Chapter 4: Synthesis, antimalarial activity and cytotoxicity of novel aminoferrocenyl-chalcone amides - Article 2

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Synthesis, antimalarial and cytotoxic activity of novel aminoferrocenyl-chalcone amides

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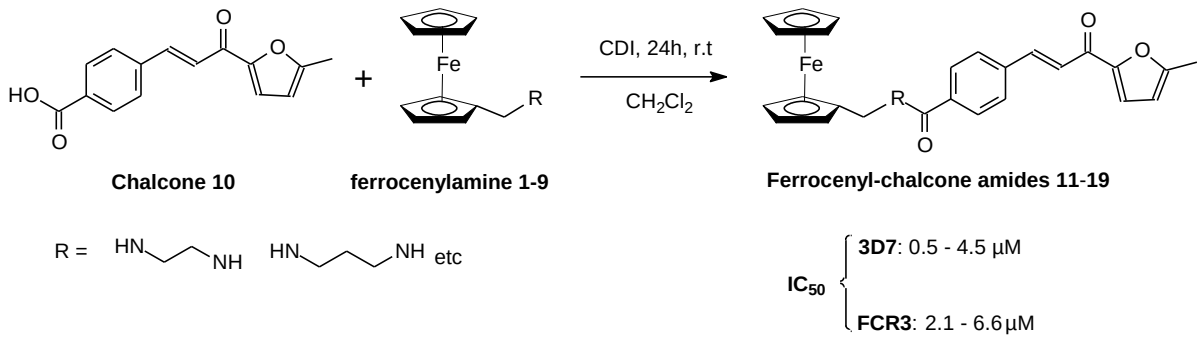
A series of aminoferrocenyl-chalcone amides were synthesized and their *in vitro* antimalarial activity against 3D7 and FCR3 strains of *Plasmodium falciparum*, as well as their cytotoxicity against WI-38 cell line determined.



Abstract

A series of aminoferrocenyl-chalcone amides **11** - **19** were synthesized through condensation of carboxylic acid-functionalized chalcone with aminoferrocenyl, using 1,1'-carbonyldiimidazole as coupling agent. These compounds were screened against the chloroquine sensitive (3D7), and antifolate- and chloroquine resistant (FCR3) strains of *Plasmodium falciparum*. Their cytotoxicity was determined against the WI-38 cell line of normal human fetal lung fibroblast. All compounds were found active, with IC_{50} values ranging between 0.5 - 4.5 µM and 2.1 - 6.6 µM against 3D7 and FCR3, respectively. Despite favourable drug-like properties, the observed activity was relatively low. They demonstrated low selectivity towards *plasmodia*, compared to mammalian cells. However, amide **11**, featuring the 1,2-diaminoethane linker, was the most active of all, with IC_{50} values of 2.6 µM and 2.1 µM against the 3D7 and FCR3 strains, respectively and a resistance index of 0.8. The redox potentials of all target compounds were found to be between 0.55 - 0.60 V.

Keywords: Chalcone; aminoferrocenyl; Plasmodium falciparum; malaria

1. Introduction

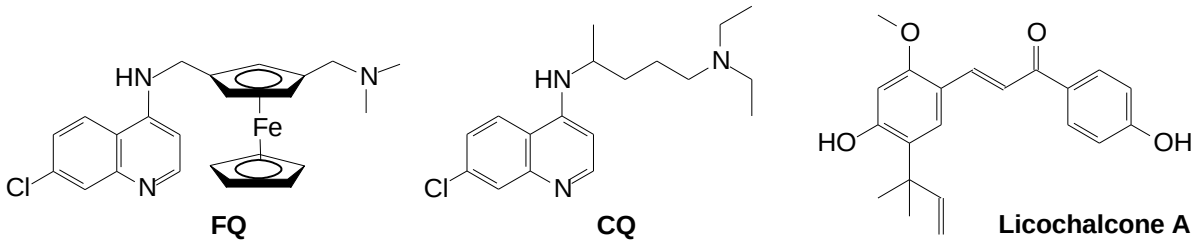
Malaria is commonly prevalent in tropical and subtropical regions, such as Africa and Asia,[1] and is endemic in 106 countries, worldwide. The disease is caused by a parasite from the genus *Plasmodium*. Of the five species that can infect humans, *P. falciparum* is by far the deadliest, with over three billion people at risk globally and around 660 000 deaths reported in 2011.[2,3] Of these reported deaths, 91% occurred in the African region, while 86% were children under the age of five.[3,4] In light of the widespread resistance of these malaria parasites against the classic known antimalarial drugs, such as chloroquine and now the established tolerance towards the commonly used artemisinins,[4] an immense need exists for identifying and developing new and effective antiplasmodial drugs.

Ever since reports of ferroquine (**FQ**), a ferrocene derivative of chloroquine (**CQ**), being active against resistant strains of *P. falciparum*, interest in organometallic compounds has attracted much attention.[5,6] Ferrocene has the remarkable ability to be recognized by many biological systems, including amino acids, proteins, DNA and carbohydrates.[7,8] The ferrocene moiety has also been proven to be a successful addition to biological compounds, such as penicillin, cephalosporine, tamoxifen, or known malaria therapeutics, by increasing the efficacy towards CQ resistant strains of the parasite, as is the case with FQ for example.[6] Additionally, reports of ferrocene related organometallic compounds having antitumour,[8] antimicrobial[5] and antimalarial[9-11] activities, make these compounds perfect candidates for possible use in the combat against malaria.

Conflicting reports on the role of ferrocene in biological systems exist. Some suggest ferrocene to be that of a mere spacer group,[12] while others attribute a more prominent role, i.e. that of contributing to the overall oxidative stress, or cytotoxicity.[10] Numerous studies have been conducted on ferrocene-containing chalcones. In one such study, one of the aryl groups of chalcone was replaced with ferrocene in order to evaluate the biological activities of the resulting derivatives, as well as the function of ferrocene.[11,13] These studies resulted in limited success. Of the more than 60 ferrocenyl-chalcone derivatives, Attar *et al*.[11] found the ferrocenyl-chalcones to be less active than their organic analogues. An investigation of another series of ferrocenyl-chalcones by Wu *et al*.[13] resulted in the 1-ferrocenyl-3-(4-nitrophenyl)prop-2-en-1-one being the most active compound, with an IC_{50} of 4.5 µM against the multi-drug resistant K1 strain of *P. falciparum*.

In light of the fact that in 4-aminoquinolines, analogous to CQ, the length of the methylene spacer between two nitrogens in the side chain is a major determinant of activity against CQ

resistant strains of *P. Falciparum*,[14] this study aimed at investigating the combination of ferrocenyl-chalcone derivatives, separated by methylene spacers of various lengths. To the best of our knowledge, there are currently limited, if any, published data on ferrocene-chalcone derivatives, containing methylene spacers between two nitrogens in the side chain, such as in CQ. Additionally, the Fe(II) center may undergo redox reactions, thus influencing the redox cycling of the malaria parasite. The ease of oxidation of Fe(II) in the ferrocenyl moiety may be influenced by chemical groups in its vicinity, which in turn may impact on the electrochemical potential of a ferrocene-containing compound and eventually its antimalarial activity.



FQ **CQ** **Licochalcone A**

Figure 1: Chemical structures of ferroquine (**FQ**), chloroquine (**CQ**) and Licochalcone A.

The first reported chalcone with antimalarial activity was Licochalcone A (**Fig. 1**), a natural product isolated from Chinese liquorice roots, with an IC_{50} of 6.5 µM against 3D7 clones.[15,16] It is generally recognized that chalcones exert their activity through inhibition of malarial cysteine proteases,[4] specifically falcipain-2,[17], which can be explicitly targeted with minimal toxicity to the host, although other targets or modes of action have also been suggested. Cysteine protease mediates protein hydrolysis through nucleophillic attack on a carbonyl of a susceptible peptide bond. The main function of malarial cysteine protease is the hydrolysis of haemoglobin in the food vacuole.[18] Cysteine protease is also presumed to be involved in the rupture of the erythrocyte membrane.[19] Recently, it has been reported that chalcones also possess radical-scavenging properties, resulting in oxidative stress.[7,20] Besides antimalarial activity, an array of biological functions, such as antibacterial, antifungal, antiviral, anti-inflammatory and antitumour activities have been attributed to the chalcones.[21-25] For this reason, the chalcone moiety could be beneficial when coupled with other pharmacophores, including ferrocene.

As part of our program focusing on the discovery of novel antimalarial compounds, and in light of the above considerations, this study investigated amide compounds formed by conjugating chalcone moiety (with a 5-methylfuran as ring B) to ferrocene pharmacophore,

through methylene spacers of various lengths. In this paper, the synthesis, *in vitro* antimalarial activity and cytotoxicity of these novel aminoferrocenyl-chalcone amides are reported.

2. Materials and Methods

2.1 Materials

4,7-dichloroquinoline, 4-formylbenzoic acid, 2-cetyl-5-methylfuran, hydrochloric acid, sodium hydroxide, 1,1'-Carbonyldiimidazole (CDI), ethylenediamine, propylenediamine, butylenediamine, piperazine, 1,2-diaminopropane, 2-aminoethylpiperazine, N-methyl-1,3-propylenediamine, 2,2'-(ethylenedioxy)bis(ethylamine), *N*-(3-aminopropyl)-*N*-methylpropane-1,3-diamine, magnesium sulphate, tertabutylammonium hexafluorophosphate and sodium bicarbonate were purchased from Sigma-Aldrich (South-Africa). All solvents used were purchased from Associated Chemical Enterprises (ACE, South Africa). All chemicals and reagents were of analytical grade and were used without further purification, except for dichloromethane (DCM) that was distilled with calcium hydride and kept on molecular sieves (4Å) prior to use in reactions.

2.2 General procedures

The ^{1}H and ^{13}C NMR spectra were recorded on a BrukerAvance™ III 600 spectrometer at a frequency of 600 and 150 MHz, respectively, in deuterated dimethyl sulfoxide (DMSO-d_6), or deuterated chloroform ($CDCl_3$). Chemical shifts are reported in parts per million δ (ppm), with the residual protons of the solvent as reference. The splitting pattern abbreviations are as follows: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), pentet (p) and multiplet (m). High resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer that had an APCI or an ESI source set at 300°C, or 180°C respectively, using Bruker Compass DataAnalysis 4.0 software. A full scan, ranging between 50 - 1500 m/z, was generated at a capillary voltage of 4500 V, an end plate offset voltage of -500 V and a collision cell RF voltage of 100 Vpp.

Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument. Melting points (mp) were determined on a BÜCHI melting point B-545 instrument and are uncorrected.

Thin layer chromatography (TLC) was performed using silica gel plates ($60F_{254}$). Column chromatography was performed using MN silica gel 60; 70 - 230 mesh ASTM, supplied by Macerey-Nagel (Germany).

A conventional three electrode system, consisting of a platinum disc as a working electrode, platinum wire as an auxiliary electrode and silver chloride ($Ag/AgCl_{(sat)}$) as reference electrode, was used for electrochemical measurements. The solution was purged with nitrogen for 5 minutes prior to each experiment. 0.05 M tetrabutylammonium hexafluorophosphate was used as supporting electrolyte in all experiments. The cyclic voltammograms were recorded at a scan rate of 0.10 Vs^{-1} and scanning was done over a range of -0.2 - 1.2 V. A millimolar (1 mM) solution in DMSO was prepared of all test compounds.

2.3 Biological testing

2.3.1 *In vitro* antimalarial assay

The *in vitro* antimalarial activity of test samples against the 3D7 and FCR3 strains of the malaria parasite, *Plasmodium falciparum,* was measured by assessing parasite survival after drug exposure, using the parasite lactate dehydrogenase (pLDH) colorimetric enzyme assay.[26] Lactate dehydrogenase is an enzyme found in all the cells and catalyses the formation of pyruvate from lactate by reducing the co-enzyme, nicotinamide adenine dinucleotide (NAD^+) to nicotinamide adenine dinucleotide hydrogenase (NADH).

In the pLDH assay, the NAD^+ analogue, 3-acetylpyridine adenine nucleotide (APAD), is reduced to 3-acetylpyridine adenine nucleotide hydrogenase (APADH) and in turn a yellow, nitro-blue tetrazolium/phenazine ethosulphate (NBT/PES) reagent is converted into purple, formazan crystals. The absorbance was recorded at 620 nm, using a multiwell spectrophotometer (Tecan Infinite F500). Formazan formation is directly proportional to pLDH activity, which in turn is indicative of the number of parasites in the cultures, following drug exposure. Assay specificity is ensured by the inability of human LDH, found in the host red blood cells, to use APAD as a co-factor. Compound inhibitory activity was determined by preparing test samples in parasite culture medium in transparent 96-well, flat bottom plates (Greiner Bio-one), with a 100 µM starting concentration in three-fold serial dilutions, to obtain eleven (11) decreasing concentrations (n = 2 for each data point). Parasitized red blood cells were added to a final concentration of 1% haematocrit, 2% parasitaemia and the plates incubated for 48 hours, before proceeding with the pLDH assay. Percentage parasite survival in each well was calculated relative to control wells that were not exposed to the drug.

The results are presented in **Table 2** as the 50% inhibitory concentrations (IC_{50}) of individual compounds, calculated from fitted sigmoidal dose-response curves.

2.3.2 *In vitro* cytotoxicity assay

The cytotoxic effects of the prepared compounds were tested, using sulforhodamine B (SRB) assay on the WI-38 cell line. The SRB assay was developed by Skehan Skehan *et al.*[27] to measure drug induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye, sulforhodamine B (Acid Red 52), to bind electrostatically in a pH dependent manner to basic protein amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilized for measurement.[28] The SRB assay was performed at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for the screening tests of this study.

The WI-38 cell line (normal human fetal lung fibroblast) from ECACC was routinely maintained as a monolayer cell culture at 37 °C, 5% CO_2, 95% air and 100% relative humidity in EMEM containing 10% fetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. For this screening experiment, the cells (21 - 50 passages) were inoculated in 96-well microtiter plates at plating densities of 10 000 cells/well and were incubated for 24 hours. After 24 hours, the cells were treated with the experimental drugs, which had previously been dissolved in DMSO and diluted in medium to produce five concentrations. Neat cells served as control. The blank contained complete medium without cells. Parthenolide was used as a standard. The plates were incubated for 48 hours after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein bound dye was extracted with 10 mM Tris base for optical density determination at a wavelength of 540 nm, using a multiwell spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC_{50}) was determined by non-linear regression. The results are also summarized in **Table 3**.

2.4 Synthesis

2.4.1 Condensation of diamines with ferrocene carboxaldehyde

Aminoferrocenyls ***1*** *–* ***8***: A mixture of ferrocene carboxaldehyde (9.3 mmol, 2 g, 1 eq.) and the appropriate primary diamine (46.5 mmol, 5 eq.) was stirred in anhydrous MeOH (50 ml) for 4 h at room temperature. An excess of sodium borohydride, $NaBH_4$ (93 mmol, 3.5 g, 10 eq.) was added and stirring was continued for an additional 2 h at room temperature (Scheme 1). Afterwards, the reaction mixture was acidified with HCl (1M, 50 ml), diluted with water (50 ml) and extracted with ethyl acetate EtOAc (2 x 50 ml) to remove any unreacted ferrocene carboxaldehyde. The aqueous layer was basified to pH 10 with a solution of

saturated sodium bicarbonate ($NaHCO_3$) and extracted with EtOAc (3 x 50 ml). The combined organic layers were dried over $MgSO_4$ and the solvent was removed *in vacuo*. The residue was subjected to column chromatography on silica gel, eluting successively with DCM:MeOH (9:1, v/v) and then with DCM:MeOH:NH_4OH (4:1:0.1, v/v/v) to afford the pure compounds **1** - **8** in high yields.

*Aminoferrocenyl **9***: Ferrocene carboxaldehyde (9.3 mmol, 2 g, 1 eq.), piperazine (46.5 mmol, 3.9 g, 5 eq.) and sodium triacetoxyborohydride, $NaBH(OAc)_3$ (23 mmol, 5 g, 2.5 eq.) were added to anhydrous THF (50 ml) and stirred at room temperature for 24 h (Scheme 1), after which the solvent was removed *in vacuo*. The residue was dissolved in EtOAc (50 ml), basified to pH 10 with NaOH (1M) and extracted with EtOAc (3 x 50 ml). The combined organic layers were dried over $MgSO_4$ and the solvent was removed *in vacuo*. The residue was purified with DCM:MeOH (9:1,v/v) and then with DCM:MeOH:NH_4OH (4:1:0.1, v/v/v) to afford the pure compound **9** in moderate yield.

2.4.1.1. N-ferrocenyl-1,2-diaminoethane, 1

The reaction of ethylenediamine and ferrocene carboxaldehyde afforeded a yellow powder; yield: 2.3 g (91%); mp: 128.9 - 134.5 °C; IR (ATR) ν_{max}/cm^{-1}: 3203, 3089, 2926, 2878, 1613, 1459, 1229, 1152, 1035, 979, 919, 806, 610; ^1H NMR (600 MHz, $CDCl_3$) δ ppm: 4.16 (t, *J* = 1.9 Hz, 2H, H-3a), 4.10 (s, 5H, H-4a), 4.07 (t, *J* = 1.8 Hz, 2H, H-2a), 3.49 (s, 2H, H-1b), 2.77 (t, *J* = 5.8 Hz, 2H, H-2b), 2.66 (t, *J* = 5.8 Hz, 2H, H-3b); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 86.91 (C-1a), 68.34 (C-3a, C-4a), 67.71 (C-2a), 52.10 (C-3b), 48.88 (C-1b), 41.67 (C-2b); HRMS (APCI) *m/z* $[M]^+$ 258.0819 (Calcd for $C_{13}H_{18}FeN_2$: 258.0820).

2.4.1.2. N-ferrocenyl-1,3-diaminopropane, 2

The reaction of propylenediamine and ferrocene carboxaldehyde afforded a yellow powder; yield: 2.5 g (88%); mp: 94.2 - 101.5 °C; IR (ATR) ν_{max}/cm^{-1}: 3262, 3098, 2862, 1620, 1472, 1332, 1271, 1224, 1104, 1035, 1020, 950, 813; ^1H NMR (600 MHz, $CDCl_3$) δ ppm: 4.14 (s, 2H, H-3a), 4.08 (s, 5H, H-4a), 4.06 (s, 2H, H-2a), 3.48 (s, 2H, H-1b), 2.73 (t, *J* = 6.8 Hz, 2H, H-2b), 2.66 (t, *J* = 7.1 Hz, 2H, H-4b), 1.60 (p, *J* = 7.0 Hz, 2H, H-3b); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 86.78 (C-1a), 68.33 (C-3a and C-4a), 67.68 (C-2a), 49.03 (C-3b), 47.37 (C-1b), 40.49 (C-2b), 33.63 (C-4b); HRMS (APCI) *m/z* $[M]^+$ 272.0964 (Calcd for $C_{14}H_{20}FeN_2$: 272.0975).

2.4.1.3. N-ferrocenyl-1,4-diaminobutane, 3

The reaction of butylenediamine and ferrocene carboxaldehyde afforded a yellow powder; yield: 2.3 g (86%); IR (ATR) ν_{max}/cm^{-1}: 3087, 2924, 2855, 1628, 1544, 1451, 1319, 1103, 999, 809, 614; ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 4.16 (t, *J* = 1.8 Hz, 2H, H-3a), 4.08 (m, 7H, H-2, H-4a, H-2a), 3.51 (s, 2H, H-1b), 2.68 (t, *J* = 6.2 Hz, 2H, H-2b), 2.60 (t, *J* = 6.7 Hz, 2H, H-5b), 1.50 (m, 4H, H-3b and H-4b); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 85.21 (C-1a), 68.69 (C-3a), 68.39 (C-4a), 67.92 (C-2a), 48.57 (C-1b and C-5b), 41.21 (C-2b), 30.00 (C-4b), 26.86 (C-3b); HRMS (APCI) *m/z* $[M+H]^+$ 287.1200 (Calcd for $C_{15}H_{23}FeN_2$: 287.1210).

2.4.1.4. N-ferrocenyl-2-aminoethylpiperazine, 4

The reaction of 2-aminoethylpiperazine and ferrocene carboxaldehyde afforded a yellow powder; yield: 2.4 (78%); mp: 87.8 - 90.1 °C; IR (ATR) ν_{max}/cm^{-1}: 3286, 2942, 2817, 1454, 1422, 1324, 1272, 1142, 1102, 1063, 1035, 984, 888, 815, 768, 600; ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 4.16 (t, *J* = 1.9 Hz, 2H, H-3a), 4.11 (s, 5H, H-4a), 4.07 (t, *J* = 1.9 Hz, 2H, H-2a), 3.47 (s, 2H, H-1b), 2.84 (t, *J* = 4.9 Hz, 2H, H-4b), 2.70 (t, *J* = 6.0 Hz, 2H, H-2b), 2.45 (t, *J* = 6.0 Hz, 2H, H-3b), 2.42 – 2.33 (m, 2H, H-5b), 1.92 (s, 1H, N-H); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 87.06 (C-1a), 68.35 (C-3a), 68.18 (C-4a), 67.63 (C-2a), 58.20 (C-3b), 54.52 (C-5b), 48.86 (C-1b), 46.04 (C-4b), 45.70 (C-2b); HRMS (APCI) *m/z* $[M+H]^+$ 328.1476 (Calcd for $C_{17}H_{26}FeN_3$: 328.1476).

2.4.1.5. N-ferrocenyl-1,2-diaminopropane, 5

The reaction of 1,2-diaminopropane and ferrocene carboxaldehyde afforded a yellow powder of two isomers; yield: 2.30 g (90%); mp: 104.2 - 109.1 °C; IR (ATR) ν_{max}/cm^{-1}: 3089, 2923, 1556, 1526, 1444, 1279, 1226, 1103, 999, 813; Major compound: ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 4.21 – 4.02 (m, 9H, H-2a, H-3a and H-4a), 3.48 (m, 2H, H-1a), 2.98 – 2.91 (m, 1H, H-3a), 2.59 (dd, *J* = 11.5, 3.4 Hz, 1H , H-2b-α), 2.35 (dd, *J* = 11.8, 7.7 Hz, 1H, H-2b-β), 1.04 (s, 3H, H-4b); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 86.83 (C-1a), 68.33 (C-2a and C-4a), 67.67 (C-3a), 57.62 (C-2b), 48.97 (C-1b), 46.45 (C-3b), 21.87 (C-4b); HRMS (APCI) *m/z* $[M]^+$ 272.0967 (Calcd for $C_{14}H_{20}FeN_2$: 272.0975).

2.4.1.6. N-ferrocenyl-N'-methyl-1,3-diaminopropane, 6

The reaction of N-methyl-1,3-propylenediamine and ferrocene carboxaldehyde afforded a yellow powder; yield: 2.1 g (81%); mp: 104.9 - 106.5 °C; IR (ATR) ν_{max}/cm^{-1}: 3243, 2929, 2797, 1638, 1532, 1434, 1413, 1388, 1322, 1262, 1220, 1104, 1036, 1017, 996, 807, 642; ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 4.15 (t, *J* = 1.8 Hz, 2H, H-3a), 4.09 (s, 5H, H-4a), 4.07 (t, *J* = 1.8 Hz, 2H, H-2a), 3.47 (s, 2H, H-1b), 2.66 (t, *J* = 7.0 Hz, 2H, H-2b), 2.60 (t, *J* = 6.8 Hz,

2H, H-4b), 2.39 (s, 3H, H-5b), 1.84 (s, 2H, NH_2), 1.65 (p, *J* = 6.9 Hz, 2H, H-3b); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 86.82 (C-1a), 68.33 (C-2a and C-4a), 67.68 (C-3a), 50.54 (C-4b), 48.98 (C-1b), 47.95 (C-2b), 36.48 (C-5b), 29.93 (C-3b); HRMS (APCI) *m/z* $[M]^+$ 286.1138 (Calcd for $C_{15}H_{22}FeN_2$: 286.1132).

2.4.1.7. N-ferrocenyl-2,2'-(ethylenedioxy)bis(ethylamine), 7

The reaction of 2,2'-(ethylenedioxy)bis(ethylamine) and ferrocene carboxaldehyde afforded a yellow oil; yield 2.4 g (77%); IR (ATR) ν_{max}/cm^{-1}: 3089, 2861, 1582, 1450, 1348, 1301, 1103, 999, 811; ^1H NMR (600 MHz, $CDCl_3$) δ ppm: 4.16 (t, *J* = 1.8 Hz, 2H, H-3a), 4.10 (s, 5H, H-4a), 4.06 (t, *J* = 1.8 Hz, 2H, H-2a), 3.58 (m, 6H, H-3b, H-4b and H-5b), 3.48 (s, 2H, H-1b), 3.45 (t, *J* = 5.1 Hz, 2H, H-6b), 2.80 (m, 4H, H-2b and H-7b), 1.90 (s, 2H, NH_2); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 86.77 (C-1a), 73.28 (C-6b), 70.33 (C-4b), 70.27 (C-5b), 70.18 (C-3b), 68.32 (C-3a and C-4a), 67.67 (C-2a), 48.88 (C-2b), 48.77 (C-1b), 41.64 (C-7b); HRMS (APCI) *m/z* $[M+H]^+$ 347.1414 (Calcd for $C_{17}H_{27}FeN_2O_2$: 347.1421).

2.4.1.8. N-ferrocenyl-N-(3-aminopropyl)-N-methylpropane-1,3-diamine, 8

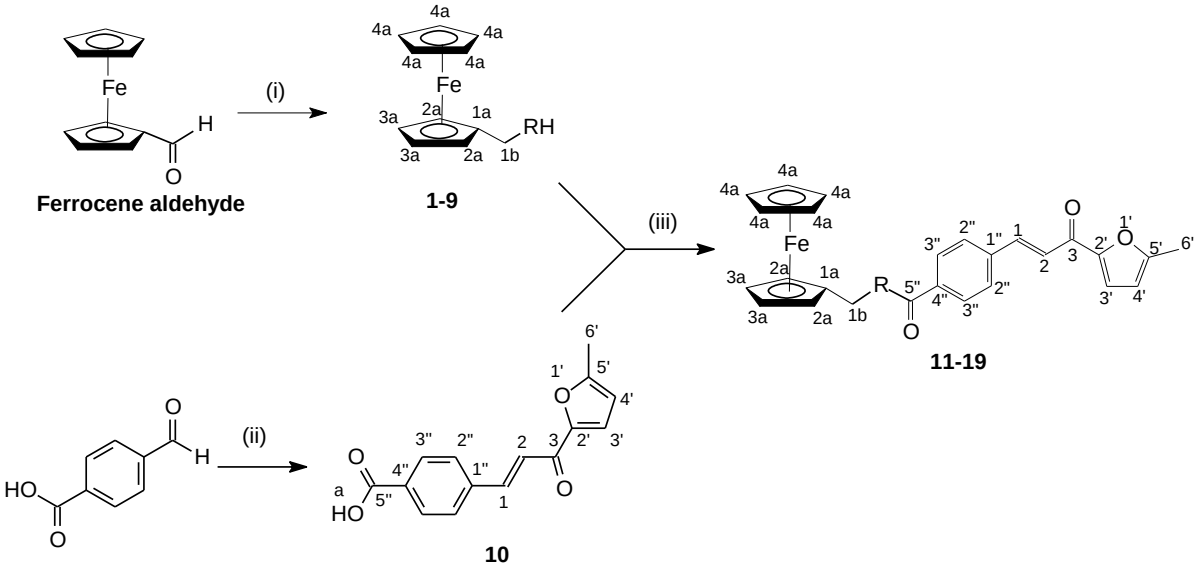
The reaction of N-(3-aminopropyl)-N-methylpropane-1,3-diamine and ferrocene carboxaldehyde afforded a yellow oil; yield: 2.3 g (71%) IR (ATR) ν_{max}/cm^{-1}: 3285, 3090, 2936, 2793, 1567, 1459, 1312, 1104, 999, 815; ^1H NMR (600 MHz, $CDCl_3$) δ ppm: 4.16 (s, 2H, H-3a), 4.09 (s, 5H, H-4a), 4.07 (s, 2H, H-2a), 3.49 (s, 2H, H-1b), 2.66 (dt, *J* = 35.4, 8.0 Hz, 4H, H-2b and H-6b), 2.34 (dt, *J* = 7.2, 2.6 Hz, 4H, H-4b and H-5b), 2.16 (s, 3H, H-8b), 1.60 (dp, *J* = 36.4, 6.8 Hz, 4H, H-3b and H-6b); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 85.54 (C-1a), 68.62 (C-3a), 68.41 (C-4a), 67.91 (C-2a), 56.12 (C-5b), 55.60 (C-4b), 48.77 (C-1b), 47.77 (C-7b), 42.15 (C-8b), 40.49 (C-2b), 29.97 (C-3b), 26.86 (C-6b); HRMS (APCI) *m/z* $[M+H]^+$ 344.1789 (Calcd for $C_{18}H_{30}FeN_3$: 344.1789).

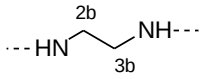
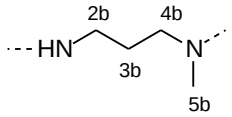
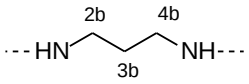
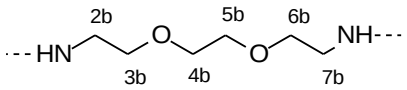
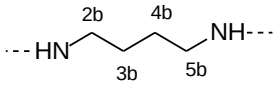
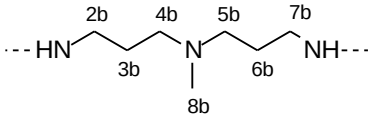
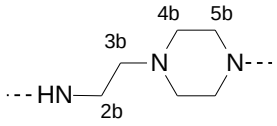
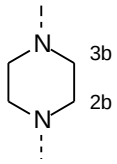
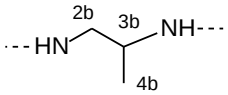
2.4.1.9. N-ferrocenyl-piperazine, 9

The reaction of piperazine and ferrocene carboxaldehyde afforded a yellow oil; yield: 0.71 g (61%); mp: 120.1 – 122.6 °C; IR (ATR) ν_{max}/cm^{-1}: 3260, 3070, 2928, 2802, 1451, 1328, 1280, 1136, 1104, 1059, 1037, 1023, 1001, 908, 811, 594; ^1H NMR (600 MHz, $CDCl_3$) δ ppm: 4.14 (t, *J* = 1.9 Hz, 2H, H-3a), 4.08 (s, 7H, H-2a and H-4a), 3.34 (s, 2H, H-1b), 2.86 (t, *J* = 5.0 Hz, 4H, H-2b), 2.39 (s, 4H, H-3b); ^{13}C NMR (151 MHz, $CDCl_3$) δ ppm: 82.12 (C-1a), 70.31 (C-3a), 68.48 (C-4a), 68.08 (C-2a), 58.77 (C-1b), 53.17 (C-3b), 45.48 (C-2b); HRMS (APCI) *m/z* $[M]^+$ 284.0965 (Calcd for $C_{15}H_{20}FeN_2$: 284.0975).

2.4.2 4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzoic acid

Formylbenzoic acid (10 mmol, 1.5 g, 1 eq.) and 2-acetyl-5-methylfuran (10.3 mmol, 1.3 g, 1.2 ml, 1.03 eq.) were added to MeOH (60 ml) while stirring. A solution of NaOH (1M, 20 ml) was slowly added, which resulted in a dark red colour change (Scheme 1). After 12 h of stirring, the pH was adjusted to 2 with an HCl (1M) solution upon which an off-white yellow precipitate formed. The precipitate was collected by suction filtration and washed with water, then with a 10% MeOH solution. The precipitate was dried and recrystallized from MeOH to yield the desired compound, chalcone **10**.[22] off-white yellow powder; yield: 2.30 g (89%); mp: 221 - 228 ºC; IR (ATR) ν_{max}/cm^{-1}: 3104, 3076, 2982, 2924, 2931, 1684, 1654, 1601, 1509, 1288, 1211, 1065, 1027, 845, 772, 754; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 13.14 (s, 1H, H-COOH), 7.97 (d, *J* = 8.4 Hz, 2H,H-2”), 7.94 (d, *J* = 8.4 Hz, 2H, H-3”), 7.80 (d, *J* = 3.5 Hz, 1H, H-3’), 7.75 (d, *J* = 15.7 Hz, 1H, H-1), 7.72 (d, *J* = 15.7 Hz, 1H, H-2), 6.44 (d, *J* = 3.3 Hz, 1H, H-4’), 2.40 (s, 3H, H-6’); ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 175.46 (C-3), 166.86 (C-5”), 158.97 (C-2’), 151.83 (C-5’), 140.75 (C-1), 138.65 (C-4”0, 131.98 (C-1”), 129.73 (C-3”), 128.74 (C-2”), 124.16 (C-2), 122.02 (C-3’), 109.64 (C-4’), 13.78 (C-6’); HRMS (APCI) *m/z* $[M+H]^+$ 257.0890 (Calcd for $C_{15}H_{13}O_4$: 257.0814).



Amides	R	Amides	R
11		**16**	
12		**17**	
13		**18**	
14		**19**	
15			

Scheme 1: Multi-step synthesis of aminoferrocenyl-chalcone amides **11** – **19**.

Reagents and conditions: (i) **1** - **8**: diamine, MeOH, 4 h, r.t. then $NaBH_4$ MeOH, 2 h, r.t.; **9**: piprazine, $NaBH(OAc)_3$, THF, 24 h, r.t.; (ii) **10**: 2-acetyl-5-methylfuran, MeOH, NaOH, 12 h, r.t.; (iii) **11** - **19**: chalcone **10**, CDI, DCM, 3h, r.t. then **1** - **9**, 24 h, r.t.

2.4.3 Condensation of chalcone with aminoferrocenyl

Chalcone **10** (3.9 mmol, 1 g, 1 eq.) and CDI (4.9 mmol, 0.8 g, 1.3 eq.) were stirred in DCM (50 ml) for 3 h at 25 ºC. Afterwards, the appropriate amino-functionalized ferrocene **1** - **9** (5.85 mmol, 1.5 eq.) was added and was the solution stirred for an additional 24 h. The reaction was quenched with the addition of water (50 ml). The organic phase was separated and the aqueous phase was extracted with DCM (3 x 50 ml). The combined organic layers were washed with water (50 ml), saturated $NaHCO_3$ solution (50 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The purification by column chromatography, eluting with DCM:MeOH (9:1, v/v) afforded the free amine, which was converted into the corresponding oxalate salt as follows:

Oxalic acid was dissolved in diethyl ether (1 g/100 ml). The free amine was dissolved while stirring in the oxalic solution at room temperature, until precipitation. The precipitate was then filtered to deliver the salt as a yellow powder. The procedure was used for the synthesis of all **11** - **19** compounds.

2.4.3.1 N-[2-(ferrocenylamino)ethyl]-4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzamido oxalate, 11

Yield: 0.6 g (36%); R_f = 0.42 (DCM:MeOH, 9:1, v/v); mp: 155.0 – 159.6 °C; IR (ATR) ν_{max}/cm^{-1}: 2945, 2772, 1643, 1597, 1542, 1507, 1438, 1409, 1370, 1300, 1239, 1206, 1067, 1025, 981, 958, 838, 802, 766; ^{1}H NMR (600 MHz, DMSO-d6) δ ppm: 9.02 (d, *J* = 83.5 Hz, 2H, N-H$^+$), 8.07 – 7.88 (m, 4H, H-2" and H-3"), 7.83 (d, *J* = 3.5 Hz, 1H, H-3'), 7.79 – 7.68 (m, 2H, H-1 and H-2), 6.46 (d, *J* = 3.2 Hz, 1H, H-4'), 4.44 (s, 2H, H-b), 4.24 (s, 2H, H-c), 4.21 (s, 5H, H-d), 4.00 (s, 2H, H-a'), 3.59 (s, 2H, H-c'), 3.05 (s, 2H, H-b'), 2.41 (s, 3H, H-6'); ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 175.54 (C-3), 166.08 (C-5"), 163.38 (oxalic acid), 158.94 (C-2'), 151.86 (C-5'), 140.97 (C-1), 137.33 (C-1"), 135.12 (C-4"), 128.59 (C-2"), 127.95 (C-3"), 123.62 (C-2), 122.01 (C-3'), 109.64 (C-4'), 76.81 (C-a), 70.57 (C-b), 68.87 (C-c), 68.77 (C-d), 46.28 (C-a'), 45.51 (C-c'), 35.87 (C-b'), 13.81 (C-6'); HRMS (ESI) *m/z* [M+H$^+$] 497.1520 (Calcd for $C_{28}H_{29}FeN_2O_3$: 497.1527).

2.4.3.2 N-[3-(ferrocenylamino)propyl]-4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzamido oxalate, 12

Yield: 0.25 g (15%); R_f = 0.54 (DCM:MeOH, 9:1, v/v); mp: 120.0 – 128.3 °C; IR (ATR) ν_{max}/cm^{-1}: 3392, 2944, 2834, 1721, 1648, 1598, 1543, 1509, 1303, 1186, 1070, 1024, 838, 765, 682; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 8.84 (s, 2H, N-H$^+$), 8.04 – 7.85 (m, 4H, H-2" and H-3"), 7.81 (s, 1H, H-3'), 7.73 (m, 2H, H-1 and H-2), 6.45 (s, 1H, H-4'), 4.59 – 3.85 (m,

9H, H-2a, H-3a and H-4a), 3.45 – 3.21 (m, 2H, H-4b), 2.88 (s, 2H, H-2b), 2.40 (s, 3H, H-6'), 1.88 (m, 2H, H-3b); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 175.54 (C-3), 165.85 (C-5"), 163.15 (oxalic acid), 158.91 (C-2'), 151.84 (C-5'), 140.99 (C-1), 137.18 (C-1"), 135.43 (C-4"), 128.60 (C-2"), 127.77 (C-3"), 123.52 (C-2), 121.97 (C-3'), 109.62 (C-4'), 76.88 (C-1a), 70.47 (C-2a), 68.82 (C-3a), 68.75 (C-4a), 46.24 (C-1b), 43.90 (C-4b), 36.45 (C-2b), 25.83 (C-3b), 13.79 (C-6'); HRMS (ESI) *m/z* $[M+H]^+$ 511.1665 (Calcd for $C_{29}H_{31}FeN_2O_3$: 511.1684).

2.4.3.3 N-[4-(ferrocenylamino)butyl]-4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzamido oxalate, 13

Yield: 0.67 g (28%); R_f = 0.39 (DCM:MeOH, 9:1, v/v); mp: 133.4 – 136.2 °C; IR (ATR) ν_{max}/cm^{-1}: 3392, 2945, 2834, 1721, 1648, 1598, 1543, 1304, 1187, 1071, 1024, 838, 766, 682; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 8.65 (s, 1H, NH^+), 8.07 – 7.84 (m, 4H, H-2" and H-3"), 7.80 (s, 1H, H-3'), 7.72 (s, 2H, H-1 and H-2), 6.45 (s, 1H, H-4'), 5.87 – 4.80 (m, 2H, NH), 4.38 (s, 2H, H-2a), 4.20 (m, 7H, H-3a and H-4a), 3.92 (s, 2H, H-1b), 3.27 (s, 2H, H-5b), 2.85 (s, 2H, H-2b), 2.40 (s, 3H, H-6'), 1.55 (s, 4H, H-4b and H-3b); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 175.57 (C-3), 165.59 (C-5"), 162.12 (oxalic acid), 158.93 (C-2'), 151.86 (C-5'), 141.05 (C-1), 137.07 (C-1"), 135.76 (C-4"), 128.61 (C-2"), 127.74 (C-3"), 123.46 (C-2), 121.96 (C-3'), 109.64 (C-4"), 76.92 (C-1a), 70.47 (C-2a), 68.87 (C-3a), 68.77 (C-4a), 46.32 (C-1b) 45.82 (C-5b), 38.61 (C-2b), 26.30 (C-4b), 23.04 (C-3b), 13.80 (C-6'); HRMS (ESI) *m/z* $[M+H]^+$ 525.1823 (Calcd for $C_{30}H_{33}FeN_2O_3$: 525.1841).

2.4.3.4 (2E)-3-[4-({4-[2-(ferrocenylamino)ethyl]piperazin-1-yl}carbonyl)phenyl]-1-(5-methylfuran-2-yl)prop-2-en-1-one oxalate, 14

Yield: 0.45 g (41%); R_f = 0.7 (DCM:MeOH, 9:1, v/v); mp: 1883 – 192.5 °C; IR (ATR) ν_{max}/cm^{-1}: 3017, 1725, 1634, 1603, 1509, 1428, 1284, 1206, 1105, 1065, 983, 834, 778, 706; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 7.90 (d, *J* = 6.9 Hz, 2H, H-2"), 7.79 (s, 1H, H-3'), 7.71 (s, 2H, H-1 and H-2), 7.45 (s, 2H, H-3"), 6.45 (s, 1H, H-4'), 4.54 – 2.56 (m, 19H, H-1a – H-4a, H-1b – H-5b), 2.40 (s, 3H, H-6'); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 175.60 (C-3), 168.37 (C-5"), 162.50 (oxalic acid), 158.87 (C-2'), 151.85 (C-5'), 141.16 (C-1), 137.35 (C-1"), 135.64 (C-4"), 128.73 (C-2"), 127.54 (C-3"), 123.13 (C-4'), 121.86 (C-3'), 109.62 (C-4'), 76.96 (C-1a), 70.50 (C-2a), 68.89 (C-3a), 68.77 (C-4a), 52.77 (C-5b); HRMS (ESI) *m/z* $[M+H]^+$ 566.2078 (Calcd for $C_{32}H_{36}FeN_3O_3$: 566.2106).

2.4.3.5 N-[1-(ferrocenylamino)propan-2-yl]-4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzamido oxalate, 15

Yield: 0.38 g (39%); R_f 0.33 = (DCM:MeOH, 9:1, v/v); mp: 151.8 – 155.3 °C; IR (ATR) ν_{max}/cm^{-1}: 2981, 2802, 1721, 1596, 1506, 1405, 1329, 1205, 1064, 831, 766, 707, 628; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 8.05 – 7.63 (m, 7H, H-2", H-3", H-3', H-1 and H-2), 6.45 (s, 1H, H-4'), 4.57 – 4.10 (m, 19H, NH$^+$, H-1a – H-4a, H-1b and H-3b), 3.69 – 2.82 (m, 2H, H-2b), 2.40 (s, 3H, H-6'), 1.22 (d, *J* = 41.9 Hz, 3H, H-4b); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 175.52 (C-3), 165.65 (C-5"), 164.57 (oxalic acid), 158.91 (C-2'), 151.83 (C-5'), 141.00 (C-1), 137.20 (C-1'), 135.42 (C-4"), 128.49 (C-2"), 128.07 (C-3"), 123.51 (C-2), 121.98 (C-3'), 109.62 (C-4'), 76.72 (C-1a), 70.62 (C-2a), 68.85 (C-3a), 68.74 (C-4a), 46.63 (C-2b), 42.43 (C-3b), 18.45 (C-4b), 13.78 (C-6'); HRMS (ESI) *m/z* [M+H]$^+$ 511.1662 (Calcd for $C_{29}H_{31}FeN_2O_3$: 511.1684).

2.4.3.6 N-[3-(ferrocenylamino)propyl]-N-methyl-4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzamido oxalate, 16

Yield: 0.42 g (42%); R_f: 0.67; 124.7 – 131.5 °C; IR (ATR) ν_{max}/cm^{-1}: 2951, 2802, 1721, 1596, 1506, 1405, 1329, 1205, 1064, 831, 766, 707, 628; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 7.91 (d, *J* = 7.6 Hz, 2H, H-2"), 7.80 (d, *J* = 3.6 Hz, 1H, H-3'), 7.72 (s, 2H, H-1 and H-2), 7.53 (s, 1H, NH), 7.46 (d, *J* = 7.5 Hz, 2H, H-3"), 6.45 (d, *J* = 3.2 Hz, 1H, H-4'), 4.41 (d, *J* = 9.2 Hz, 2H, H-2a), 4.28 – 4.14 (m, 8H, H-3a and H-4a), 3.96 (s, 2H, H-1b), 3.86 (s, 2H, H-4b), 3.49 (s, 2H, H-2b), 2.87 (s, 3H, H-5b), 2.41 (s, 3H, H-6'), 1.92 (s, 2H, H-3b); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 175.59 (C-3), 170.01 (C-5"), 163.46 (oxalic acid), 158.84 (C-2'), 151.84 (C-5'), 141.20 (C-1), 128.59 (C-2"), 127.44 (C-3"), 123.06 (C-2), 121.83 (C-3'), 109.60 (C-4'), 70.38 (C-2a), 68.83 (C-3a), 68.74 (C-4a), 46.13 (C-1a), 45.94 (C-4b), 43.89 (C-2b), 36.94 (C-5b), 23.18 (C-3b), 13.78 (C-6'); HRMS (ESI) *m/z* [M+H]$^+$ 525.1812 (Calcd for $C_{30}H_{33}FeN_2O_3$: 525.1841).

2.4.3.7 N-(2-{2-[2-(ferrocenylamino)ethoxy]ethoxy}ethyl)-4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzamido oxalate, 17

Yield: 0.7 g (61%); R_f = 0.61 (DCM:MeOH, 9:1, v/v); mp: 91.0 – 92.0 °C; IR (ATR) ν_{max}/cm^{-1}: 3325, 3032, 1717, 1633, 1601, 1509, 1299, 1207, 1067, 840, 768, 710; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm: 8.68 (s, 1H, NH$^+$), 7.92 (s, 4H, H-2" and H-3"), 7.81 (d, *J* = 3.7 Hz, 1H, H-3'), 7.78 – 7.67 (m, 2H, H-1 and H-2), 6.45 (d, *J* = 3.2 Hz, 1H, H-4'), 4.39 (s, 2H, H-2a), 4.20 (m, 7H, 3a and 4a), 3.93 (s, 2H, H-1b), 3.51 (m, 8H, H-3b – H-6b), 3.26 – 3.10 (m, 2H, H-2b), 2.95 (s, 2H, H-7b), 2.40 (s, 3H, H-6'); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 175.54 (C-3), 165.66 (C-5"), 162.25 (oxalic acid), 158.91 (C-2'), 151.85 (C-5'), 141.01 (C-1), 137.10 (C-

1”), 135.57 (C-4”), 128.59 (C-2”), 127.74 (C-3”), 123.49 (C-2), 121.94 (C-3), 76.70 (C-1a), 70.58 (C-2a), 69.72 (C-4b), 69.46 (C-5b), 69.04 (C-6b), 68.85 (C-3a), 68.72 (C-4a), 67.61 (C-6b), 65.68 (C-3b), 46.34 (C-1b), 45.58 (C-7b), 45.10 (C-2b), 13.79 (C-6’); HRMS (ESI) *m/z* $[M+H]^+$ 585.2019 (Calcd for $C_{32}H_{37}FeN_2O_5$: 585.2051).

2.4.3.8 N-(3-{[3-(ferrocenylamino)propyl](methyl)amino}propyl)-4-[(1E)-3-(5-methylfuran-2-yl)-3-oxoprop-1-en-1-yl]benzamido oxalate, 18

Yield: 0.32 g (29%); R_f = 0.65 (DCM:MeOH, 9:1, v/v); mp: 150.2 – 155.2 °C; IR (ATR) ν_{max}/cm^{-1}: 3392, 2944, 2834, 1721, 1648, 1598, 1543, 1509, 1303, 1186, 1070, 1024, 838, 765, 682; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 8.73 (s, 1H, NH^+), 8.16 – 7.53 (m, 7H, H-2”, H-3”, H-3’, H-1 and H-2), 6.45 (s, 1H, H-4’), 4.19 (m, 11H, H-2a, H-3a, H-4a and H-1b), 3.30 (s, 2H, H-2b), 2.79 (m, 4H, H-4b and H-5b), 2.40 (s, 6H, H-6’ and H-8b), 1.79 (s, 4H, H-3b and H-6b); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm 175.54 (C-3), 165.65 (C-5”), 164.97 (oxalic acid), 158.91 (C-2’), 151.84 (C-5’), 141.03 (C-1), 137.06 (C-1”), 135.68 (C-4”), 128.61 (C-2”), 127.76 (C-3”), 123.45 (C-2), 121.94 (C-3’), 109.62 (C-4’), 77.19 (C-1a), 70.34 (C-2a), 68.80 (C-3a), 68.72 (C-4a), 54.04 (C-4b), 53.44 (C-5b), 46.28 (C-1b), 44.12 (C-7b), 40.04 (C-8b), 37.17 (C-2b), 25.30 (C-3b), 21.76 (C-6b), 13.78 (C-6’); HRMS (ESI) *m/z* $[M+H]^+$ 582.2382 (Calcd for $C_{33}H_{40}FeN_3O_3$: 582.2419).

2.4.3.9 (2E)-3-{4-[(4-ferrocenylpiperazin-1-yl)carbonyl]phenyl}-1-(5-methylfuran-2-yl)prop-2-en-1-one oxalate, 19

Yield: 0.5 g (36%); R_f = 0.41(DCM:MeOH, 9:1, v/v); mp: 153.6 – 158.5 °C; IR (ATR) ν_{max}/cm^{-1}: 3011, 1722, 1634, 1602, 1508, 1427, 1370, 1332, 1285, 1206, 1105, 1068, 945, 834, 765, 698; ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 7.91 (d, *J* = 7.7 Hz, 2H, H-2”), 7.78 (d, *J* = 3.6 Hz, 1H, H-3’), 7.71 (s, 2H, H-1 and H-2), 7.47 (d, *J* = 7.6 Hz, 2H, H-3”), 6.48 – 6.41 (m, 1H, H-4’), 4.34 (s, 2H, H-2a), 4.24 (s, 2H, H-3a), 4.19 (s, 5H, H-4a), 3.98 (s, 2H, H-1b), 2.97 (m, 4H, H-3b and H-2b), 2.40 (s, 3H, H-6’); ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm 175.57 (C-3), 168.54 (C-5”), 163.34 (oxalic acid), 158.89 (C-2’), 151.84 (C-5’), 141.07 (C-1), 136.50 (C-1”), 135.96 (C-4”), 128.75 (C-2”), 127.67 (C-3”), 123.26 (C-2), 121.88 (C-3’), 109.62 (C-4’), 76.00 (C-1a), 70.95 (C-2a), 69.06 (C-3a), 68.74 (C-4a), 55.76 (C-1b), 50.19 (C-3b and C-2b), 13.78 (C-6’); HRMS (ESI) *m/z* $[M+H]^+$ 523.1656 (Calcd for $C_{30}H_{31}FeN_2O_3$: 523.1684).

3. Results

3.1 Chemistry

The amino-ferrocenyl intermediates **1** - **9** were easily prepared in high yields, through reductive amination of ferrocene carboxaldehyde with $NaBH_4$ for the primary amines, and with $NaBH(OAc)_3$ for the secondary amine (piperazine). The synthesis of the intermediate **10** was attained through Claisen-Schmidt condensation of 2-acetyl-5-methylfuran and 4-formylbenzoic acid in basic methanolic water. The presence of the carboxylic acid of the chalcone was observed by a highly deshielded proton at 13.07 ppm and a carbon at 166 ppm. The hybrid compounds **11** - **19** were synthesized in low to moderate yields through amide formation between the carboxylic acid of the chalcone and the free amine of the ferrocene intermediates, using CDI as coupling agent. For stability reasons, the free base target compounds were converted into oxalate salts.

3.2 Reduction potential and physicochemical properties

The compounds in DMSO showed only one oxidation peak, whilst the corresponding reduction peak was observed when the sweep was reversed. All synthesized target compounds showed higher redox potentials than ferrocene and ferrocene carboxaldehyde (**FcA**), while a comparison of the nine compounds showed similar potential (**Table 1**).

Table 1: Electrochemical potential of synthesized compounds

Compound	E_{pa} (V)[a]	E_{pc} (V)[b]	$E_{1/2}$ (V)[c]
Fc	0.55	0.40	0.47
FcA	0.67	0.37	0.52
11	0.68	0.54	0.60
12	0.66	0.55	0.60
13	0.64	0.54	0.59
14	0.65	0.55	0.60
15	0.66	0.55	0.60
16	0.66	0.55	0.60
17	0.65	0.55	0.60
18	0.65	0.55	0.60
19	0.61	0.49	0.55

[a] anodic potential; [b] cathodic potential; [c] $E_{1/2} = (E_{cat} + E_{an})/2$; Fc = ferrocene; FcA = ferrocene carboxaldehyde.

Preliminary ADMET calculations were performed, using Discovery Studio 3.1 in order to determine the drug-likeness of the synthesized compounds. **Table 2** illustrates that the synthesized ferrocenyl-chalcone amides had similar to lower levels of calculated water solubilities, compared to CQ at neutral pH value. They also showed exceptionally high absorption levels, similar to CQ, which was consistent with their lower log *P* values, falling in the ideal range. However, none of the compounds showed better calculated solubility and intestinal absorption levels than the intermediate chalcone, **10**. Contrary, **FcA** had low solubility and moderate absorption levels.

Table 2: Calculated ADMET properties of synthesized compounds

Compound	Log P[a]	ADMET Solubility level[b]	ADMET Absorption level[c]
10	2.9	3	0
11	2.1	2	0
12	2.2	2	0
13	2.8	2	0
14	2.3	2	0
15	2.5	1	0
16	2.4	2	0
17	1.8	2	0
18	2.4	2	0
19	2.7	1	0
FcA	nd	2	1
CQ	3.1	2	0

[a] Calculated with Accelrys Discovery Studio 3.1; [b] ADMET aqueous solubility level (Log S_w) at 25 °C and pH 7, values = 1, 2 and 3 indicate very low, low and good solubility, respectively as determined using Accelrys Discovery Studio 3.1; [c] ADMET human intestinal absorption, values = 0, 1 and 2 indicate good, moderate and poor absorption, respectively as determined using Accelrys Discovery Studio 3.1, FA = ferrocene carboxaldehyde, nd = not defined.

3.3 *In vitro* antimalarial activity and cytotoxicity

The aminoferrocenyl-chalcone amides **11** - **19** and chalcone **10** were screened *in vitro*, alongside ferrocene carboxaldehyde (**FcA**) and an equimolar combination **M** (**FcA**-**10**), against the 3D7 and FCR3 strains of *P. falciparum*. Compounds **11** - **19** were found active with IC_{50} values ranging between 0.5 - 4.5 µM against 3D7 and between 2.1 - 6.6 µM against the FCR3 strain (**Table 3**). Compound **18** demonstrated the highest activity against the 3D7 strain, while compound **11** showed the highest activity against the FCR3 strain, with IC_{50}

values of 0.5 µM and 2.1 µM, respectively. Compound **11** was found slightly more active against the FCR3 strain than against the 3D7 strain, resulting in a resistance index (RI) of 0.8. The combination **M** was inactive against the 3D7 strain, but showed activity against FCR3, having an IC_{50} of 12 µg/ml. However, none of the amides showed activities comparable to that of CQ, regardless of the strain being considered. They were found cytotoxic to mammalian cells in the presence of parasitic cells, which resulted in poor selectivity towards parasitic cells, generating selectivity index (SI) values of 1 – 16. The amides also possessed lower cytotoxicity than the reference, emetine. The combination **M**, chalcone **10** and **FcA** were all found non-toxic towards the mammalian cells.

Table 3: *In vitro a*ntimalarial activity and cytotoxicity of screened compounds

	Activity IC_{50} (µM)[a]		**Resistance Index**	**Cytotoxicity IC_{50} (µM)[b]**	**Selectivity Index**
Compd	**3D7**	**FCR3**	**RI[d]**	**WI-38 HFLF[b]**	**SI**
10	38.1	32.4	0.8	104.7	2.7
11	2.6	2.1	0.8	3.6	1.4
12	2.4	6.6	2.7	3.0	1.2
13	3.0	5.2	1.7	3.3	1.1
14	3.0	4.1	1.4	3.8	1.3
15	3.2	4.2	1.3	3.7	1.2
16	3.2	5.2	1.6	4.6	1.4
17	4.5	4.6	1.0	41.9	9.4
18	0.5	2.8	5.3	3.4	6.5
19	3.5	4.9	1.4	55.4	16.0
FcA	4.6	14.5	3.2	>100	nd
M*	>100*	12.2*	nd	>100	nd
CQ	0.02	0.05	2.5	nd	nd
Em	nd	nd	nd	0.05	nd

* Expressed in µg/ml; [a] Resistance Index (RI) = IC_{50}W2/IC_{50}3D7; [b] WI-38 cell line of normal human fetal lung fibroblast; [c] Selectivity Index (SI) = IC_{50}WI-38-HFLF/IC_{50}3D7, nd = not determined, **M** = equimolar combination of Fc and **10**, FcA = ferrocene carboxaldehyde, Em = emetine.

4. Discussion

4.1 Chemistry

The IR spectra of all intermediates **1** - **9** showed a distinctively broad N-H stretching band between 3200 - 3000 cm^{-1}, as well as the disappearing of the conjugated carbonyl peak of the ferrocene carboxaldehyde at around 1650 cm^{-1}. The presence of the ferrocene moiety in these intermediates was clearly identified by nine protons between 4.2 - 4 ppm on the ^{1}H NMR spectra. The peaks found between 68 – 67 ppm were assigned to the signals of C-2a – C-4a and the quaternary carbon (C-1a) was found at 86 ppm on the ^{13}C NMR spectra. Compounds **1**, **2**, **5** – **8** showed only three distinguishable carbon signals, while **3**, **4** and **9** displayed an additional fourth distinguishable carbon signal. The various aliphatic parts of the intermediates were identified with C peaks on the ^{13}C/DEPT135 between ca. 25 - 75 ppm and the ^{1}H peaks found in the 3.8 - 1.7 ppm regions on the ^{1}H NMR spectra.

The synthesis of chalcones *via* Claisen-Schmidt condensation led to the exclusive formation of the E-stereoisomer, as ascertained by the large *J* value of 15.7 Hz, typically associated with vinylic protons in an *anti* periplanar orientation. The formation of such a compound is a result of the more favourable *anti* periplanar elimination of the hydroxide group from the carbanion, intermediate in this reaction.[13] The vinylic carbon C-1 had a larger chemical shift than C-2 and was therefore found further downfield. This could have been explained by the existence of a formal positive charge on C-1, as a result of the resonance of the unsaturated aryl system in the vicinity.

The carbon C-3 of **10** was shielded and was assigned the peak at 175 ppm, which is commonly associated with a conjugated carbonyl carbon. The IR spectra showed a strong stretching band at 1654 cm^{-1}, which is generally attributed to an unsaturated ketone. Additional evidence, such as six conjugated tertiary carbons (as determined with DEPT90) and six doublets (^{1}H NMR) further confirmed the structure of chalcone **10**. The integration of the two doublets at 7.9 ppm accounted for four protons, which were assigned to H-2” and H-3”, indicating that the *ortho*- and *meta* potions were non-equivalent. Due to the electronic effect of both the ketone and heterocyclic oxygen in the furan ring, quaternary carbon C-2’ was deshielded and was assigned to the peak at 158 ppm. The presence of the methyl group was indisputably assigned to the only aliphatic signal at 13 ppm.

The target amides possessed a free amine group in their structures, which caused instability, due to its hygroscopic/reactive nature. All target amides were therefore converted into oxalate salt to protect/protonate this group, thus rendering them more stable. The IR spectra

of **11** - **19** displayed a weak absorption frequency between 3100 - 2800 cm^{-1}, due to N-H stretching of the protonated amine. The ^{1}H NMR spectra showed eight protons between 6.5 - 8.5 ppm and eleven protons between 4 - 5 ppm, which were consistent with the presence of chalcone moiety (8H), the ferrocenyl moiety (9H) and the methylene 1b (2H), respectively. The ^{13}C NMR also supported the presence of both moieties with twelve peaks between 109 - 180 ppm for the chalcone part, as well as one CH_3 at 13 ppm (C-6'), while the carbons of the ferrocene moiety showed peaks between 65 - 80 ppm. A highly deshielded proton peak, perceived in the 8.5 - 9.2 ppm region, was assigned the acidic proton of the oxalate salts.

4.2 Reduction potential and physicochemical properties

Ferrocene undergoes a reversible one-electron oxidation at a low potential of ~0.55 V to produce the ferricenium (Fe^{3+}) cation, which has an unpaired electron in one of the non-bonding e_{2g} orbitals, which therefore results in a free radical of high stability.[29] Contrary, ferrocene carboxaldehyde has an oxidation potential of ~0.67 V, indicating that this compound does resist oxidation, which was consistent with the expected result from this study. Indeed, because of the electron withdrawing effect of the aldehyde carbonyl, the substituted cyclopentadiene ring (C_p) in FcA is already more electron deficient than it is in ferrocene, which causes its reluctance to donate an additional electron. On the contrary, if electron donating groups, such as methyl or amino groups were attached to the C_p-ring, the oxidation potential would decrease, due to the additional electron density being imparted by such groups, which would ultimately result in favourable oxidation of ferrocene.

A methylene amino group was attached to one C_p-ring of ferrocenyl moiety of each amide. This should have resulted in a decrease in the oxidation potential, as compared to ferrocene and ferrocene carboxyaldehyde. However, the experimentally determined $E_{1/2}$ values, all higher than those of both Fc and FcA, contradicted expectations (**Table 1**). A plausible explanation could be that when amino groups are protonated (as was the case during this study), they become electron withdrawing, rather than electron donating groups. Subsequently, resistance of the ferrocenyl moiety to oxidation occurred during this study.

In **Table 1**, all of the target compounds showed similar redox potentials, despite having structural differences, due to different lengths of spacers used. Thus, neither the spacer types, nor spacer lengths influenced the redox potentials of these compounds. Furthermore, the presence of different chemical moieties in the vicinity of Fe(II) influenced the ease of oxidation of Fe(II) containing compounds,[14] However, during this study, the chemical entity, containing the ferrocenyl moiety and the moiety itself would in fact convey analogous properties (**Fig. 2**). This could have been ascribed to the fact that the first four atoms were

similar in amides **11** - **18**, thus having the same impact on the ferrocenyl moiety. In the case of **19**, the tertiary amine influenced the redox potential of the Fe(II), as could be observed from the lower half-potential of 0.55 V *versus* ~0.6 V for the secondary amines. This finding supported the findings of Chibale *et al.*[14] Since the half-potential of all target compounds were still considered to be low, these compounds could still undergo oxidization, as seen from the reversible cyclic voltammograms (see Annexure).

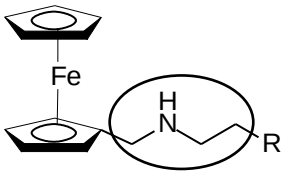


Figure 2: General structure of targeted compounds **11 – 18**, indicating the same chemical environment.

Drug development is largely hampered by poor pharmacokinetics and toxicity.[30] It is therefore essential to determine drug-like properties already during the early stages of the development of any drug. The aqueous solubility of any compound largely influences its ability to be absorbed when taken orally. Poor water solubility is generally associated with high lipophilicity, while hydrophilic compounds generally show poor permeability and hence low absorption, which to a large extent influence the drug's bioavailability.[30] These two properties are thus of high importance when designing new drugs.

The *n*-octanol/water partition coefficient (log *P*) offers a reliable measure of the balance between lipophilicity and aqueous solubility, with values between 1 - 5 being targeted, and values between 1 - 3 being ideal.[31,32] The predicted log *P* values of all the synthesized target compounds were comparable to that of CQ and fell comfortably into the ideal range. The predicted solubility levels at pH 7 were also comparable to that of CQ. These compounds were characterized by good predicted absorption levels. Overall, the amides had favourable predicted drug-like properties and were expected to possess antimalarial activities, comparable to that of chloroquine.

4.3 *In vitro* antimalarial activity and cytotoxicity

The *P. falciparum* 3D7 strain, a line cloned from NF54, is of African origin and is CQ susceptible. It has often been used to evaluate *in vitro* antigametocytes activity of trial antimalarial compounds.[33] In contrast, the FCR3 clone, from Gambia in Western Africa, is a

multi-drug resistant strain. It has shown resistance against traditional antimalarial drugs, including chloroquine, cycloguanil and pyrimethamine (antifolates).[34]

Since the amides prepared during this study were tested *in vitro* and not metabolized by metabolic enzymes, they would act as new entities and not as prodrugs. The observed IC_{50} values were therefore most likely to be those of the hybrids, rather than that of any active metabolite.

Compared to the IC_{50} of 6.5 µM of Licochalcone A against 3D7 clone,[16] chalcone **10** was found to be five-fold less active against both the 3D7 and FCR3 strains. Chalcones, containing electron withdrawing groups on ring A and a heterocyclic ring B, had been reported to show overall better antimalarial activities,[23] which was contradicted by the performance of chalcone **10** against the 3D7 and FCR3 strains during this study.

With the limited number of compounds being prepared during this study, a realistic structure activity relationship (SAR) could not be depicted. There were no observed structure activity trends. As indicated above, the incorporation of different methylene spacers may influence both the activity and the redox cycling of the target compounds, but in this study, no significant differences were observed from the $E_{1/2}$ and IC_{50} data, suggesting that the spacer type had limited impact on the activity of these compounds.

With similar predicted drug-like properties, all of these amides were found to be 25 to 150-fold less potent than CQ, irrespective of the strain of *P. falciparum* being considered. This confirmed that the antimalarial activities of these ferrocenyl chalcones were influenced by other factors than those governing the activity of CQ and physicochemical properties.[14]

Ferrocene carboxaldehyde was found to be as active as the amides against the 3D7 strain, whilst having lost most of its activity against the resistant FCR3 strain, resulting in a resistance index of 3.2. Amide **18**, the most active of all these amides against the 3D7 strain, suffered the same fate. With the exception of **12** and **18**, other amides retained their activities against the FCR3 strain. The combination **M**, was found inactive against the CQS 3D7 strain (IC_{50} > 100 µg/ml), whilst showing an IC_{50} of 12 µg/ml against FCR3, indicating a major gain in activity against the resistant strain. This observed activity may have been indicative of a synergistic effect of the two entities and that chemically binding of these ferrocene and chalcone pharmacophores into a single hybrid, may not be the best route for achieving optimum antimalarial activity. However, the fact that amide **12**, the least active (IC_{50} = 6.6 µM or 3.4 µg/ml) and amide **11**, the most active (IC_{50} = 2.1 µM or 1 µg/ml)

demonstrated four- and fifteen-fold higher activities than the combination **M**, respectively, while also being more active than both chalcone **10** and **FcA**, confirmed the possibility of synergism. These findings could have been indicative thereof that the hybrids strategy should perhaps not be ruled out with regards to achieving optimum activity from these two moieties. An interesting future prospect might thus be to investigate molar combinations of the intermediate aminoferrocenyl compounds, with chalcones alongside the hybrids, in order to generate a conclusive outcome.

All compounds were found to have low cytotoxicity against mammalian cells, compared to the reference compound, emetine. However, results from the selectivity index (SI), which is an indication of selective toxicity towards parasitic cells in the presence of mammalian cells, gave different outcomes. All of the nine compounds prepared during this study were found cytotoxic, with low to moderate SI values below 20. This suggested that the observed antimalarial activities of these compounds against the parasitic cells were not intrinsic, i.e. the resultant mode of action, such as ion trapping, was independent of their structural features, but rather due to cytotoxicity, emanating from the oxidation of Fe(II) into cytotoxic ferricenium ion, as reported by numerous authors.[29,35] Indeed, ferricenium cation reacts with hydrogen peroxide (Fenton reaction) to generate reactive oxygen species (superoxide and hydroxyl radical), known to inflict considerable damage to DNA to ultimately cause cell death.

Chloroquine does not possess redox properties, and yet it was more potent than all of the amides prepared during this study, suggesting that the reported mechanisms of action of CQ, such as heme inhibition and ion trapping, responsible for its antimalarial activity, were more important than the redox induced mechanism.

5. Conclusion

During this study, a series of nine, novel aminoferrocenyl-chalcone amides, **11** – **19**, were synthesized in a three-step process, involving amino-functionalizing ferrocenes, a carboxylic acid-functionalized chalcone and 1,1'-carbonyldiimidazole as coupling reagent. Routinely used techniques, such as NMR, HRMS and IR served to confirm their structures. Preliminary ADMET predictions served to envisage their drug-likeness properties. Despite favourable calculated drug-like properties, their observed antimalarial activities were all relatively low. During antimalarial screen alongside CQ, all of the amides proved to be active, with IC_{50} values ranging between 0.5 - 4.5 µM and 2.1 - 6.6 µM against the 3D7 and FCR3 strains of *P. falciparum*, respectively. The amides overall displayed retention of activity against the FCR3 strain, compared to the CQS, except for compounds **12** and **18**. All compounds

furthermore demonstrated low selective toxicity towards the parasitic cells in the presence of mammalian cells. No clear SAR could be drawn, since no trend was observed in terms of activity with an increase in the chain length. It was concluded that the amines' observed activities were not intrinsic, but rather attributable to cytotoxicity, most likely due to the formation of a ferricenium cation.

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