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Synthesis, characterization and *in vitro* anti-cancer activity of *N*-(ferrocenyl)benzoyl tri- and tetrapeptide esters

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ABSTRACT

N-ortho, *N-meta* and *N-para*-(ferrocenyl)benzoyl tri- and tetrapeptide esters (**2–7**) were prepared by coupling *ortho*, *meta* and *para*-ferrocenyl benzoic acids to the tri- and tetrapeptide ethyl esters of GlyGlyGly(OEt) and GlyGlyGlyGly(OEt) in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride and 1-hydroxybenzotriazole. The compounds were characterized by a range of NMR spectroscopic techniques, mass spectrometry and cyclic voltammetry. The anti-proliferative effects of the *ortho* derivatives **2** and **5** were measured *in vitro* against H1299 lung cancer cells and both gave IC_{50} values greater than 50 μM. Therefore, extending the length of the peptide chain had a negative effect on activity, relative to *N*-(ferrocenyl)benzoyl amino acid and dipeptide derivatives.

1. Introduction

The metallocene ferrocene has several novel applications due to its ease of derivatization, stability, spectroscopic properties and redox activity. Research in the area of ferrocenyl derivatives has focused on their potential as sensors, peptide mimetic models and unnatural drugs [1–7]. Amino acids and peptides play diverse roles in biological systems, hence the synthesis of *N*-ferrocenyl and *N*-ferrocenoyl amino acid and peptide derivatives has been extensively reported [8–21]. The medicinal application of ferrocene is currently an active area of research with many reports showing the activity of ferrocene derivatives *in vivo* and *in vitro*. The main attention has focused on their use as anti-malarial and anti-cancer drugs [22]. It has also been shown that *N*-(ferrocenylmethyl)fluorobenzene-carboxamide derivatives display anti-cancer activity against ER (+) MDA-MB-435-S-F breast cancer cells [23].

We have previously reported the synthesis and structural characterization of *N-ortho, N-meta* and *N-para*-(ferrocenyl)benzoyl derivatives incorporating natural amino acids and dipeptide derivatives [24–30,32,33]. The compounds are composed of three key moieties, namely, (i) an electroactive core, (ii) a conjugated aromatic linker that lowers the redox potential and (iii) an amino acid or peptide derivative that can interact with other molecules *via* hydrogen bonding. The ferrocenyl benzoyl derivatives have lower redox potentials when compared to the corresponding ferrocenoyl dipeptide derivatives. This fact can be explained in terms of substituent effects. The benzoyl moiety offers extended conjugation to the pi electrons of the ferrocene rings making these derivatives easier to oxidise to the ferricenium species thus making them suitable as anti-cancer agents. The novel ferrocenyl benzoyl dipeptide derivatives were shown to be cytotoxic. An *ortho*-ferrocenylbenzoyl amino acid derivative of glycine, *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycine ethyl ester was initially tested for its *in vitro* anti-cancer activity towards H1299 lung cancer cells. This compound was found to be cytotoxic and had an IC_{50} value of 48 μM, whereas the starting material, *ortho*-ferrocenyl ethyl benzoate, was completely inactive against the cell lines. This indicates that the amino acid or dipeptide derivative of these compounds is essential for biological activity. Therefore, other derivatives were evaluated for their anti-cancer activity against H1299 lung cancer cells. The dipeptide derivative *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycine-glycine ethyl ester was shown to have an IC_{50} value of approximately 20 μM. From this it may be assumed that the glycine residue of the dipeptide that is attached to the benzoyl group is important for activity. As the dipeptide derivative was more active than the amino acid derivative, a logical extension of this study was the preparation of longer peptide chains. Therefore, the peptide moiety was chain extended by additional glycine residues. Herein, we now report the synthesis and structural characterization of *N-ortho*, *N-meta* and *N-para*-(ferrocenyl)benzoyl tri- and tetrapeptide esters. The *in vitro* anti-proliferative activity for *N-ortho*-(ferrocenyl)benzoyl tri- and tetrapeptide esters **2** and **5** against H1299 lung cancer cells is also presented.

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2. Results and discussion

2.1. Synthesis

The arylation of ferrocene is readily achieved by reacting ferrocene with an aryl diazonium salt. For the synthesis of the *N*-(ferrocenyl)benzoyl peptide derivatives (**2–7)** ethyl-2, ethyl-3 and ethyl-4-aminobenzoate were used in order to generate the starting materials, *ortho*, *meta* and *para*-ferrocenyl ethyl benzoates, respectively. These compounds were isolated as viscous oils. The ethyl ester group was efficiently cleaved to yield the three ferrocenyl benzoic acids by saponification using 10% sodium hydroxide. This procedure is outlined in Scheme 1.

Coupling reactions were used to facilitate the introduction of the ferrocenyl benzoyl group onto the peptide esters. *Ortho*, *meta* and *para*-ferrocenyl benzoic acids **1** were treated with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), and triethylamine (TEA) in dichloromethane at 0 °C in the presence of glycyl-glycyl-glycine ethyl ester hydrochloride and glycyl-glycyl-glycyl-glycine ethyl ester hydrochloride (Scheme 1). The resulting *N*-(ferrocenyl)benzoyl peptide derivatives (**2–7**) gave spectroscopic data in accordance with the proposed structures in yields ranging from 29% to 55%.

2.2. Characterization

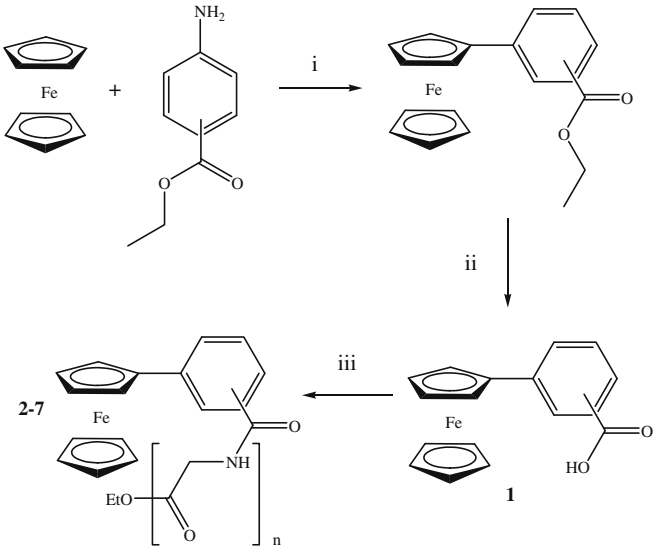
The *N*-(ferrocenyl)benzoyl peptide derivatives (**2-7)** were characterized by a combination of ^{1}H NMR, ^{13}C NMR and DEPT-135 spectroscopy, mass spectrometry and cyclic voltammetry. All the proton and carbon chemical shifts for compounds **2–7** were unambiguously assigned by a combination of DEPT-135 and ^{1}H–^{13}C–COSY (HMQC). The aromatic signals in the ^{1}H NMR spectra of the *N*-(ferrocenyl)benzoyl peptide esters (**2–7)** varied depending on whether *ortho*, *meta* or *para*-ferrocenyl benzoic acid was used as a starting material. For the *ortho* derivatives the aromatic region showed a doublet, triplet, multiplet splitting pattern, integrating for one, one and two protons, respectively. In the *meta* derivatives the pattern observed was a singlet that integrated for one proton, a multiplet that integrated for two protons and a triplet that integrated for one proton. The *para* substituted splitting pattern consisted of two doublets that each integrated for two protons. The chemical shift of the amide proton that forms the amide bond between the benzoyl group and the peptide chain was present between δ 8.9 and δ 8.5 for the tripeptides and δ 8.9 and δ 8.6 for the tetrapeptides. For example in the case of *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycyl-glycyl-glycyl-glycine ethyl ester **5** the amide protons appear downfield between δ 8.50 and δ 8.17. There are two triplets that integrate for one proton each and a multiplet that integrates for two protons. The signals in the aromatic region confirm the presence of four protons, observed as a doublet, triplet and multiplet between δ 7.85 and δ 7.50. The multiplet integrates for two protons while the triplet and doublet both integrate for one proton. The protons in the *ortho* position of the (η^5-C_5H_4) ring appear at δ 4.71 and the *meta* protons occur at δ 4.33. The singlet at δ 4.1 represents the unsubstituted cyclopentadienyl (η^5-C_5H_5) ring. This peak overlaps with the methylene protons of the ethyl ester. The methylene groups of the peptide chain appear as a triplet at δ 3.91 and two doublets at δ 3.86 and 3.81, respectively. The doublets integrate for two protons each, while the triplet integrates for four protons. The methyl group of the ethyl ester appears as a triplet at δ 1.25.

In the ^{13}C NMR spectra of the *N*-(ferrocenyl)benzoyl peptide esters the amide and ethyl ester carbonyl carbon atoms appear between δ 170.1 and δ 166.4. In the aromatic region the pattern observed depended on whether the derivatives were *ortho*, *meta* or *para* substituted. The *ortho* and *meta* derivatives give rise to six carbon peaks as all six carbon atoms are non-equivalent. The *para* derivatives displays four carbon signals, two of these being quaternary carbon atoms that were easily identified by DEPT-135.

The ferrocenyl carbon atoms are present between δ 84.4 and δ 68.2 indicative of a monosubstituted ferrocene unit. The *ipso* carbon on the substituted cyclopentadienyl (η-C_5H_4) ring appears in the narrow range of δ 84.3 to δ 83.1. The unsubstituted cyclopentadienyl (η^5-C_5H_5) ring occurs as an intense peak at approximately δ 69, while the *ortho* and *meta* carbon atoms of the substituted cyclopentadienyl (η-C_5H_4) ring have chemical shifts between δ 68 and δ 66. The methylene group of the ethyl ester appears at δ 60.4 in all the spectra. This methylene group of the ethyl ester and the methylene groups of the peptide chain are easily recognised by their negative resonance in DEPT-135 spectra. The methylene carbon atoms of the tripeptide chain appear in the range δ 42.7 and δ 40.0 and are in the range of δ 42.2 and δ 39.9 for the tetrapeptide derivatives. The methyl group of the ethyl ester appears at δ 14.0 in all the spectra. A summary of selected chemical shifts (δ) for the ^{13}C NMR spectra of compounds (**2**–**7**) is presented in Table 1.

All *N*-(ferrocenyl)benzoyl peptide derivatives (**2–7**) exhibit a one electron reversible redox process in the cyclic voltammograms (CVs) similar to ferrocene, under the same conditions. The $E^{\circ\prime}$ values range from 39 to 75 mV versus the ferrocene/ferricenium redox couple (Fc/Fc^{+}). A notable trend is observed whereby the orientation around the central benzoyl moiety effects the redox potentials in the order *ortho* < *meta* < *para*. Oxidation of the ferrocenyl unit in the *ortho* derivatives occurs more readily compared to the *meta* and *para* derivatives. It is possible that the *ortho* orientation around the benzoyl moiety imparts electron density to the ferrocene and therefore makes the iron centre more susceptible to oxidation. This electron density is less pronounced in the *meta* and *para* derivatives.

Soft ionization techniques such as electrospray ionization (ESI) mass spectrometry permit the analysis of thermolabile and non-volatile analytes [31]. Electrospray ionization (ESI) was employed in the analysis of compounds (**2–7**) and confirmed the correct relative molecular mass for all the compounds. Examination of the mass spectra revealed the presence of both radical-cations $[M]^{+}$**.** as well as $[M+H]^{+}$ species. Intense adducts due to sodium were also present 22 Da higher than the protonated molecular ion species. Similar observations were made in the analysis of the *N*-(ferrocenyl)benzoyl dipeptide ester derivatives [27–30,32,33]. Sequence



Scheme 1. Synthesis of *N-ortho*, *N-meta* and *N-para* ferrocenyl benzoyl tri- and tetrapeptide esters **2**–**7**. i = $NaNO_2$, HCl, 5 °C, ii = NaOH/MeOH, HCl, iii = EDC, HOBt, TEA, GlyGlyGlyOEt.HCl (**2**–**4**) *n* = 3, GlyGlyGlyGlyOEt.HCl (**5**–**7**) *n* = 4.

Table 1
^{13}C spectroscopic data for compounds (**2**–**7**).

Compound	C=O	Ipso (η-C_5H_4)	(η-C_5H_5)	O-CH_2CH_3	Peptide CH_2
2	170.1–169.1	84.4	69.4	60.4, 14.0	42.3–40.6
3	170.0–166.6	83.9	69.4	60.4, 14.0	42.8–40.6
4	169.7–166.5	83.1	69.5	60.4, 14.0	42.7–40.6
5	170.1–169.1	84.4	69.4	60.4, 14.0	42.3–40.6
6	169.6–166.6	84.0	69.4	60.4, 14.0	42.8–40.6
7	169.6–166.5	83.2	69.5	60.4, 14.0	42.8–41.2

specific fragment ions were not observed or were of low intensity in the mass spectra.

2.3. In vitro anti-proliferation activity

In previous biological testing it was observed that the IC_{50} decreased as the peptide chain increased from one to two amino acid residues [32]. Therefore, it was of interest to prepare longer peptide chains of three and four amino acid residues. The *in vitro* cytotoxicity of the *N*-(ferrocenyl)benzoyl derivatives **2** and **5** against the human lung carcinoma cell line H1299 was evaluated by the acid phosphatase assay. A plot of cell survival versus compound concentration for compounds **2**, **5**, the dipeptide *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycine-glycine ethyl ester and *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycine ethyl ester is presented in Fig. 1. The synthesis of *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycine-glycine ethyl ester and *N*-{*ortho*-(ferrocenyl)-benzoyl}-glycine ethyl ester has previously been reported [32]. Compounds **2** and **5** both have IC_{50} values >50 μM, thus increasing the length of the peptide chain has a negative effect on the anti-proliferative effect of the ferrocenyl derivatives. Compound **2** had an IC_{50} value of 63 μM (RSD 8%), whereas compound **5** did not register an IC_{50} value in the concentration range used against H1299 lung cancer cells. From Fig. 1 it can be concluded that a dipeptide chain is required for optimum activity. The production of Reactive Oxygen Species (ROS) has been implicated in the biological activity of ferrocenyl compounds, these species result from the easily accessible ferrocene/ferricenium redox couple. As compounds **2–7** have very similar redox potentials to *N*-(ferrocenyl)benzoyl dipeptide esters that are highly active *in vitro*, it may be assumed that the peptide chain imparts a secondary mechanism of action to these compounds [32,33]. However, further studies regarding the mode of action must be undertaken, before any definitive conclusions may be drawn.

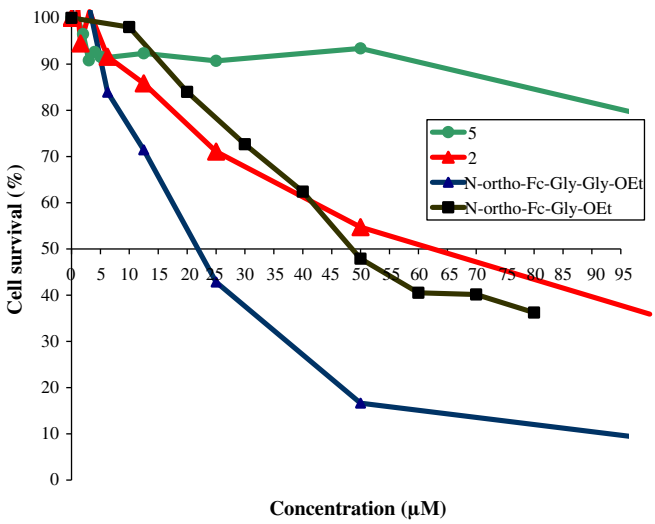


Fig. 1. IC_{50} plot of compounds **2**, **5** and selected *N*-(ferrocenyl)benzoyl derivatives *in vitro* vs. H1299 lung cancer cells.

3. Conclusion

In conclusion *N*-(ferrocenyl)benzoyl tri-and tetrapeptide esters have been prepared using standard peptide chemistry. The products (**2–7)** gave spectroscopic data in accordance with the proposed structures in yields ranging from 29% to 55%. The *ortho* tri- and tetrapeptide derivatives **2** and **5** were tested *in vitro* against H1299 lung cancer cells and showed IC_{50} values greater than those of *N*-(ferrocenyl)benzoyl amino acid and dipeptide esters. Therefore, extending the length of the peptide chain had a negative effect on anti-cancer activity, relative to *N*-(ferrocenyl)benzoyl amino acid and dipeptide 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 8452A diode array UV–Vis spectrophotometer. NMR spectra were obtained on a Bruker AC 400 NMR spectrometer operating at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. The ^1H and ^{13}C NMR chemical shifts (ppm) are relative to TMS and all coupling constants (*J*) are in Hertz. Electrospray ionization mass spectra were obtained on a Brüker Daltonics 3000 Esquire-LC ion trap mass spectrometer. Elemental Analysis was carried out by the Microanalytical Laboratory at University College Dublin. Cyclic voltammograms were recorded in acetonitrile (Sigma–Aldrich), with 0.1 M tetrabutylammonium perchlorate (TBAP) as a supporting electrolyte, using a CH Instruments Electrochemical Analyzer (Pico-Amp Booster and Faraday Cage). The experiments were carried out at room temperature. A three-electrode cell consisting of a glassy carbon working-electrode, a platinum wire counter-electrode and an Ag/Ag^+ reference electrode was used. The scan rate was 0.1 V/s. The concentration range of the ferrocene compounds was 1.0 mMol in acetonitrile. The $E^{o\prime}$ values obtained for the test samples were referenced to the Fc/Fc^+ couple.

4.2. General procedure for the preparation of N-(ferrocenyl)benzoyl tripeptide esters

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

1-Hydroxybenzotriazole (0.189 g, 1.4 mmol) was added to a solution of *ortho*-ferrocenyl benzoic acid (0.245 g, 0.8 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.267 g 1.4 mmol) and triethylamine (2 mls) in dichloromethane (40 mls) at 0 °C. After 30 min glycyl-glycyl-glycine ethyl ester hydrochloride (0.229 g, 0.9 mmol) was added and the reaction was stirred at room temperature for 48 h. The reaction mixture was washed with water, 10% potassium hydrogen carbonate and

5% citric acid. The organic layer was dried over $MgSO_4$ and the solvent was removed *in vacuo*. The product was purified by column chromatography (eluant: ethyl acetate). Recrystallization from ethyl acetate furnished the product as an orange solid. (0.24 g, 59%). m.p. 138–140 °C; $E^{\circ\prime}$ = 39 mV (vs Fc/Fc^+).

UV–Vis λ_{max} MeCN: 335, 450 nm.

I.R. ν_{max} (KBr): 3293, 2927, 2851, 1737, 1691, 1516, 1426, 1277, 1104 cm^{-1}.

^{1}H NMR (400 MHz) δ (DMSO): 8.54 (1H, t, *J* = 6 Hz, –CON*H*–), 8.32 (1H, t, *J* = 5.6 Hz, –CON*H*–), 8.13 (1H, t, *J* = 6 Hz, –CON*H*–), 7.85 (1H, d, *J* = 7.6 Hz ArH), 7.42 (1H, t, *J* = 4.8 Hz ArH), 7.22–7.38 (2H, m, ArH), 4.65 {2H, t, *J* = 1.6 Hz, *ortho* on (η-C_5H_4)}, 4.26 {2H, t, *J* = 1.6 Hz, *meta* on (η-C_5H_4)}, 4.00–4.05 {7H, m, (η-C_5H_5), –OCH_2CH$_3$}, 3.78–3.83 (6H, m, –NHCH_2CO–), 1.17 (3H, t, *J* = 7.2 Hz, –OCH$_2$CH_3).

^{13}C NMR (100 MHz) δ (DMSO): 170.1, 169.6, 169.3, 169.1, 136.2, 136.0, 130.6, 128.7, 127.4, 125.4, 84.4, 69.4, 68.8, 68.2, 60.4 (–ve DEPT), 42.3 (–ve DEPT), 41.7 (–ve DEPT), 40.6 (–ve DEPT), 14.0.

Anal. Calc. for $C_{25}H_{27}FeN_3O_5$: C, 59.42; H, 5.39; N, 8.32. Found: C, 59.72; H, 5.56; N, 8.75%.

Mass spectrum: $[M+Na]^+$ found: 528.20.

$C_{25}H_{27}N_3O_5FeNa$ requires: 528.12.

4.2.2. N-{meta-(ferrocenyl)-benzoyl}-glycyl-glycyl-glycine ethyl ester (**3**)

For compound **3** *meta*-ferrocenyl benzoic acid (0.24 g, 0.8 mmol) was used as a starting material. Recrystallization from ethyl acetate furnished the product as a yellow solid. (0.26 g, 64%). m.p. 166–168 °C; $E^{\circ\prime}$ = 55 mV (vs Fc/Fc^+).

UV–Vis λ_{max} MeCN: 325, 450 nm.

I.R. ν_{max} (KBr): 3229, 3079, 1831, 1725, 1740, 1603, 1335, 1118, 1105 cm^{-1}.

^{1}H NMR (400 MHz) δ (DMSO): 8.89 (1H, t, *J* = 6 Hz, –CON*H*–), 8.29–8.32 (2H, m, –CON*H*–), 8.01 (1H, s, ArH), 7.75 (2H, t, *J* = 1.6 Hz, ArH), 7.43 (1H, t, *J* = 8 Hz, ArH), 4.86 {2H, t, *J* = 2 Hz, *ortho* on (η-C_5H_4)}, 4.40, {2H, t, *J* = 1.6 Hz, *meta* on (η^5-C_5H_4)}, 4.12, (2H, q, *J* = 7.2 Hz, –OCH_2CH$_3$), 4.04 {5H, s, (η^5-C_5H_5)}, 3.95 (2H, d, *J* = 6 Hz, –NHCH_2CO–), 3.86 (2H, d, *J* = 6 Hz, –NHCH_2CO–), 3.77 (2H, d, *J* = 6 Hz, –NHCH_2CO–), 1.20 (3H, t, *J* = 7.2 Hz, –OCH$_2$CH_3).

^{13}C NMR (100 MHz) δ (DMSO): 170.0, 169.7, 169.4, 166.6, 139.2, 133.9, 128.8, 128.4, 124.9, 124.3, 83.9, 69.4, 69.1, 66.4, 60.4 (–ve DEPT), 42.8 (–ve DEPT), 41.7 (–ve DEPT), 40.6 (–ve DEPT), 14.0.

Anal. Calc. for $C_{25}H_{27}FeN_3O_5$: C, 59.42; H, 5.39; N, 8.32. Found: C, 59.08; H, 5.22; N, 8.64%.

Mass spectrum: $[M+Na]^+$ found: 528.20.

$C_{25}H_{27}N_3O_5FeNa$ requires: 528.12.

4.2.3. N-{para-(ferrocenyl)benzoyl}-glycyl-glycyl-glycine ethyl ester (**4**)

For compound **4** *para*-ferrocenyl benzoic acid (0.21 g, 0.7 mmol) was used as a starting material. Recrystallization from ethyl acetate furnished the product as an orange solid. (0.22 g, 62%). m.p. 206–208 °C; $E^{\circ\prime}$ = 73 mV (vs Fc/Fc^+).

UV–Vis λ_{max} MeCN: 355, 450 nm.

I.R. ν_{max} (KBr): 3275, 3090, 2987, 2345, 1751, 1607, 1519, 1378, 1249, 1028, 993 cm^{-1}.

^{1}H NMR (400 MHz) δ (DMSO): 8.79 (1H, t, *J* = 6 Hz, –CON*H*–), 8.30 (2H, t, *J* = 6.4 Hz, –CON*H*–), 7.83 (2H, d, *J* = 8.4 Hz, ArH), 7.65 (2H, d, *J* = 8.4 Hz, ArH), 4.90 {2H, t, *J* = 2 Hz, *ortho* on (η^5-C_5H_4)}, 4.41 {2H, t, *J* = 1.6 Hz, *meta* on (η^5-C_5H_4)}, 4.10 (2H, q, *J* = 7.2 Hz, –OCH_2CH$_3$), 4.02 {5H, s, (η^5-C_5H_5)}, 3.92 (2H, d, *J* = 5.6 Hz, –NHCH_2CO–), 3.86 (2H, d, *J* = 6 Hz, –NHCH_2CO–), 3.76 (2H, d, *J* = 6 Hz, –NHCH_2CO–), 1.19 (3H, t, *J* = 6.8 Hz –OCH$_2$CH_3).

^{13}C NMR (100 MHz) δ (DMSO): 169.7, 169.5, 169.4, 166.5, 142.8, 130.9, 127.5, 125.3, 83.1, 69.5, 66.6, 66.4, 60.4 (–ve DEPT), 42.7 (–ve DEPT), 41.7 (–ve DEPT), 40.6 (–ve DEPT), 14.0.

Anal. Calc. for $C_{25}H_{27}FeN_3O_5$: C, 59.42; H, 5.39; N, 8.32. Found: C, 59.32; H, 5.47; N, 7.91%.

Mass spectrum: $[M+Na]^+$ found: 528.20.

$C_{25}H_{27}N_3O_5FeNa$ requires: 528.12.

4.3. General procedure for the preparation of N-(ferrocenyl)benzoyl tetrapeptide esters

4.3.1. N-{ortho-(ferrocenyl)-benzoyl}-glycyl-glycyl-glycyl-glycine ethyl ester (**5**)

1-Hydroxybenzotriazole (0.09 g, 0.65 mmol) was added to a solution of *ortho*-ferrocenyl benzoic acid (0.18 g, 0.6 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.267 g 1.4 mmol) and triethylamine (2 mls) in dichloromethane (40 mls) at 0 °C. After 30 min glycyl-glycyl-glycyl-glycine ethyl ester hydrochloride (0.18 g, 0.6 mmol) was added and the reaction was stirred at room temperature for 48 h. The reaction mixture was washed with water, 10% potassium hydrogen carbonate and 5% citric acid. The organic layer was dried over $MgSO_4$ and the solvent was removed *in vacuo*. The product was purified by column chromatography (eluant 9:1 ethyl acetate:methanol). Recrystallization from ethyl acetate furnished the product as an orange solid. (0.10 g, 30%). m.p. 168–170 °C; $E^{\circ\prime}$ = 44 mV (vs Fc/Fc^+).

UV–Vis λ_{max} MeCN: 332, 445 nm.

I.R. ν_{max} (KBr): 3293, 3083, 2346, 1522, 1430, 1407, 1211, 1105, 1011 cm^{-1}.

^{1}H NMR (400 MHz) δ (DMSO): 8.50 (1H, t, *J* = 6 Hz, –CON*H*–), 8.29–8.35 (2H, m, –N*H*CO–), 8.17 (1H, t, *J* = 5.6 Hz, –N*H*CO–), 7.87 (1H, d, *J* = 7.6 Hz, ArH), 7.47 (1H, t, *J* = 4.8 Hz, ArH), 7.31–7.35 (2H, m, ArH), 4.71 {2H, *J* = 2 Hz, *ortho* on (η^5-C_5H_4)}, 4.33 {2H, t, *J* = 2 Hz, *meta* on (η^5-C_5H_4)}, 4.11–4.17 {7H, m, (η^5-C_5H_5), –OCH_2CH$_3$}, 3.91 (4H, t, *J* = 4.8 Hz, –NHCH_2CO–), 3.86 (2H, d, *J* = 5.6 Hz, –NHCH_2CO–), 3.81 (2H, d, *J* = 6 Hz, –NHCH_2CO–), 1.25 (3H, t, *J* = 7.2 Hz, –OCH$_2$CH_3).

^{13}C NMR (100 MHz) δ (DMSO): 170.1, 169.6, 169.3, 169.2, 169.1, 136.2, 136.0, 130.0, 128.7, 127.4, 125.4, 84.4, 69.4, 68.7, 68.2, 60.4 (–ve DEPT), 42.3 (–ve DEPT), 42.1 (–ve DEPT), 41.7 (–ve DEPT), 40.6 (–ve DEPT), 14.0.

Anal. Calc. for $C_{27}H_{30}FeN_4O_6$ requires: C, 57.66; H, 5.38; N, 9.96. Found: C, 57.18; H, 5.53; N, 9.75%.

Mass spectrum: $[M+Na]^+$ found: 585.20.

$C_{27}H_{30}N_4O_6FeNa$ requires: 585.14.

4.3.2. N-{meta-(ferrocenyl)-benzoyl}-glycyl-glycyl-glycyl-glycine ethyl ester (**6**)

For compound **6** *meta*-ferrocenyl benzoic acid (0.18 g, 0.6 mmol) was used as a starting material. Recrystallization from ethyl acetate furnished the product as a yellow solid. (0.09 g, 27%). m.p. 171–173 °C; $E^{\circ\prime}$ = 58 mV (vs Fc/Fc^+).

UV–Vis λ_{max} MeCN: 330, 450 nm.

I.R. ν_{max} (KBr): 3280, 3084, 2366, 1735, 1559, 1458, 1376, 1283, 1204, 1148 cm^{-1}.

^{1}H NMR (400 MHz) δ (DMSO): 8.95 (1H, t, *J* = 4.4 Hz, –CON*H*–), 8.34 (2H, q, *J* = 3.2 Hz, –CON*H*–), 8.29 (2H, t, *J* = 6 Hz, –CON*H*–), 8.01 (1H, s, ArH), 7.79–7.87 (2H, m, ArH), 7.47 (1H, t, *J* = 7.6 Hz, ArH), 4.93 {2H, t, *J* = 1.6 Hz, *ortho* on (η^5-C_5H_4)}, 4.46 {2H, t, *J* = 1.6 Hz, *meta* on (η^5-C_5H_4)}, 4.15 (2H, q, *J* = 7.2 Hz, –OCH_2CH$_3$), 4.10 {5H, s, (η^5-C_5H_5)}, 4.02 (2H, d, *J* = 5.6 Hz, –NHCH_2CO–), 3.82–3.89 (6H, m, –NHCH_2CO–), 1.25 (3H, t, *J* = 7.2 Hz, –OCH$_2$CH_3).

^{13}C NMR (100 MHz) δ (DMSO): 169.6, 169.5, 169.3, 169.1, 166.6, 139.2, 133.9, 128.8, 128.4, 124.9, 124.3, 84.0, 69.4, 69.1, 66.4, 60.4 (–ve DEPT), 42.8 (–ve DEPT), 42.1 (–ve DEPT), 41.7 (–ve DEPT), 40.6 (–ve DEPT), 14.0.

Anal. Calc. for $C_{27}H_{30}FeN_4O_6$: C, 57.66; H, 5.38; N, 9.96. Found: C, 57.57; H, 5.74; N, 10.09%.

Mass spectrum: $[M+Na]^+$ found: 585.20.

$C_{27}H_{30}N_4O_6FeNa$ requires: 585.14.

*4.3.3. N-{para-(ferrocenyl)-benzoyl}-glycyl-glycyl-glycyl-glycine ethyl ester (**7**)*

For compound **7** *para*-ferrocenyl benzoic acid (0.18 g, 0.6 mmol) was used as a starting material. Recrystallization from ethyl acetate furnished the product as an orange solid. (0.10 g, 30%). m.p. 160–162 °C; $E^{\circ\prime}$ = 75 mV (vs Fc/Fc^+).

UV–Vis λ_{max} MeCN: 349, 450 nm.

I.R. ν_{max} (KBr): 3270, 2937, 2739, 2345, 1719, 1542, 1474, 1283, 1120, 1035 cm^{-1}.

^1H NMR (400 MHz) δ (DMSO): 8.83 (1H, t, J = 5.6 Hz, –CON*H*–), 8.21–8.29 (3H, m, –CON*H*–), 7.83 (2H, d, J = 8.4 Hz ArH), 7.64 (2H, d, J = 8.4 Hz ArH), 4.90 {2H, t, J = 1.6 Hz, *ortho* on (η^5-C_5H_4)}, 4.41 {2H, t, J = 1.6 Hz, *meta* on (η^5-C_5H_4)}, 4.01 (2H, q, J = 6.8 Hz, –OC$H_2$$CH_3$), 3.93 (2H, d, J = 5.6 Hz –NHCH_2CO–), 3.82 (2H, d, J = 6 Hz, –NHCH_2CO–), 3.76 (4H, t, J = 6.4 Hz, –NHCH_2CO–) 1.18 (3H, t, J = 7.2 Hz, –OCH_2CH_3).

^{13}C NMR (100 MHz) δ (DMSO): 169.60, 169.57, 169.3, 169.2, 166.5, 142.8, 131.1, 127.6, 125.3, 83.2, 69.53, 69.47, 66.6, 60.4 (−ve DEPT), 42.8 (−ve DEPT), 42.1 (−ve DEPT), 41.7 (−ve DEPT), 41.2 (−ve DEPT), 14.0.

Anal. Calc. for $C_{27}H_{30}FeN_4O_6$: C, 57.66; H, 5.38; N, 9.96. Found: C, 57.96; H, 5.77; N, 10.18%.

Mass spectrum: $[M+Na]^+$ found: 585.20.

$C_{27}H_{30}N_4O_6FeNa$ requires: 585.14.

4.4. General in vitro anti-proliferation assay procedure

Compounds **2** and **5** were dissolved in 100 μl of DMSO and drug dilutions at two times their final concentrations were prepared in cell culture media. 100 μl of these drug dilutions were added to plates containing cells that had been incubated for 24 h in a 37 °C, 5% CO_2 incubator. The plates were then incubated in the same conditions for 6–7 days or until cell confluency reached 80–90%. The assessment of cell survival in the presence of the drug was then measured using an acid phosphatase assay. Media was removed from the plates and each well on the plate was washed with 100 μl PBS. This was removed and 100 μl of freshly prepared phosphatase substrate in 0.1 M sodium acetate was added to each well. The plates were then incubated in the dark for 2 h at 37 °C. Colour development was monitored during this time then the enzymatic reaction was stopped by adding 50 μl of 1 N NaOH. The fluorescence of the plate was measured at 405 nm with a reference wavelength of 620 nm. The IC_{50} value was determined by plotting cell survival percentage (relative to control cells) against drug concentration.

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