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Note

N-ortho-Ferrocenyl benzoyl dipeptide esters: Synthesis, structural characterization and *in vitro* anti-cancer activity of *N*-{*ortho*-(ferrocenyl)benzoyl}-glycine-L-alanine ethyl ester and *N*-{*ortho*-(ferrocenyl)benzoyl}-L-alanine-glycine ethyl ester

Alan J. Corry [a], Alok Goel [a,b], Steven R. Alley [a,b], Paula N. Kelly [a,b], Dermot O'Sullivan [b], David Savage [a], Peter T.M. Kenny [a,b,*]

[a] *School of Chemical Sciences, Dublin City University, Dublin 9, Ireland*
[b] *National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland*

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Abstract

N-ortho-ferrocenyl benzoyl dipeptide esters **2**–**6** were prepared by coupling *ortho*-ferrocenyl benzoic acid **1** to the dipeptide ethyl esters GlyGly(OEt) (**2**), GlyAla(OEt) (**3**), GlyPhe(OEt) (**4**), AlaGly(OEt) (**5**) and AlaPhe(OEt) (**6**). The compounds were fully characterized by a range of NMR spectroscopic techniques, mass spectrometry and cyclic voltammetry. The cytotoxicity of **3** and **5** towards lung cancer cells has been determined.

Keywords: Ferrocene; Bioorganometallic chemistry; Dipeptides; Cytotoxicity; Lung cancer

1. Introduction

The organometallic compound ferrocene is a promising candidate for biological applications due to its stability, electrochemical properties, and ease of use [1,2]. Ferrocene can be conjugated to drugs such as antibiotics, aspirin, anti-malarials and anti-cancer drugs, such as tamoxifen [3–6]. Ferricenium salts of the form $Fc^{+}X^{-}$ ($X{=}CCl_3CO_2$) have demonstrated anti-tumour activity [7]. The relatively low reduction potential of these compounds is responsible for generating reactive oxygenated species (ROS) under physiological conditions and results in anti-cancer activity [8,9]. Thus, there is increased interest in the synthesis of ferrocene derivatives with low oxidation potentials. The synthesis and structural characterization of *N*-ferrocenoyl and *N*-ferrocenyl amino acid and peptide derivatives have been previously reported [10–27]. Herein, we report the synthesis and structural characterization of *N-ortho*-ferrocenyl benzoyl dipeptide esters and *in vitro* anti-cancer activity of *N*-{*ortho*-(ferrocenyl)benzoyl}-glycine-L-alanine ethyl ester and *N*-{*ortho*-(ferrocenyl)benzoyl}-L-alanine-glycine ethyl ester. The *N-ortho*-ferrocenyl dipeptide esters **2**–**6** consist of three key components: (i) the electroactive core; (ii) a conjugated linker that lowers the oxidation potential and (iii) a peptide derivative that can interact with other biomolecules via secondary interactions, such as hydrogen bonding. We have previously reported *N-para* and *N-meta*-ferrocenyl benzoyl dipeptide esters [28–30].

2. Results and discussion

ortho-Ferrocenyl benzoic acid **1** was prepared as previously reported [31]. The dipeptide ethyl ester hydrochloride

* Corresponding author. Address: School of Chemical Sciences, Dublin City University, Dublin 9, Ireland. Tel.: +353 1 7005689; fax: +353 1 7005503.
E-mail address: peter.kenny@dcu.ie (P.T.M. Kenny).

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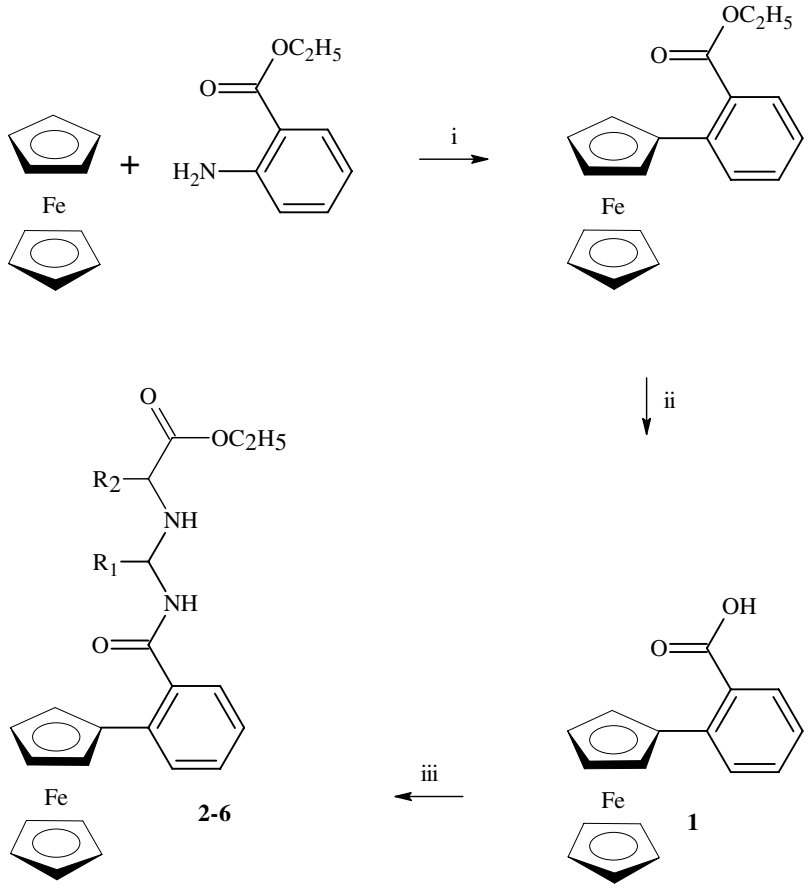
salts of GlyGly, GlyAla, GlyPhe, AlaGly and AlaPhe were coupled to *ortho*-ferrocenyl benzoic acid using 1,3-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) in the presence of excess triethylamine in dichloromethane (Scheme 1). Purification by column chromatography gave the desired products in yields of between 25% and 47%, and all compounds gave spectroscopic data in accordance to the proposed structures. The yields for this series of compounds are lower than those reported for the related *meta* and *para* derivatives [28–30]. This could be attributed to steric hindrance of the *ortho* substituent with the unsubstituted (η^5-C_5H_5) ring. Also in contrast to the related *para* and *meta* derivatives the compounds are not particularly stable and decompose over a period of time.

The *N*-*ortho*-ferrocenyl benzoyl dipeptide derivatives **2**–**6** were characterized by a combination of ^{1}H NMR, ^{13}C NMR, DEPT-135 and ^{1}H-^{13}C COSY (HMQC) spectroscopy, mass spectrometry and cyclic voltammetry. The ^{1}H and ^{13}C NMR spectra for compounds **2**–**6** showed peaks in the ferrocene region that are typical of a ferrocenyl benzoyl group [32–34]. The aromatic protons appear from δ 7.15 to δ 7.8. The protons in the *ortho* position of the (η^5-C_5H_4) ring appear between δ 4.52 and δ 4.76, the *meta* peaks appear between δ 4.29 and δ 4.46, while the unsubstituted (η^5-C_5H_5) ring appears between δ 4.06 and δ 4.21.

For example in the ^{1}H NMR spectrum of **5** (obtained in DMSO-d_6) two amide protons were present at δ 8.35 and δ 8.18. Both of these signals integrate for one proton each and appear as a doublet and a triplet; the alanine amide proton as a doublet at δ 8.35 and the glycine amide proton as a triplet at δ 8.18. The signals in the aromatic region confirm the presence of four protons, which appear between δ 7.24 and δ 7.80.

In the ferrocenyl region, the typical mono substituted ferrocene splitting pattern is observed. The peaks of the *ortho* protons on the (η^5-C_5H_4) ring appear as the furthest downfield ferrocenyl signal. In compound **5**, this signal appears at δ 4.58 and the *meta* (η^5-C_5H_4) protons at δ 4.27. The quintet at 4.41 ppm, which integrates for one proton, represents the α-proton of the alanine amino acid. There is a multiplet at δ 4.01–4.13 that integrates for seven protons. This corresponds to the unsubstituted (η^5-C_5H_5) ring of the ferrocene and the methylene group of the ethyl ester. The CH_2 group of the glycyl moiety is seen at δ 3.78–3.93. The methyl group of the alanine moiety and the methyl group of the ethyl ester of the dipeptide appear as a doublet at δ 1.25 and as a triplet at δ 1.17, respectively.



Scheme 1. Synthesis of *N*-{*ortho*-(ferrocenyl)-benzoyl} dipeptide ethyl esters **2**–**6**, (i) $NaNO_2$, HCl, 5 °C; (ii) NaOH/MeOH; (iii) DCC, HOBt, triethylamine, dipeptide ethyl ester (GlyGly **2**, GlyAla **3**, GlyPhe **4**, AlaGly **5** and **6** AlaPhe **6**).

The ^{13}C spectra of compounds **2**–**6** show typical mono-substituted ferrocene peaks between δ 68.4 and δ 85.6. The *ipso* carbon of the (η^5-C_5H_4) ring is present in the narrow range between δ 84.7 and δ 85.6. This peak is absent from the DEPT 135 spectrum. The quaternary carbons and the methylene carbons of the dipeptide derivatives **2**–**6** were identified by the DEPT 135 spectrum.

In the ^{13}C NMR spectrum of compound **5**, there are three peaks seen between δ 169.7 and δ 173.0. These are due to the three carbonyl groups. Six inequivalent carbons are present in the aromatic region. This is typical for an *ortho* disubstituted benzene group with two different substituents. The quaternary carbons were identified by DEPT 135 are seen at δ 136.5 and δ 136.6 and the four remaining carbons are at δ 125.8, 127.8, 129 and 130.5, respectively. The peak at δ 85 absent from the DEPT 135 spectrum is the *ipso* ferrocenyl carbon. The carbons of the unsubstituted (η^5-C_5H_5) ring appear at δ 69.8. The *meta* carbons of the ferrocenyl group are at δ 69.5 and the *ortho* carbons appear at δ 68.4. The methylene groups can be assigned from the DEPT 135 spectrum. The methylene of the ester is seen at δ 60.8, while the methylene group of the glycine appears at δ 41.1. The remaining methyl carbons appear at δ 18.0 and δ 14.4, respectively.

Both matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) confirmed the correct relative molecular mass for all the compounds. The ESI mass spectra displayed both radical cation and $[M+H]^+$ species. Sodium and potassium adducts were also observed 22 Da and 38 Da higher than the protonated molecular ion species.

In contrast to the corresponding mass spectra of the *meta* and *para* derivatives an important fragment ion at *m*/*z* $[M\text{-}65]^+$ is present in all the spectra. This corresponds to the loss of the unsubstituted (η^5-C_5H_5) ring. The formation of this ion, which is unique for the *N-ortho*-ferrocenyl benzoyl derivatives, is possibly due to steric hindrance between the *ortho* substituted benzoyl substituents and the unsubstituted (η^5-C_5H_5) ring.

The CV curves for compounds **2**–**6** exhibit quasi-reversible behaviour similar to the Fc/Fc^+ redox couple. The $E°$ (oxidation potential) values were in 49–52 mV (versus Fc/Fc^+) range. These values are significantly lower than ferrocenoyl dipeptide ester derivatives. For ferrocenoyl-Ala-Ala-OMe the $E°$' value was reported as 230 mV (versus Fc/Fc^+) [35], while the corresponding value for ferrocenoyl-Ala-Phe-OMe was reported as 190 mV (versus Fc/Fc^+) [26]. It is explicable in terms of substituent effects. The benzoyl moiety offers extended conjugation to the pi electrons of the Cp rings and makes ferrocenyl benzoyl derivatives **2**–**6** easier to oxidize.

The cytotoxicity of compounds **3** and **5** were measured against highly invasive/superinvasive, H1299, lung cancer cells. Compound **5** showed an IC_{50} value of 21 μM (RSD 15%), while compound **3** showed an IC_{50} value of 5.3 μM (RSD 8%). *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycine ethyl ester was initially tested against the above cell line and showed an IC_{50} value of 48 μM (RSD 13%), while the *meta* analogue of **5** had an IC_{50} value of 26 μM (RSD 20%) [36]. A comparison between the *ortho* amino acid and dipeptide compounds **3** and **5** is seen in Fig. 1. From these results it can be seen that the order of the amino acids in the dipeptide is crucial for activity. Both compounds tested have similar structures, however **3** is much more active against H1299 cancer cells than compound **5**. From this is may be assumed that the glycine moiety of the dipeptide that is attached to the benzoyl group is important for activity. The benefits of glycine in treating various diseases have been previously reported [37].

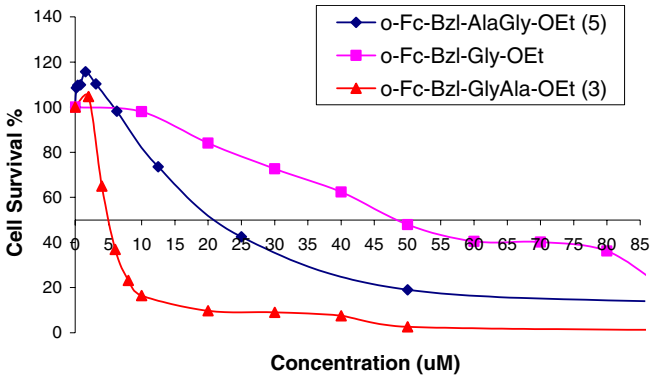


Fig. 1. Cytotoxicity of compounds **3** and **5** and the corresponding *ortho*-glycine derivative.

The possible mechanism for their anti-cancer activity originates from the low redox potential of these derivatives and their ability to catalyze the generation of reactive oxygenated species (ROS), under physiological conditions, that can oxidatively modify cellular components (e.g., DNA), disturb the redox balance in the cell, and/or interfere with the redox-related cellular signalling pathways [38]. Alternatively, cytotoxicity may arise from direct interactions of these derivatives with other cellular components, such as proteins [39]. Since, preliminary results imply some structure activity relationship, it is envisaged that the peptide chain of these derivatives may have a secondary mode of action. These preliminary results are encouraging and further studies are in progress to elucidate the mechanism of action and for the selection of a series that has maximum cytotoxicity against lung cancer cells.

3. Conclusions

In conclusion, the novel *N-ortho* ferrocenyl benzoyl dipeptides **2**–**6** were prepared using standard peptide coupling protocols. The compounds were characterized using NMR spectroscopic techniques, mass spectrometry and cyclic voltammetry. Compounds **3** and **5** were tested *in vitro* against H1299 lung cancer cells and showed IC_{50} values of 5.3 μM and 21 μM, respectively, which is higher than the corresponding amino acid derivatives.

4. Experimental

4.1. General procedures

All chemicals were purchased from Sigma/Aldrich and used as received. Commercial grade reagents were used without further purification, however, solvents were purified prior to use. Melting points were determined using a Griffin melting point apparatus and are uncorrected. Infrared spectra were recorded on a Nicolet 405 FT-IR spectrometer and UV–Vis spectra on a Hewlett–Packard 8452 A diode array UV–Vis spectrophotometer. NMR spectra were obtained on a Bruker AC 400 NMR spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The ^{1}H and ^{13}C NMR chemical shifts (ppm) are relative to TMS and all coupling constants (J) are in Hz. Matrix assisted laser desorption ionization mass spectra were obtained on a Bruker Ultraflex TOF/TOF mass spectrometer employing a nitrogen laser at 337 nm. Electrospray ionization mass spectra were performed on a Bruker Esquire ion trap mass spectrometer.

4.2. General procedure for the synthesis of N-{ortho-(ferrocenyl)benzoyl} dipeptide esters

4.2.1. N-{ortho-(ferrocenyl)-benzoyl}-glycine-glycine ethyl ester 2

Glycine-glycine ethyl ester hydrochloride (0.2 g, 1.0 mmol) was added to a solution of *ortho*-ferrocenyl benzoic acid (0.3 g, 1.0 mmol), 1-hydroxybenzatriazole (0.2 g, 1.5 mmol), triethylamine (0.5 ml) and dicyclohexylcarbodiimide (0.45 g, 2.1 mmol) in 50 ml of dichloromethane at 0 °C. After 30 min the temperature was raised to room temperature and the reaction was allowed to proceed for 48 h. The precipitated *N*-*N*′-dicyclohexylurea was removed by filtration and the filtrate was washed with water, 10% potassium hydrogen carbonate and 5% citric acid. The dichloromethane was then dried over $MgSO_4$ and the solvent was removed *in vacuo*. The product was purified by column chromatography {eluant 2:3 petroleum ether (40–60 °C): ethyl acetate}. Recrystallization from petroleum ether (40–60 °C)-ethyl acetate furnished the title compound as orange needles (0.198 g, 44%), m.p. 69–71 °C, $E^{\circ\prime} = 49$ Mv (versus Fc/Fc$^+$). Mass spectrum: found: $[M]^{+\cdot}$, 448.106; $C_{23}H_{24}N_2O_4Fe$ requires, 448.109. IR ν_{max}(KBr): 3397, 2983, 1737, 1657, 1650, 1524, 1379, 1202 cm^{-1}. UV–Vis λ_{max} EtOH; 326 (ε 1430), 439 (ε 290) nm.

^{1}H NMR (400 MHz) δ (DMSO): 8.50 (1 H, t, $J = 6$ Hz, –CON*H*–), 8.70 (1H, t, $J = 6$ Hz, –CON*H*–), 7.79 (1H, d, $J = 7.6$ Hz, ArH), 7.40 (1H, t, $J = 7.6$ Hz, ArH), 7.24–7.29 (2H, m, ArH), 4.64 {2H, t, $J = 2$ Hz, *ortho* on (η^5-C_5H_4)}, 4.27 (2H, t, $J = 2$ Hz), *meta* on (η^5-C_5H_4), 4.22 (2H, q, $J = 7.2$ Hz, –OC*H*$_2$CH$_3$), 4.06 (5H, s, η^5-C_5H_5), 3.87 (2H, d, $J = 5.6$ Hz, –NHC*H*$_2$CO–), 3.83 (2H, d, $J = 5.6$ Hz, –NHC*H*$_2$CO–), 1.28 (3H, t, $J = 7.2$ Hz, –OCH$_2$C*H*$_3$). ^{13}C NMR (100 MHz) δ (DMSO): 170.5, 170.1, 169.7, 136.6, 136.4, 130.4, 129.1, 127.8, 125.8, 84.8, 69.8, 69.1, 68.6, 60.9 (−ve DEPT), 42.3 (−ve DEPT), 41.0 (−ve DEPT), 14.4.

4.2.2. N-{ortho-(ferrocenyl)-benzoyl}-glycine-L-alanine ethyl ester 3

For the compound **3** glycine-L-alanine ethyl ester hydrochloride (0.2 g, 1.0 mmol) was used as a starting material. The product was purified by column chromatography {eluant 2:3 petroleum ether (40–60 °C): ethyl acetate}. Recrystallization from petroleum ether (40–60 °C): ethyl acetate furnished the title compound as an orange solid (0.136 g, 29%). m.p. 102–104 °C, $E^{\circ\prime} = 50$ mV (versus Fc/Fc$^+$), $[\alpha]_D^{20} = -4^\circ$ (c 1.9, EtOH). Mass spectrum: found: $[M]^+$ 462.113, $C_{24}H_{26}N_2O_4Fe$ requires: 462.124. IR ν_{max}(KBr): 3376, 3281, 1720, 1647, 1480, 1370, 1293 cm^{-1}. UV–Vis λ_{max} MeCN; 224 (ε 340), 445 (ε 210) nm.

^{1}H NMR (400 MHz) δ ($CDCl_3$): 7.80 (1H, d, $J = 7.6$ Hz, ArH), 7.40–7.44 (2H, m, ArH), 7.27 (1H, t, $J = 7.6$ Hz, ArH), 6.72 (1H, d, $J = 6.8$ Hz, –CON*H*–), 6.15 (1H, br.s, –CON*H*–), 4.50–4.52 {3H, m, *ortho* on (η^5-C_5H_4), –C*H*(CH$_3$)}, 4.29 {2H, s, *meta* on (η^5-C_5H_4)}, 4.20 (2H, q, $J = 6.8$ Hz, –OC*H*$_2$CH$_3$), 4.11 {5H, s, (η^5-C_5H_5)}, 3.97 (2H, t, $J = 4.8$ Hz, –CONHC*H*$_2$–), 1.39 {3H, d, $J = 7.2$ Hz, –CH(C*H*$_3$)}, 1.28 (3H, t, $J = 6.8$ Hz, –OCH$_2$C*H*$_3$).

^{13}C NMR (100 MHz) δ ($CDCl_3$):173.0, 171.3, 168.4, 136.9, 135.5, 131.3, 130.0, 128.3, 126.7, 85.6, 70.2, 69.7, 69.6, 69.2, 61.9 (−ve DEPT), 48.5, 44.0 (−ve DEPT), 18.7, 14.5.

4.2.3. N-{ortho-(ferrocenyl)-benzoyl}-glycine-L-phenylalanine ethyl ester 4

For the compound **4** glycine-L-phenylalanine ethyl ester hydrochloride (0.2 g, 0.7 mmol) was used as a starting material. The product was purified by column chromatography {eluant 2:3 petroleum ether (40–60 °C): ethyl acetate}. The resultant product was recrystallized from petroleum ether (40–60 °C): ethyl acetate and furnished the title compound as orange needles, (0.105 g, 28%), m.p. 51–53 °C, $E^{\circ\prime} = 51$ mV (versus Fc/Fc$^+$), $[\alpha]_D^{20} = +2^\circ$ (c 2.1, EtOH). Mass spectrum: found: $[M]^+$ 538.168, $C_{30}H_{30}N_2O_4Fe$ requires: 538.156. IR ν_{max}(KBr): 3331, 2933, 1735, 1654, 1648, 1523, 1376, 1216 cm^{-1}. UV–Vis λ_{max} EtOH; 323 (ε 1570), 440 (ε 360) nm.

^{1}H NMR (400 MHz) δ (DMSO): 8.29 (1H, t, $J = 6$ Hz, –CON*H*–), 8.18 (1H, d, $J = 7.6$ Hz, –CON*H*–), 7.62 (1H, d, $J = 8$ Hz, ArH), 7.32 (1H, t, $J = 8$ Hz, ArH), 7.03–7.15 (7H, m, ArH), 4.48 {2H, s, *ortho* on (η^5-C_5H_4)}, 4.30–4.37 {1H, m, $J = 6.4$ Hz, –C*H*(CH$_2$Ph)}, 4.09 {2H, s, *meta* on (η^5-C_5H_4)}, 3.86–3.92 {7H, m, (η^5-C_5H_5), –OC*H*$_2$CH$_3$}, 3.64 (2H, t, $J = 5.2$ Hz, –NHC*H*$_2$CO–), 2.80–2.85 {2H, m, –CH(C*H*$_2$Ph)}, 0.92 (3H, t, $J = 7.2$ Hz, –OCH$_2$C*H*$_3$). ^{13}C NMR (100 MHz) δ (DMSO): 171.7, 170.3, 169.2, 137.3, 136.6, 136.4, 130.7, 129.5, 129.1, 128.6, 127.7, 127.0, 125.7, 84.7, 69.8, 69.1,

69.1, 68.6, 60.9 (−ve DEPT), 54.1, 42.2 (−ve DEPT), 37.3 (−ve DEPT), 14.3.

4.2.4. N-{ortho-(ferrocenyl)-benzoyl}-L-alanine-glycine ethyl ester 5

For compound **5** L-alanine-glycine ethyl ester hydrochloride (0.2 g, 1.0 mmol) was used as a starting material. The product was purified by column chromatography {eluant 2:3 petroleum ether (40–60 °C): ethyl acetate}. Recrystallization from petroleum ether (40–60 °C): ethyl acetate furnished the title compound as orange needles (0.181 g, 39%). m.p 55–57 °C, $E^{\circ\prime} = 51$ mV (versus Fc/Fc^{+}), $[\alpha]_D^{20} = +0.3^{\circ}$ (c 2.05, EtOH). Mass spectrum: found: $[M]^{+}$ 462.09, $C_{24}H_{26}N_2O_4Fe$ requires: 462.12. IR ν_{max}(KBr): 3326, 2933, 1752, 1655, 1638, 1509, 1200 cm^{-1}. UV–Vis λ_{max} EtOH; 321 (ε 1050), 441 (ε 240) nm.

^{1}H NMR (400 MHz) δ (DMSO): 8.35 (1H, d, $J =$ 7.2 Hz, –CON*H*–), 8.18 (1H, t, $J = 7.2$ Hz, –CON*H*–), 7.80 (1H, d, $J = 8$ Hz, ArH), 7.41 (1H, t, $J = 8$ Hz, ArH), 7.24–7.38 (2H, m, ArH), 4.57–4.59 {2H, m, *ortho* on (η^5-C_5H_4)}, 4.41 {1H, quint, $J = 7.6$ Hz, –C*H*(CH_3)}, 4.25–4.29 {2H, m, *meta* on (η^5-C_5H_4)}, 4.01–4.13 {7H, m, –O*C*$H_2$$CH_3$, ($\eta^5$-$C_5H_5$)}, 3.78–3.93 (2H, m, –CONH-C*H*$_2$–), 1.25 {3H, d, $J = 7.6$ Hz, –CH(C*H*$_3$)}, 1.17 (3H, t, $J = 7.2$ Hz, –OCH_2C*H*$_3$). ^{13}C NMR (100 MHz) δ (DMSO): 173.0, 170.1, 169.7, 136.6, 136.5, 130.5, 129.0, 127.8, 125.8, 85.0, 69.8, 69.5, 68.8, 68.5, 68.4, 60.8 (−ve DEPT), 48.7, 41.1 (−ve DEPT), 18.0, 14.4.

4.2.5. N-{ortho-(ferrocenyl)benzoyl}-L-alanine-L-phenylalanine ethyl ester 6

For the compound **6** L-alanine-L-phenylalanine ethyl ester hydrochloride (0.2 g, 0.7 mmol) was used as starting material. The product was purified by column chromatography {eluant 2:3 petroleum ether (40–60 °C): ethyl acetate}. Recrystallization from petroleum ether (40–60 °C): ethyl acetate furnished the title compound as an orange solid (0.102 g, 25%), m.p. 137–139 °C, $E^{\circ\prime} = 52$ mV (versus Fc/Fc^{+}), $[\alpha]_D^{20} = +2^{\circ}$ (c 2.05, EtOH). Mass spectrum: found: $[M]^{+}$ 552.158, $C_{31}H_{32}N_2O_4Fe$ requires: 552.171. IR ν_{max}(KBr): 3397, 3309, 2932, 1742, 1645, 1509, 1496, 1210 cm^{-1}. UV–Vis λ_{max} EtOH; 323 (ε 1570), 440 (ε 340) nm.

^{1}H NMR (400 MHz) δ(DMSO): 8.37–8.46 (2H, m, –CON*H*-), 7.80 (1H, d, $J = 7.2$ Hz, ArH), 7.60 (1H, t, $J = 7.2$ Hz, ArH), 7.38–7.47 (7H, m, ArH), 4.76 {2H, t, $J = 1.2$ Hz, *ortho* on (η^5-C_5H_4)}, 4.60–4.76 {2H, m, –C*H*(CH_3), –C*H*(CH_2Ph)}, 4.42–4.46 {2H, m, *meta* on (η^5-C_5H_4)}, 4.21–4.26 {7H, m, (η^5-C_5H_5), –O*C*$H_2$$CH_3$}, 3.19–3.23 {2H, m, –CH(C*H*$_2$Ph)}, 1.35 {3H, d, $J = 7.2$ Hz, –CH(C*H*$_3$)}, 1.27 (3H, t, $J = 7.6$ Hz, –OCH_2C*H*$_3$).

^{13}C NMR (100 MHz) δ (DMSO): 172.6, 171.7, 169.5, 137.3, 136.5, 130.5, 129.5, 129.1, 128.6, 128.5, 127.8, 126.9, 125.8, 85.0, 69.8, 69.5, 68.7, 68.5, 60.9 (−ve DEPT), 53.9, 48.6, 33.7 (−ve DEPT), 18.1, 14.4.

4.3. General procedure for in vitro cytotoxicity assays

Lung cancer cells (H1299, highly invasive/superinvasive; H1299 carboplatin resistant variant) were harvested by trypsinisation and a cell suspension of 1×10^4 cells/ml was prepared in a cell culture medium. The cell suspension (100 μl) was added to a flat bottom 96-well plate (Costar, 3599), plates were agitated gently in order to ensure even dispersion of cells over the surface of the wells, and then cells were incubated for an initial 24 h in a 37 °C, 5% CO_2 incubator, to allow cell attachment to the wells. A stock solution of a test sample was prepared in dimethyl sulfoxide; dilute solutions of the test sample were prepared by spiking the cell culture medium with a calculated amount of the stock solution. 100 μl aliquot of the each dilute solution was added to each well of the plate, plate was gently agitated, and then incubated at 37 °C, 5% CO_2 for 6–7 days, until cell confluency reached 80–90%. Assessment of cell survival in the presence of the ferrocenyl derivatives (**2**–**6**) was determined by the acid phosphatase assay. The concentration of drug that kills 50% of the cells (the IC_{50} value) was determined by plotting % survival of cells (relative to the control cells) against concentration of the ferrocenyl derivative.

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