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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

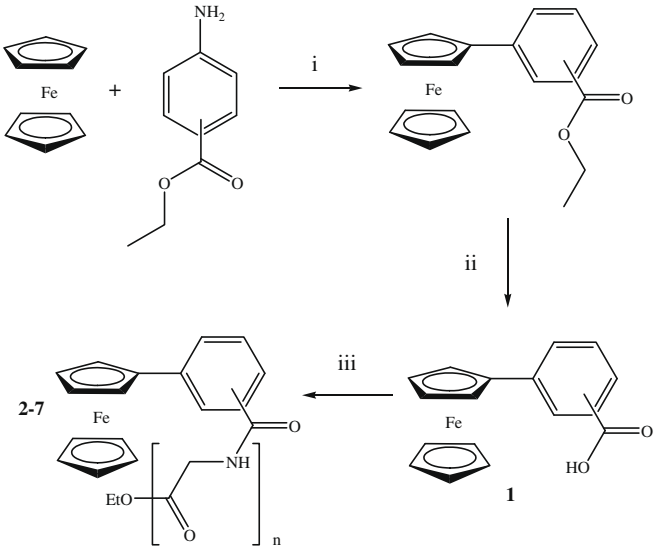
The *N*-(ferrocenyl)benzoyl peptide derivatives (**2-7)** were characterized by a combination of ^{1}H NMR, ^{13}C NMR and DEPT-135 spectroscopy, mass spectrometry and cyclic voltammetry. All the proton and carbon chemical shifts for compounds **2–7** were unambiguously assigned by a combination of DEPT-135 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.