			HPβCD · Fc(5) (**10**)		20.0 ± 1.1

[a] Concentration required to reduce to 50% the number of cells with respect to the untreated control.

properties. Better results were obtained for compound **4**, in which one of the CHO groups in **3** has been replaced by a tertiary amine group. The highest activity (IC_{50} 71.2 μM) was exhibited by compound **5**, which has an amide group instead of the CHO group in **4**. The other two ferrocenes studied differ in the nature of the substituent adjacent to the $CONH_2$ group. The methyl acetate derivative **6** gave an IC_{50} value of 91.6 μM.

The poor solubility of compounds **1**–**6** in water will obviously limit their use in biological systems. One approach to improve the water solubility of metallocene anticancer drugs is to prepare the corresponding ionic salts with the charge linked to the cyclopentadienyl ligands. McGowan and co-workers have, for example, prepared several amino-functionalized ferrocene salts [63,64]. We therefore synthesized an ammonium iodide salt **7** by reaction of **5** with CH_3I. Compound **7** is water soluble and a cytotoxicity test was carried out using an aqueous solution. However, the observed activity was not significantly different from that observed when a 1.5% DMSO aqueous solution was used. It is noteworthy that compound **7** eventually decomposes when dissolved in water.

The IC_{50} value for the β-CD inclusion compound **8** was identical to that obtained for the nonincluded compound **5** (Table 1). Much better results were obtained for the two inclusion compounds containing the chemically modified CDs. The IC_{50} values for **9** and **10** were 25 and 20 μM, respectively. The use of permethylated β-CD for the encapsulation of drug molecules could result in artificially lower IC_{50} values due to the moderate toxicity of this CD. In fact, TRIMEB is a strong solubilising agent known to induce cell lysis in tests with erythrocytes [49]. HPβCD, on the other hand, has a very safe toxicological profile [49,65], and has been demonstrated to be able to lower the hemolytic activity of the parent CD [49]. Thus, taking into account the similarity of the two IC_{50} values for **9** and **10**, it is reasonable to conclude that the solubilising effect of the CD carriers is the main factor responsible for increasing the cytotoxic activity of compound **5**, rather than any potential intrinsic cytotoxicity of the CD hosts.

2.4. Cyclic voltammetry

It is generally agreed that the ferrocene/ferrocenium couple is central to the antitumor activity of ferrocene-based drug molecules. In most cases, ferrocenium salts are reported to be more potent anticancer agents than the neutral molecules. However, as mentioned in the introduction, it may be irrelevant whether a ferrocene-based anticancer drug is administered in the original (ferrocene) or in the oxidized (ferrocenium) state, as long as the compound possesses sufficient water solubility to enter the vascular system rapidly [32]. The ferrocene/ferrocenium equilibrium distribution should be dictated by the compound's reduction potential and by environmental conditions such as pH. In support of this, electrochemical studies by Jaouen and co-workers have shown that the cytotoxic effect of a series of ferrocenyl diphenol complexes on prostate and breast cancer cell lines correlates with the ease of oxidation of the ferrocene group [66]. We therefore decided to characterize the redox properties of the ferrocene derivatives, and look for any relationship between these results and the biochemical data.

Table 2
Electrochemical data in CH_2Cl_2 at room temperature[a]

Compound	E_{pa}[b]	E_{pc}[c]	$E_{1/2}$[d]
Fc	0.55	0.37	0.46
1	0.71	0.60	0.66
2	0.48	0.33	0.40
3	1.06	0.99	1.03
4[e]	0.78		
	1.09	0.90	0.99
5[e]	0.31		
	0.62		
	0.82	0.73	0.77
6	0.77	0.64	0.70
7[e]	0.45		
	0.88	0.74	0.81
8	0.67		
	1.10		
		0.20	
9	0.69	0.61	
	0.94	0.83	
10	0.70		
	0.93	0.77	0.85

[a] Potentials in V vs. Fc/Fc^+; scan rate 250 mV s^{-1}.
[b] E_{pa}, anodic sweep (peak potentials, V).
[c] E_{pc}, cathodic sweep (peak potentials, V).
[d] $E_{1/2}$, average of the anodic and cathodic peak potentials.
[e] Scan rate 100 mV s^{-1}.

Redox potentials obtained from the cyclic voltammetric data of the ferrocene compounds are listed in Table 2. In CH_2Cl_2, at the scan rate range used, the derivatives **1**–**3** and **6** presented one reversible $Fe^{II/III}$ oxidation, and the $E_{1/2}$ values are strongly dependent on the nature of the cyclopentadienyl substituents. The 1,2-disubstituted derivatives **4**, **5** and **7**, with amino groups, present considerable changes in the voltammograms, indicating a more complex electro-oxidation reaction scheme. The CVs of compounds **4** and **7** show a reversible oxidation wave at $E_{1/2} = 0.99$ V and 0.81 V, respectively. In addition, irreversible oxidation peaks are observed at about 0.78 V for **4** and 0.45 V for **7**, which are probably due to the oxidation of the amino group. Complex **5**, besides the expected reversible oxidation at $E_{pa} = 0.82$ V, exhibits two irreversible oxidation peaks ($E_{pa} = 0.31$ V and $E_{pa} = 0.62$ V). It is possible that the first irreversible oxidation is due to the direct oxidation of the nitrogen atom on the pendant arm, and the decomposition of the oxidized product may give rise to the second irreversible peak.

The CVs of the inclusion compounds **8**–**10** showed some significant differences compared with the CV of the nonincluded ferrocene **5**. While the amine peak observed at 0.62 V for **5** was still present at about the same potential for **8**–**10**, the ferrocene peak at 0.82 V was apparently broadened and shifted to more positive potentials. This is a

common observation for cyclodextrin–ferrocene complexes and is usually attributed to a significant host–guest interaction, i.e. noncovalent encapsulation of the ferrocene molecule within the hydrophobic cavity of the CD moiety [67–69]. Ferrocene is more difficult to oxidize in the presence of the CD host because it is more strongly bound than the oxidized form, ferrocenium (Fc^+).

3. Conclusions

A series of ferrocene derivatives were successfully prepared and characterized. Some of the compounds exhibited significant cytotoxic activity against the EAT line. However, there is no obvious relationship between the antitumor activity and redox behavior. The presence of two distinctive groups seems to be more important, and the results indicate that opportune choice of the ring substituents may allow the preparation of active and selective antitumor agents. A wider range of substituents will have to be investigated before a meaningful structure-performance relationship can be established. For the most active complex, 2-(*N,N*-dimethylaminomethyl)ferrocenecarboxamide (**5**), which is practically insoluble in water, increasing its solubility by salt formation or by inclusion in unmodified β-cyclodextrin did not lead to better results. However, significantly higher antitumor activities were obtained by using the chemically modified CDs TRIMEB and HPβCD. Since the 1,2-disubstituted ferrocenes used in this study are racemic, further studies may be oriented towards elucidation of the activity of optically pure enantiomers. This seems to be particularly interesting for pharmacological investigation.

4. Experimental

4.1. General methods and procedures

All air sensitive reactions were performed using standard Schlenk techniques under nitrogen. Solvents were dried by standard procedures (diethyl ether over Na/benzophenone ketyl; acetonitrile over CaH_2). Oxalyl chloride was freshly distilled prior to use. Ferrocene, *t*-butyl lithium, trifluoroacetic acid, methyl iodide, concentrated ammonia, acetic anhydride and deuterated solvents were purchased from commercial suppliers and used as received. Literature procedures were used to prepare *N*,*N*-dimethylaminomethylferrocene (**1**) [50], 1,2-bis(hydroxymethyl)ferrocene (**2**) [51], 1,2-diformylferrocene (**3**) [51], and 2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxaldehyde (**4**) [52]. β-CD was kindly donated by Laboratoires La Roquette, France. TRIMEB was obtained from Fluka and HPβCD, with an average degree of substitution of 3, was obtained from Cyclolab, Hungary.

Microanalyses were performed at the ITQB (by Conceição Almeida). Infrared spectra were recorded on a Unican Mattson Mod 7000 FTIR spectrophotometer in solution or as KBr pellets. Powder XRD data were collected on a Philips X'pert diffractometer using Cu Kα radiation filtered by Ni ($\lambda = 1.5418$ Å). TGA studies were performed using a Shimadzu TGA 50 system at a heating rate of 5 K min^{-1} under a static atmosphere of air. Liquid ^1H NMR and ^{13}C NMR spectra were measured at room temperature (RT) on a Bruker AMX 300 spectrometer. ^{13}C CP MAS NMR spectra were recorded at 125.72 MHz on a (11.7 T) Bruker Avance 500 spectrometer, with a 4.5 μs ^1H 90° pulse, 2 ms contact time, a spinning rate of 7 kHz and 12 s recycle delays. Chemical shifts are quoted in parts per million from tetramethylsilane. Mass spectra were acquired on a Bruker esquire 3000* (by Ana V. Coelho).

Cyclic voltammetry was performed with a BAS CV/50 voltammetric analyzer, in a closed standard three-electrode electrochemical cell that kept the solution protected from air, using CH_2Cl_2 solutions containing 0.1 mol dm^{-3} tetrabutylammonium hexafluorophosphate. The solutions were purged with argon and kept under an inert atmosphere throughout the measurements. Dichloromethane was distilled from CaH_2 and tetrabutylammonium hexafluorophosphate was dried under dynamic vacuum at 140 °C for 12 h prior to use. A BAS MF-2012 glassy-carbon electrode was used as the working electrode and a BAS MW-4130 electrode as the counter electrode. The measured potentials were not corrected for liquid junction potentials and are reported relative to that of the SCE reference electrode and to the $E_{1/2}$ value of the ferrocenium/ferrocene couple.

4.2. 2-(*N,N*-dimethylaminomethyl)ferrocenecarboxamide (*5*)

A 1.6 M pentane solution of *t*-BuLi (840 μL, 1.35 mmol) was added dropwise to a solution of dimethylaminomethylferrocene (218 mg, 0.90 mmol) in dry diethyl ether (15 mL) at RT. The reaction mixture was stirred for 15 min at RT and then cooled to −78 °C with an acetone/dry-ice bath. After 5 min, crushed dry ice (790 mg, 17.9 mmol) was added. The reaction mixture was removed from the cold bath and left to reach RT over approximately 30 min. Trifluoroacetic acid (140 μL, 1.79 mmol) was added dropwise and the mixture left to stir for 1 h to form an orange-red precipitate. This product was dissolved by the addition of acetonitrile (5 mL). Oxalyl chloride (235 μL, 2.69 mmol) was then added and the mixture stirred for 2 h at RT. The solution was filtered off and the solid residue washed several times with diethyl ether and acetonitrile. After evaporating the solvents from the combined extracts, the residue was redissolved in small amounts of acetonitrile and diethyl ether, and the solvents removed again under reduced pressure. This process was repeated in order to remove the excess of oxalyl chloride. The rubber-like dark yellow product was then dissolved in acetonitrile (5 mL) and concentrated ammonia was added (5 mL). The solution was left to stir overnight at RT. The solvent was evaporated and the pellet was dried in vacuo, and purified by flash chromatography (eluent: CH_3OH:Et_2O:Et_3N =

5:90:5) to provide 160 mg (63%) of pure amide as an orange crystalline solid. Elemental analysis calculated (%) for $C_{14}H_{18}N_2OFe$ (286.16): C, 58.76; H, 6.34; N, 9.79. Found: C, 58.30; H, 5.90; N, 9.65%. IR (KBr): $\nu = 3400$s, 3197s, 2989m, 2980m, 2970m, 2939s, 2859s, 2823s, 2777s, 1643vs ($CONH_2$), 1459vs, 1409w, 1373s, 1350m, 1278m, 1254m, 1233m, 1195m, 1178m, 1157m, 1105m, 1095m, 1048w, 1040w, 1008s, 1004s, 954m, 841m, 813s, 752m, 707m, 617m, 603m, 563m, 519s, 491s, 456m, 432m cm^{-1}. ^1H NMR (CD_3OD, 300 MHz): 4.73 (m, 1H, C_5H_3RR'), 4.29–4.25 (m, 2H, C_5H_3RR'), 4.11 (s, 5H), 4.06 (d, 1H, $J = 12.6$ Hz, CH_2N), 2.98 (d, 1H, $J = 12.6$ Hz, CH_2N), 2.11 [s, 6H, $N(CH_3)_2$]. ^{13}C NMR (CD_3OD, 75.4 MHz): 176.33, 83.24, 77.96, 75.30, 73.21, 71.61, 69.69, 59.29, 44.13. DEPT (CD_3OD, 75.4 MHz): 75.28 (+), 73.24 (+), 71.61 (+), 69.69 (+), 59.28 (−), 44.15 (+). MS (ESI): 287.0 $[M+1]^+$, (100%); 241.9 $[M-45]^+$, (28%). M.p. 164–166 °C.

4.3. 2-Carbamoylferrocenylmethyl acetate (6)

2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxamide (**5**) (154 mg, 0.54 mmol) was dissolved in acetic anhydride (30 mL) and the solution stirred at RT for 3 h. The reaction mixture was then carefully poured into an aqueous solution of sodium bicarbonate and the aqueous layer was extracted several times with diethyl ether. The combined organic layers were washed with brine, dried over anhydrous sodium sulphate, filtered and evaporated. The residue was purified by flash chromatography (eluent: $CH_3OH:Et_2O = 95:5$) to provide 110 mg (68%) of pure acetate as a red oil. IR (neat): $\nu = 3449$, 3348, 3194, 3099, 1731s, 1658s, 1605s, 1474m, 1443m, 1380m, 1249s, 1106m, 1023m, 948m, 826m cm^{-1}. ^1H NMR (CD_2Cl_2, 300 MHz): 5.16 (AB quartet, $J_{A,B} = 12$ Hz, 2H), 4.53 (m, 1H, C_5H_3RR'), 4.41 (m, 1H, C_5H_3RR'), 4.27 (m, 1H, C_5H_3RR'), 4.14 (s, 5H, Cp), 1.96 (s, 3H, CH_3). ^{13}C NMR (CD_3OD, 75.4 MHz): 176.0, 172.7, 84.7, 74.0, 71.6, 71.5, 70.7, 70.6, 63.1, 20.8. MS (ESI): 241.9 $[M-59]^+$, (100%).

4.4. N,N,N-trimethyl-N-(2-carbamoylferrocenylmethyl)ammonium iodide (7)

2-(*N*,*N*-dimethylaminomethyl)ferrocenecarboxamide (**5**) (150 mg, 0.52 mmol) was dissolved in a small quantity of acetonitrile (3 mL) and diethyl ether (5 mL). Methyl iodide (100 μL, 1.57 mmol) was added dropwise and after 30 min stirring at RT, a large amount of yellow precipitate had formed. Diethyl ether (10 mL) was added slowly to the mixture. After another 30 min stirring, the precipitate was filtered, washed several times with diethyl ether and dried in vacuo to provide 194 mg (87%) of the pure ammonium salt as a yellow powder. Elemental analysis calculated (%) for $C_{15}H_{21}N_2OFeI \cdot H_2O$ (446.11): C, 40.39; H, 5.20; N, 6.28. Found: C, 40.42; H, 5.32; N, 6.14%. IR (KBr): $\nu = 3436$vs, 1641s, 1604m, 1473m, 1384m, 1307w, 1249w, 877m cm^{-1}. ^1H NMR (D_2O, 300 MHz): 6.46 (s, 2H), 5.12–4.26 (m, 8H), 2.91 [s, 9H, $N(CH_3)_3$], 2.79 (s, 2H). ^{13}C NMR (d_6-DMSO, 75.4 MHz): 171.0, 75.0, 74.8, 74.7, 70.3, 63.3, 51.3. M.p. 117 °C (decomposition).

4.5. β-CD · Fc(5) (8)

A solution of **5** (126 mg, 0.44 mmol) in ethanol (4 mL) was added to a solution of β-CD (0.50 g, 0.44 mmol) in water (5 mL) at 65 °C. The mixture was stirred for 1 h and left to settle and cool slowly. After 24 h, a microcrystalline orange precipitate had formed. The yield increased upon further evaporation in air (0.55 g, 88%). Elemental analysis calculated (%) for $(C_{42}H_{70}O_{35}) \cdot (C_{14}H_{18}N_2OFe) \cdot 9H_2O$ (1583.3): C, 41.65; H, 6.73; N, 1.80. Found: C, 41.87; H, 6.72; N, 1.94. IR (KBr): $\nu = 3404$vs, 2930s, 1658s, 1600s, 1471s, 1419s, 1379s, 1300m, 1248m, 1156s, 1108s, 1088vs, 1034vs, 1004s, 936m, 834m, 758m, 704m, 606m, 579m cm^{-1}. ^{13}C CP MAS NMR: $\delta = 173.0$ (guest CO), 103.5 (β-CD, C-1), 80.9 (β-CD, C-4), 72.8 (β-CD, C-2,3,5), 59.8 (β-CD, C-6), 43.6 [guest $N(CH_3)_2$].

4.6. TRIMEB · Fc(5) (9)

Compound **5** (0.06 g, 0.21 mmol) was added stepwise to a solution of TRIMEB (0.30 g, 0.21 mmol) in water (1 mL), allowing each fraction to dissolve before adding the next. When guest dissolution was no longer possible, ethanol (0.5 mL) was added to obtain a clear orange solution. After stirring for 1 h, the vessel was immersed in liquid nitrogen for instant freezing and the frozen solvent was removed by lyophilization to obtain a bright orange solid product. IR (KBr): $\nu = 2980$s, 2930s, 2891sh, 2829s, 1670m, 1629m, 1460s, 1405w, 1369m, 1324w, 1304w, 1194s, 1163vs, 1142vs, 1108vs, 1073vs, 1034vs, 970s, 952m, 915sh, 861m, 822w, 755m, 707m, 601w, 558m, 518m cm^{-1}. ^{13}C CP MAS NMR: $\delta = 175.0$ (guest CO), 99.9 (TRIMEB, C-1), 82.2, (TRIMEB, C-2,3,4), 71.0 (TRIMEB, C-5,6), 60.6, 59.9, 57.9 (TRIMEB, O–CH_3), 43.5 [guest $N(CH_3)_2$].

4.7. HPβCD · Fc(5) (10)

Compound **5** (0.065 g, 0.23 mmol) was added stepwise to a solution of HPβCD (0.30 g, 0.23 mmol) in water (1 mL), allowing each fraction to dissolve before adding the next. When guest dissolution was no longer possible, ethanol (0.2 mL) was added to obtain a clear orange solution. After stirring for 1 h, the vessel was immersed in liquid nitrogen for instant freezing and the frozen solvent was removed by lyophilization to obtain a bright orange solid product. IR (KBr): $\nu = 3394$vs, 2969sh, 2930s, 2827w, 1657s, 1605m, 1468m, 1414m, 1379m, 1335m, 1300w, 1256w, 1157s, 1083vs, 1039vs, 947m, 851m, 762w, 708w, 580m, 521w cm^{-1}. ^{13}C CP MAS NMR: $\delta = 175.0$ (guest CO), 102.7 (HPβCD, C-1), 81.9 (HPβCD, C-4),

72.6 (HPβCD, C-2,3,5), 70.9 (guest Cp), 66.8 (host HP group, –O*C*H$_2$*C*H(OH)CH$_3$), 59.8 (HPβCD, C-6), 43.2 [guest N(CH$_3$)$_2$], 19.3 (host HP group, –OCH$_2$-CH(OH)*C*H$_3$).

4.8. Cell culture

The Ehrlich ascites mouse tumor cell line was purchased from ECACC (The European Collection of Cell cultures) and propagated in NCTC-135 medium (Sigma, ref. N3262), 2 mM in L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were routinely propagated in 75 cm^2 tissue culture flasks (SARSTEDT, Leicester, UK), in a humidified atmosphere of 5% CO_2 in air at 37 °C, and were trypsinized and harvested into new medium every 2–4 days, just before confluence. The cells were cultured for a minimum of two passages after thawing prior to experimentation.

4.9. MTT assay

This assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water soluble substrate (MTT) into a dark blue product which is quantified by spectrophotometric means. Briefly, exponentially growing cells were trypsinized, dispensed into 96-well tissue culture plates and allowed to attach overnight. The next day the cells were treated with the proper ferrocene with concentrations ranging from 1 to 1000 μM. By addition of an adequate volume of a freshly prepared DMSO-ferrocene solution to the medium (1.5% DMSO in aqueous medium), the desired test concentrations were obtained. After an incubation period of 3 h the cells were washed twice with phosphate buffer saline (PBS) and 100 μL of medium were added. The cells were incubated for 24 h and 10 μL of a MTT solution (5 mg/mL) were added to each well. The tetrazolium/formazan reaction was allowed to proceed for 3 h and the medium was carefully removed. The dark blue formazan crystals were dissolved by adding 150 μL of DMSO and agitating for 15 min in a plate shaker. The optical density was measured at 540 nm using a 96-well multiscanner autoreader. The percentage of survival was calculated using the formula: %survival = live cell number [test]/live cell number [control] × 100. The IC_{50} values were calculated by nonlinear regression analysis using the graphed Prism software (GraphPad Software Inc., San Diego, CA).

Acknowledgements

We are grateful to POCI 2010, FCT and FEDER (Project POCI/QUI/56109/2004) for financial support. Z.P. thanks the FCT for partial funding (BD/17264/99). We also wish to thank João Rocha for access to research facilities.

References

[1] T.W. Hambley, Coord. Chem. Rev. 166 (1997) 181.
[2] E. Wong, C.M. Giandomenico, Chem. Rev. 99 (1999) 2451.
[3] N.J. Wheate, J.G. Collins, Coord. Chem. Rev. 241 (2003) 133.
[4] K.E. Dombrowski, W. Baldwin, J.E. Sheats, J. Organomet. Chem. 302 (1986) 281.
[5] P. Köpf-Maier, Eur. J. Clin. Pharmacol. 47 (1994) 1.
[6] M.J. Clarke, F. Zhu, D.R. Frasca, Chem. Rev. 99 (1999) 2511.
[7] P. Yang, M.L. Guo, Coord. Chem. Rev. 185–186 (1999) 189.
[8] P.J. Dyson, G. Sava, Dalton Trans. (2006) 1929.
[9] K.H. Thompson, C. Orvig, Dalton Trans. (2006) 761.
[10] A. Vessières, S. Top, W. Beck, E. Hillard, G. Jaouen, Dalton Trans. (2006) 529.
[11] E.A. Hillard, A. Vessières, S. Top, P. Pigeon, K. Kowalski, M. Huché, G. Jaouen, J. Organomet. Chem. 692 (2007) 1315.
[12] M.J. Clarke, Coord. Chem. Rev. 232 (2002) 69.
[13] M. Galanski, V.B. Arion, M.A. Jakupec, B.K. Keppler, Curr. Pharm. Design 9 (2003) 2078.
[14] Y.K. Yan, M. Melchart, A. Habtemariam, P.J. Sadler, Chem. Commun. (2005) 4764.
[15] A. Habtemariam, M. Melchart, R. Fernández, S. Parsons, I.D.H. Oswald, A. Parkin, F.P.A. Fabbiani, J.E. Davidson, A. Dawson, R.E. Aird, D.I. Jodrell, P.J. Sadler, J. Med. Chem. 49 (2006) 6858.
[16] W.H. Ang, P.J. Dyson, Eur. J. Inorg. Chem. (2006) 4003.
[17] A. Bergamo, G. Sava, Dalton Trans. (2007) 1267.
[18] M.M. Harding, G. Mokdsi, Curr. Med. Chem. 7 (2000) 1289.
[19] J. Vinklárek, H. Paláčková, J. Honzíček, J. Holubová, M. Holčapek, I. Císařová, Inorg. Chem. 45 (2006) 2156.
[20] P.M. Abeysinghe, M.M. Harding, Dalton Trans. (2007) 3474.
[21] P. Köpf-Maier, H. Köpf, E.W. Neuse, J. Cancer Res. Clin. Oncol. 108 (1984) 336.
[22] P. Köpf-Maier, Z. Naturforsch, C: J. Biosci. 40 (1985) 843.
[23] E.W. Neuse, F. Kanzawa, Appl. Organomet. Chem. 4 (1990) 19.
[24] D. Osella, M. Ferrali, P. Zanello, F. Laschi, M. Fontani, C. Nervi, G. Cavigiolio, Inorg. Chim. Acta 306 (2000) 42.
[25] G. Tabbi, C. Cassino, G. Cavigiolio, D. Colangelo, A. Ghiglia, I. Viano, D. Osella, J. Med. Chem. 45 (2002) 5786.
[26] M. Gielen, Appl. Organomet. Chem. 16 (2002) 481.
[27] J.M. Rademaker-Lakhai, D. van den Bongard, D. Pluim, J.H. Beijnen, J.H.M. Schellens, Clin. Cancer Res. 10 (2004) 3717.
[28] S. Top, A. Vessières, C. Cabestaing, I. Laios, G. Leclercq, C. Provot, G. Jaouen, J. Organomet. Chem. 637 (2001) 500.
[29] S. Top, A. Vessières, G. Leclercq, J. Quivy, J. Tang, J. Vaisserman, M. Huché, G. Jaouen, Chem. Eur. J. 9 (2003) 5223.
[30] E. Hillard, A. Vessières, L. Thouin, G. Jaouen, C. Amatore, Angew. Chem., Int. Ed. 45 (2006) 285.
[31] U. Schatzschneider, N. Metzler-Nolte, Angew. Chem., Int. Ed. 45 (2006) 1504.
[32] G. Caldwell, M.G. Meirim, E.W. Neuse, C.E.J. van Rensburg, Appl. Organomet. Chem. 12 (1998) 793.
[33] E.W. Neuse, J. Inorg. Organomet. Polym. Mater. 15 (2005) 3.
[34] D.R. van Staveren, N. Metzler-Nolte, Chem. Rev. 104 (2004) 5931.
[35] M.F.R. Fouda, M.M. Abd-Elzaher, R.A. Abdelsamaia, A.A. Labib, Appl. Organomet. Chem. 21 (2007) 613.
[36] T. Loftsson, M.E. Brewster, J. Pharm. Sci. 85 (1996) 1017.
[37] R.A. Rajewski, V.J. Stella, J. Pharm. Sci. 85 (1996) 1142.
[38] R.D. Sinisterra, V.P. Shastri, R. Najjar, R. Langer, J. Pharm. Sci. 88 (1999) 574.
[39] D.R. Alston, T.H. Lilley, J.F. Stoddart, J. Chem. Soc., Chem. Commun. (1985) 1600.
[40] D.R. Alston, A.M.Z. Slawin, J.F. Stoddart, D.J. Williams, J. Chem. Soc., Chem. Commun. (1985) 1602.

[41] D.R. Alston, P.R. Ashton, T.H. Lilley, J.F. Stoddart, R. Zarzycki, A.M.Z. Slawin, D.J. Williams, Carbohydr. Res. 192 (1989) 259.
[42] I. Turel, A. Demšar, J. Košmrlj, J. Inclusion Phenom. Macrocyclic Chem. 35 (1999) 595.
[43] J. Vinklárek, J. Honzíček, J. Holubová, Central Eur. J. Chem. 3 (2005) 72.
[44] C.C.L. Pereira, M. Nolasco, S.S. Braga, F.A.A. Paz, P. Ribeiro-Claro, M. Pillinger, I.S. Gonçalves, Organometallics 26 (2007) 4220.
[45] S.S. Braga, I.S. Gonçalves, M. Pillinger, P. Ribeiro-Claro, J.J.C. Teixeira-Dias, J. Organomet. Chem. 632 (2001) 11.
[46] T. Utsuki, H. Brem, J. Pitha, T. Loftsson, T. Kristmundsdottir, B.M. Tyler, A. Olivi, J. Controlled Release 40 (1996) 251.
[47] S.S. Braga, M.P.M. Marques, J.B. Sousa, M. Pillinger, J.J.C. Teixeira-Dias, I.S. Gonçalves, J. Organomet. Chem. 690 (2005) 2905.
[48] F. Hapiot, S. Tilloy, E. Monflier, Chem. Rev. 106 (2006) 767.
[49] T. Irie, K. Uekama, J. Pharm. Sci. 86 (1997) 147.
[50] D. Lednicer, C.R. Hauser, Org. Synth. Coll. 5 (1973) 434.
[51] S.I. Goldberg, W.D. Bailey, J. Am. Chem. Soc. 96 (1974) 6381.
[52] S. Picart-Goetgheluck, O. Delacroix, L. Maciejewski, J. Brocard, Synthesis (2000) 1421.
[53] V.I. Sokolov, L.L. Troitskaya, O.A. Reutov, J. Organomet. Chem. 182 (1979) 537.
[54] W. Saenger, Angew. Chem., Int. Ed. Engl. 19 (1980) 344.
[55] M.J. Gidley, S.M. Bociek, J. Am. Chem. Soc. 110 (1988) 3820.
[56] S.J. Heyes, N.J. Clayden, C.M. Dobson, Carbohydr. Res. 233 (1992) 1.
[57] R.P. Veregin, C.A. Fyfe, R.H. Marcessault, M.G. Tayler, Carbohydr. Res. 160 (1987) 41.
[58] J.R. Johnson, N. Shankland, I.H. Sadler, Tetrahedron 41 (1985) 3147.
[59] Y. Yamamoto, M. Onda, Y. Takahashi, Y. Inoue, R. Chûjô, Carbohydr. Res. 170 (1987) 229.
[60] J. Li, A. Harada, M. Kamachi, Bull. Chem. Soc. Jpn. 67 (1994) 2808.
[61] R.W. Mason, K. McGrouther, P.R.R. Ranatunge-Bandarage, B.H. Robinson, J. Simpson, Appl. Organomet. Chem. 13 (1999) 163.
[62] V.J. Fiorina, R.J. Dubois, S. Brynes, J. Med. Chem. 21 (1978) 393.
[63] S. Bradley, K.D. Camm, X. Liu, P.C. McGowan, R. Mumtaz, K.A. Oughton, T.J. Podesta, M. Thornton-Pett, Inorg. Chem. 41 (2002) 715.
[64] S.J. Furtado, A.L. Gott, P.C. McGowan, J. Chem. Soc., Dalton Trans. (2004) 436.
[65] S. Gould, R.C. Scott, Food Chem. Toxicol. 43 (2005) 1451.
[66] E. Hillard, A. Vessières, F. Le Bideau, D. Plażuk, D. Spera, M. Huché, G. Jaouen, ChemMedChem 1 (2006) 551.
[67] C. Lu, X. Ren, L. Liu, Y. Zhang, C. Hu, H. Zhu, Q. Meng, J. Inclusion Phenom. Macrocyclic Chem. 43 (2002) 19.
[68] T. Komura, T. Yamaguchi, K. Noda, S. Hayashi, Electrochim. Acta 47 (2002) 3315.
[69] E. Coutouli-Argyropoulou, A. Kelaidopoulou, C. Sideris, G. Kokkinidis, J. Electroanal. Chem. 477 (1999) 130.