Supporting Information

Synthesis, in vitro antimalarial activities and cytotoxicities of amino-artemisinin-1, 2-disubstituted ferrocene derivatives

Christo de Lange[a], Dina Coertzen[b], Frans J. Smit[a], Johannes F. Wenzel[a], Ho Ning Wong[a], Lyn-Marie Birkholtz[b], Richard K. Haynes[a*] and David D. N'Da[a*]

[a]Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa.
bDepartment of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Pretoria 0002, South Africa.

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1. General procedures

Dihydroartemisinin (DHA) (a mixture of 10-α and 10-β epimers) was purchased from Changzhou Kaixuan Chemical Co (Chunjiang,China). Piperazine, oxalyl chloride, piperidine, morpholine, thiomorpholine, ferrocenecarboxaldehyde, potassium *tert*-butoxide, triethylamine and sodium tri-acetoxyborohydride were purchased from Sigma-Aldrich (Johannesburg, South Africa). Methanol, magnesium sulfate, diethyl ether, dichloromethane and toluene were purchased from ACE chemicals (Johannesburg, South Africa). Sodium hydroxide was purchased from Saarchem (Krugersdorp, South Africa). All the chemicals and reagents were of analytical grade. Diethyl ether and tetrahydrofuran was dried and distilled from a sodium-benzophenone distil, dichloromethane and toluene were dried and distilled from calcium hydride. Chemicals were used without further purification.

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance™ III 600 spectrometer at a frequency of 600 MHz and 150 MHz, respectively, in $CDCl_3$. Chemical shifts are reported on the δ scale (ppm) downfield from tetramethylsilane (δ=0.0 ppm) using the residual solvent signal at δ=7.26 ppm (^{1}H) or δ=77.00 ppm (^{13}C) as internal standard.

High resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer, equipped with an atmospheric pressure chemical ionisation (APCI) or an electrospray ionisation (ESI) source, set at 200 °C or 180 °C, respectively, using Bruker Compass DataAnalysis 4.0 software. A full scan from 50 to 1500 *m*/*z* was performed at a capillary voltage of 4500 V, an end plate offset voltage of −500 V, with the nebulizer set at 1.6 Bar and 0.4 Bar, respectively, and a collision cell RF voltage of 100 Vpp.

Mass spectra (MS) were recorded in positive mode on a Thermo Electron LXQ™ ion trap mass spectrometer, equipped with Xcalibur 2.2 data acquisition and analysis software. The MS had an APCI source set at 300 °C, and was direct infusion with a Harvard syringe pump utilized at a flow rate of 10 μL/min. A full scan from 100 to 1200 amu was achieved in 1 s, with a capillary voltage of 7 V, while the corona discharge was 10 μA.

Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument using the Attenuated Total Reflectance (ATR) technique. Thin layer chromatography (TLC) was performed, using silica gel plates ($60F_{254}$), obtained from Merck (Johannesburg, South Africa). Column chromatography was performed, using silica gel 60, 70-230 mesh ASTM, supplied by Macherey-Nagel (Germany).

2. Biological evaluation

2.1 *In vitro* efficacy studies on asexual *P. falciparum* parasites

The *P. falciparum* parasites were maintained at 37 °C in human erythrocytes (O+) suspended in complete culture medium [RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 200 μM hypoxanthine (Sigma-Aldrich), 0.2% sodium bicarbonate, 24 μg/mL Gentamycin (Sigma-Aldrich) and 0.5% AlbuMAX II] under an atmosphere of 90% N_2, 5% O_2, and 5% CO_2.[1] The *in vitro* ring-stage intra-erythrocytic *P. falciparum* NF54 parasite cultures (genotyped drug sensitive) (200 μL at 1% haematocrit, 1% parasitaemia) were treated with compounds at 5 and 1 μM. The controls for this assay included chloroquine disulfate (1 μM as positive control) and complete RPMI media (as negative control) and grown for 96 h at 37 °C under the 90% N_2, 5% O_2, and 5% CO_2 gas mixture in 96-well plates. At the conclusion of the 96 h growth period, equal volumes (100 μL each) of the *P. falciparum* parasite cultures were combined with SYBR Green I lysis buffer (0.2 μL/mL 10 000xSYBR Green I, Invitrogen; 20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% (w/v) saponin; 0.08% (v/v) Triton X-100). The samples were incubated at 37 °C for 1 h after which the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The 'background' fluorescence (i.e. that measured in the samples derived from chloroquine-treated iRBC samples in which parasite proliferation was completely inhibited) was subtracted from the total fluorescence measured for each sample to provide a measure of parasite proliferation. Data obtained were analyzed in Excel and sigmoidal dose-response curves were plotted using GraphPad 5.0. Experiments are always performed in technical triplicates for at least three independent biological replicates (n=3).

2.2 Determination of gametocytocidal activities [2]

The luciferase reporter assay was established to enable accurate, reliable and quantifiable investigations of the stage-specific action of gametocytocidal compounds for both the early and

late gametocytes using the NF54-PfS16-GFP-Luc marker cell line. Drug assays were set up on day 5 and 10 (representing >90% of either early stage II/III or mature stage IV/V gametocytes, respectively). In each instance, assays were set up using a 2 – 3% gametocytaemia, 1.5% haematocrit culture and 48 h drug pressure in a gas chamber (90% N_2, 5% O_2, and 5% CO_2) at 37 ºC. Luciferase activity was determined in 20 µL parasite lysates by adding 50 µL luciferin substrate (Promega Luciferase Assay System) at room temperature and detection of resultant bioluminescence at an integration constant of 10 s with the GloMax®-Multi+ Detection System with Instinct® Software. Methylene blue (5 µM) is routinely included as a control. Dual point screens are routinely performed as technical triplicates for a single biological assay.

2.3 *In vitro* anticancer and cytotoxicity screening[3]

A375 (ATCC® CRL-1619™ Human malignant melanoma) and Hek293 cells (ATCC® CRL-1573™ Human embryonic kidney cells) were cultured in Dulbecco's modified essential medium (DMEM; Hyclone, GE healthcare, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 200 mM L-Glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For compound treatment, cells were seeded in a 96 well plate and cultured until 80-90% confluent. Stock solutions for compounds were prepared in DMSO preheated to 40 °C. All subsequent dilutions were prepared in serum free DMEM and vehicle controls were included in all experiments.

The 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine *in vitro* cell viability.[4] A375 and Hek293 cells were seeded in a 96-well plate and incubated until cells were ~ 90% confluent. After 24 hours exposure to the compounds (12 - 1800 µM), growth medium was removed, cells rinsed twice with 1 x phosphate buffered saline (PBS) and 100 µL fresh serum free medium containing 5 mg/ml MTT solution was added. Cells were then incubated for 4 hours at 37 °C, after which the MTT was carefully removed and replaced with 100 µL dimethyl sulfoxide (DMSO). After 1 hour of incubation at 37 °C, cell viability was determined using a microplate reader (SpextraMac Paradigm) at an absorbance wavelength of 550 nm and background wavelength of 630 nm with DMSO measured as a blank. Cell viability is expressed as a percentage relative to the untreated control, which is assumed to be 100 % viable.

As a positive control, cells were treated with 0.01% Triton-X 100 (Sigma-Aldrich, St Louis, MO, USA) for 4 hours. Using the MTT assay data, IC_{50} values was calculated using GraphPad Prism 5. In brief, data was normalized to the negative controls (presumed to be 100% viable), followed by the log-transformation of the concentration values. The curve was fitted using the log (inhibitor) vs. response function and the IC_{50} values calculated. Experiments were done at least in triplicate.

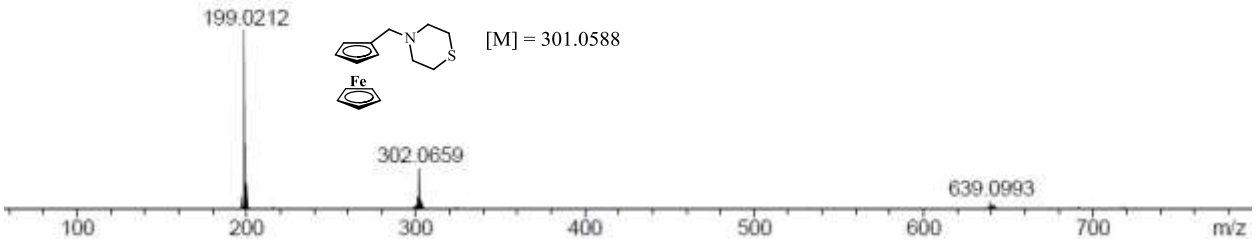
3. General experimental procedures

3.1 Reductive amination of ferrocenecarboxaldehyde

A stirred solution of ferrocenecarboxaldehyde (11 mmol, 1 equiv) in anhydrous dichloromethane (50 mL) under nitrogen was treated with the secondary amine (12.2 mmol, 1.1 equiv.). The solution was treated portionwise with sodium triacetoxyborohydride (25 mmol, 2.2 equiv) after which it was left to stir for 4 h at room temperature under N_2. The reaction mixture was poured onto ice, and basified to pH 10 with aqueous NaOH (1M, 5-10 mL), and extracted with diethyl ether until the extract was colourless. The combined extracts were dried ($MgSO_4$), and filtered. The filtrate was evaporated under reduced pressure, and the residue was submitted to column chromatography over silica gel. Eluting with dichloromethane-MeOH (9:1) afforded the derivatives.

(Thiomorpholinomethyl)ferrocene

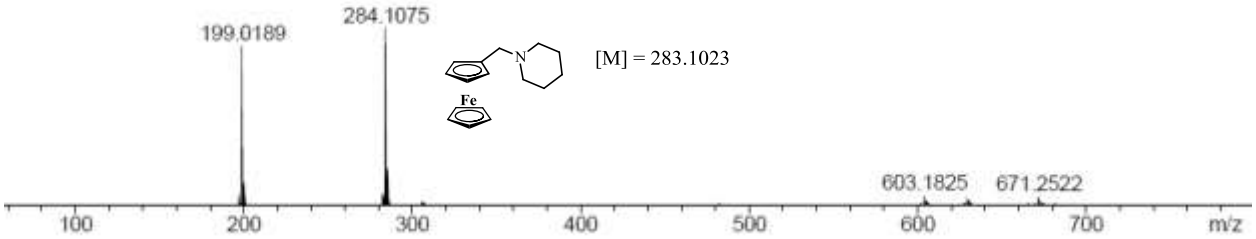
Yellow powder, 2.38 g (72%), melting point 101.4-105.3 °C.



HRMS ESI: m/z $[M+H]^+$ 302.0659 (calculated for $C_{15}H_{20}FeNS^+$: 302.0666)

(Piperidinomethyl)ferrocene

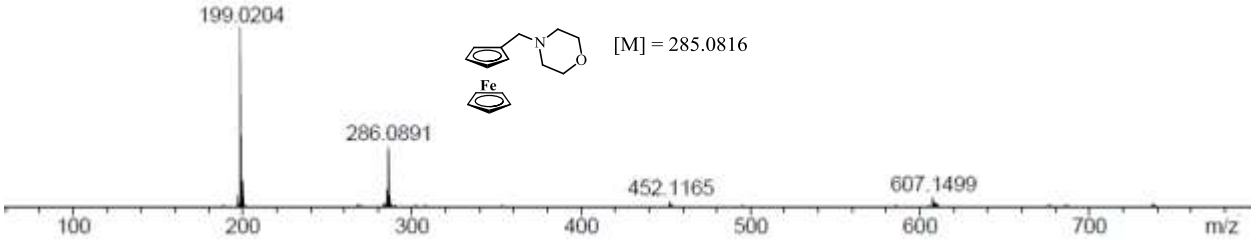
Yellow powder, 2.42 g (78%), melting point 105.7-108.2 °C.



HRMS ESI: m/z $[M+H]^+$ 284.1075 (calculated for $C_{16}H_{22}FeN^+$: 284.1102)

(Morpholinomethyl)ferrocene

Yellow powder, 2.28 g (80%), melting point 108.1-110.8 °C.



HRMS (ESI): *m/z* $[M+H]^+$ 286.0891 (calculated for $C_{15}H_{20}FeNO^+$: 286.0894)

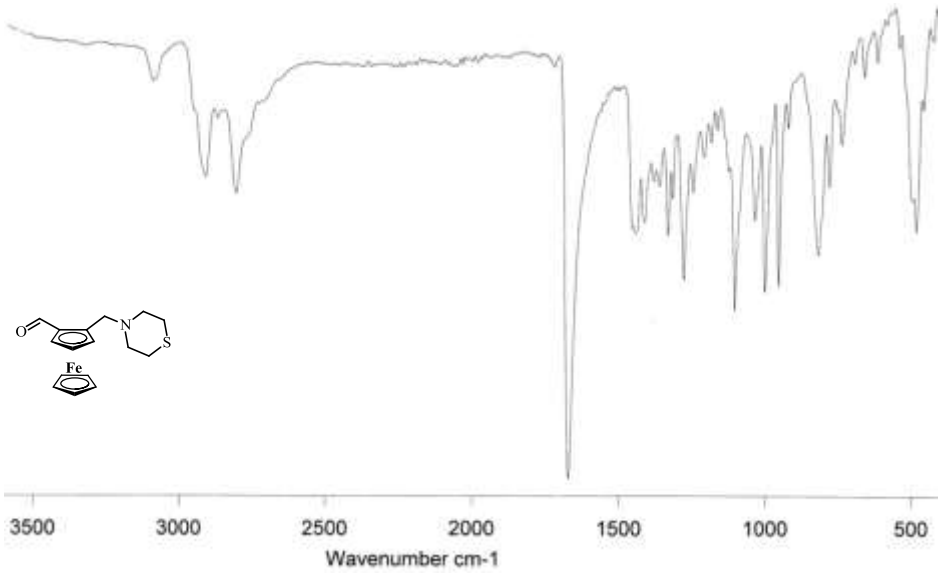
3.2 Synthesis of aminoferrocene aldehydes

The aminoferrocene (33.2 mmol, 1 equiv.) together with 0.3 mmol potassium *tert*-butoxide (0.1 equiv.) was dissolved in anhydrous diethyl ether (20 mL) under argon at room temperature, and with stirring, *n*-butyllithium (36.5 mmol, 1.1 equiv) was slowly added at room temperature. The reaction mixture was left under argon to stir for 16 h. DMF (99.6 mmol, 3 equiv) was slowly added to the reaction mixture that was left to stir for an additional 4 hr. The reaction mixture was quenched with of ice water (15 mL), and extracted with diethyl ether until the extract remained clear. The combined extracts were dried ($MgSO_4$) and then filtered. The filtrate was evaporated under reduced pressure to dryness, with the flask enclosed in foil to protect the contents from light. The residue was purified by column chromatography over silica gel, with the column also enclosed in foil; eluting with diethyl ether-hexane-triethylamine (7:2:1) afforded the derivatives. Whilst

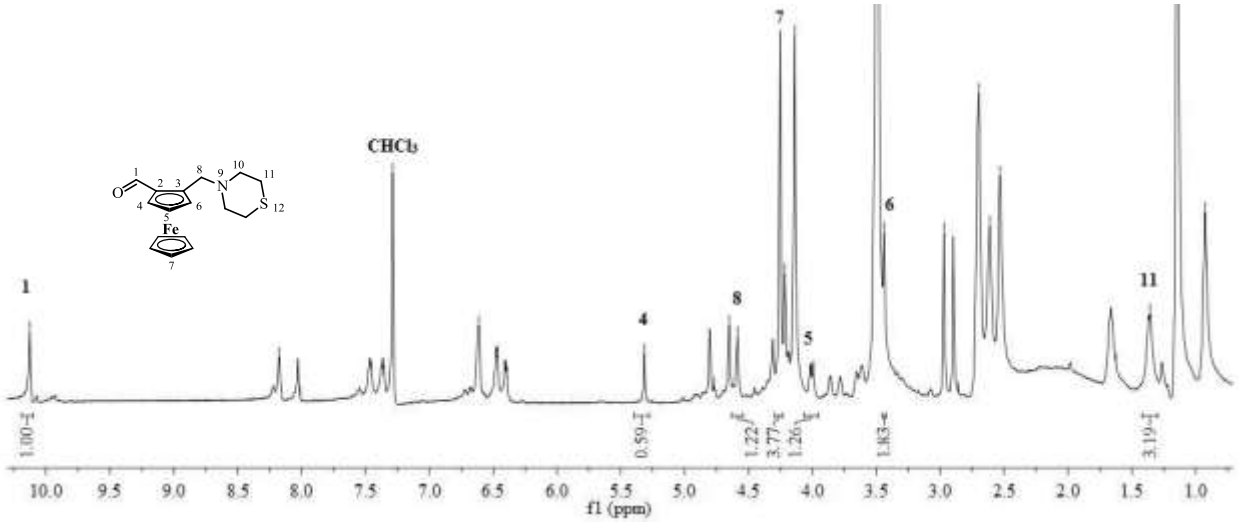
most derivatives were stable during storage, the thiomorpholine-formyl derivative described below, because of instability, was used immediately in the ensuing reductive amination reaction below.

(Thiomorpholinomethyl)-2-formylferrocene

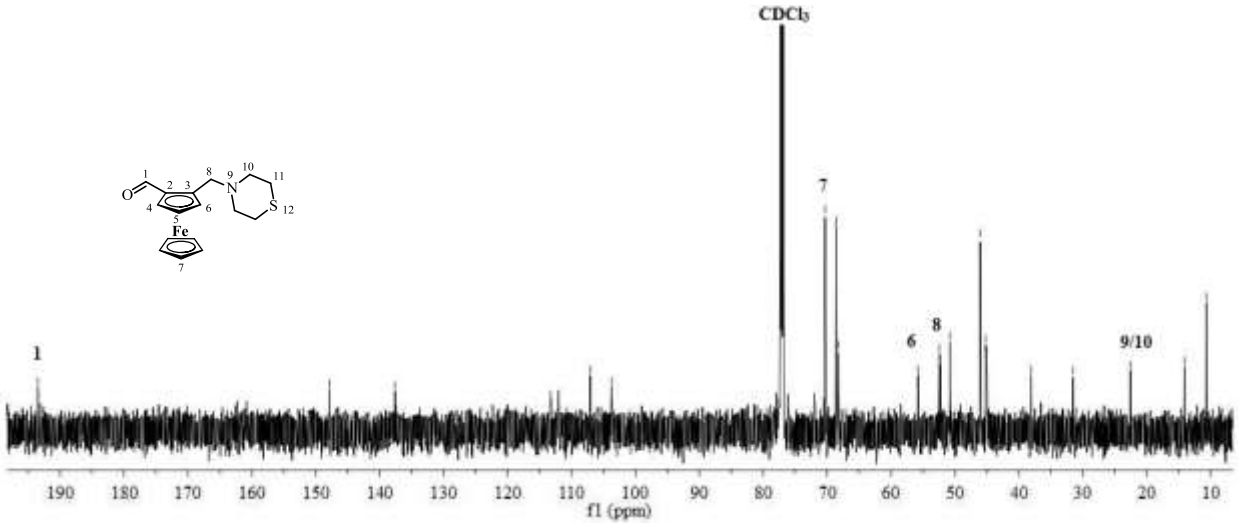
Red oil, 2.51 g (23%).



IR (ATR) ν_{max}/cm^{-1}: 3090, 2909, 2804 (H–C=O), 1670, 1412, 1332, 1278, 1104, 1036, 1001, 954, 820, 783, 742, 485.



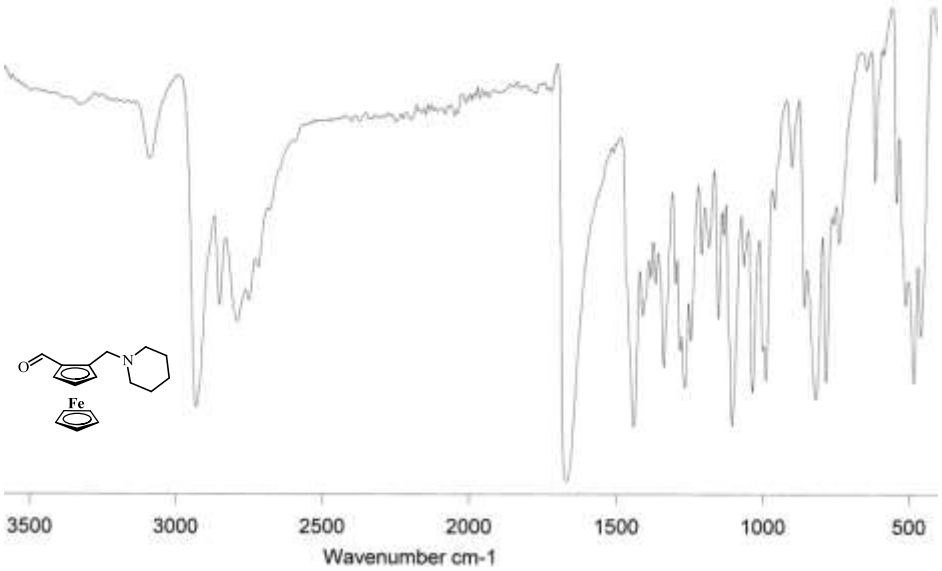
^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 10.13 (s, 1H, H-1), 5.32 (s, 1H, H-4), 4.59 (H-8), 4.22 (H-7), 4.01 (H-5), 3.44 (H-6), 1.36 (H-11).



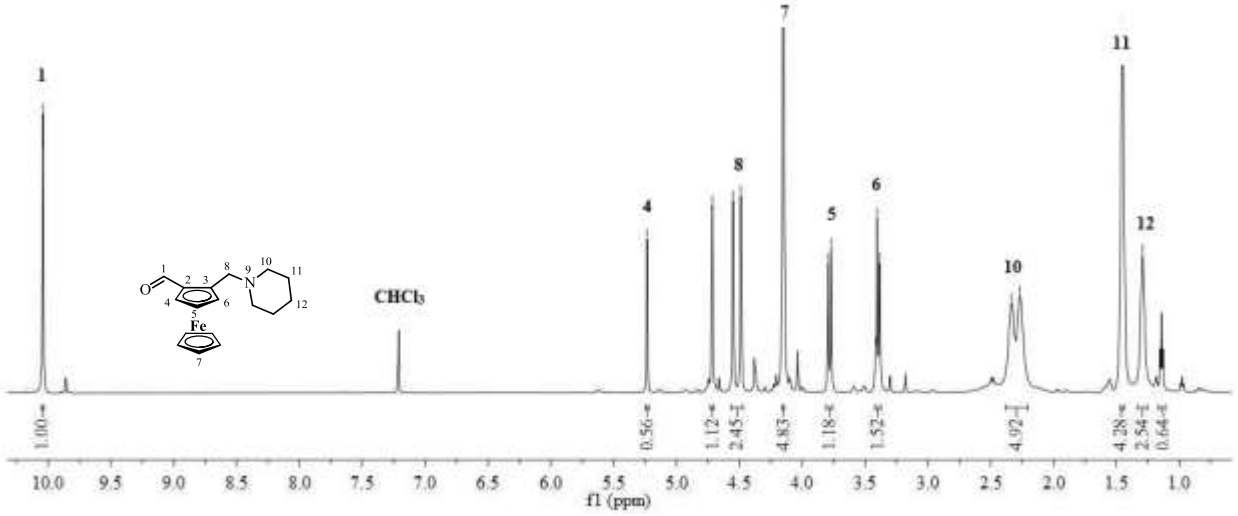
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 193.45 (C-1), 70.29 (C-7), 52.43 (C-6), 50.78 (C-8), 22.53 (C-9/C-10).

(Piperidinomethyl)-2-formylferrocene

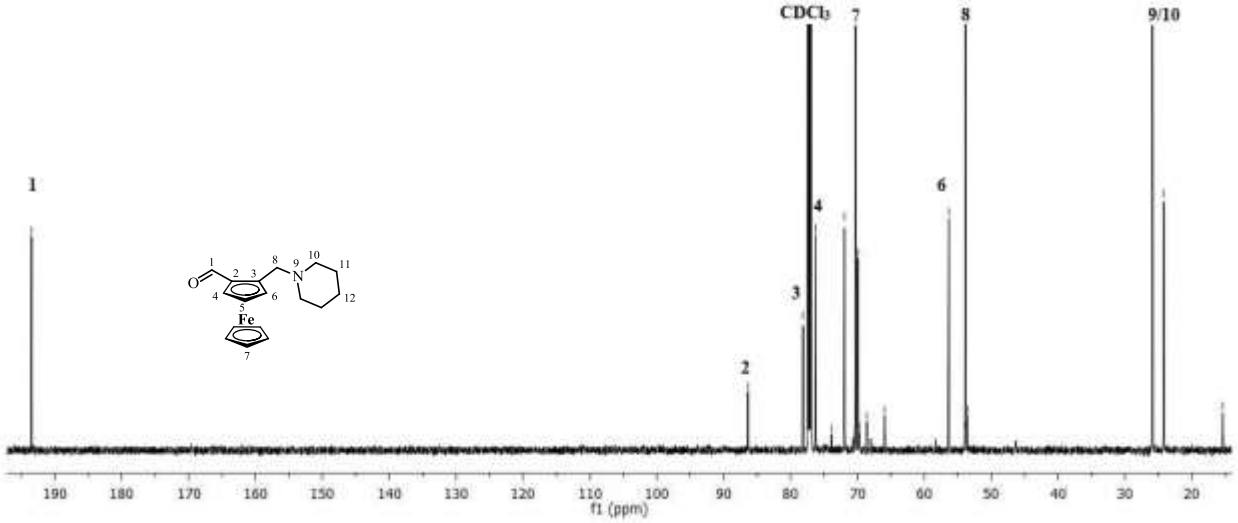
Red oil, 2.68 g (26%).



IR (ATR) ν_{max}/cm^{-1}: 3093, 2930, 2850, 2790 (H–C=O), 2750, 1669, 1439, 1409, 1337, 1265, 1246, 1151, 1035, 989, 859, 819, 785, 485, 462.



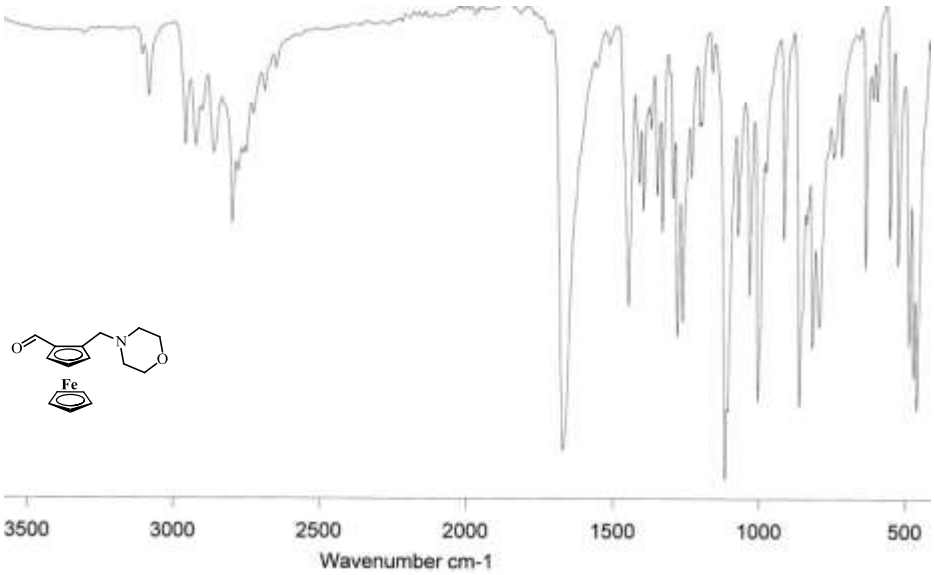
^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): .10.08 (s, 1H, H-1), 5.29 (s, 1H,), 4.77 (s, 1H,), 4.61-4.55 (d, *J* = 4.5, 2H,), 4.22 (s, 5H,), 3.94-3.92 (d, *J* = 3.9, 1H,), 3.61 (s, 4H,), 3.43 (s, 1H,), 2.48-2.41 (d, *J* = 2.4, 4H,), 1.20 (m,).



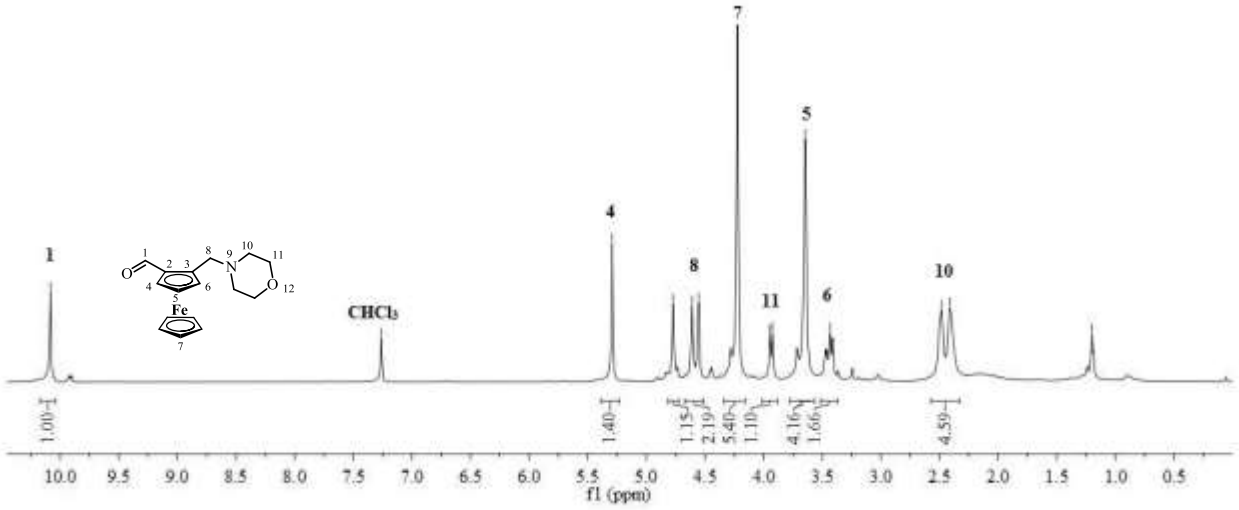
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm):193.49 (C-1), 86.38 (C-2), 78.14 (C-3), 76.19 (C-4), 71.94 (C-6), 70.29 (C-7), 69.95, 65.98, 56.33 (C-8), 53.81 (C-10), 25.91 (C-11), 24.19 (C-12), 15.38.

(Morpholinomethyl)-2-formylferrocene

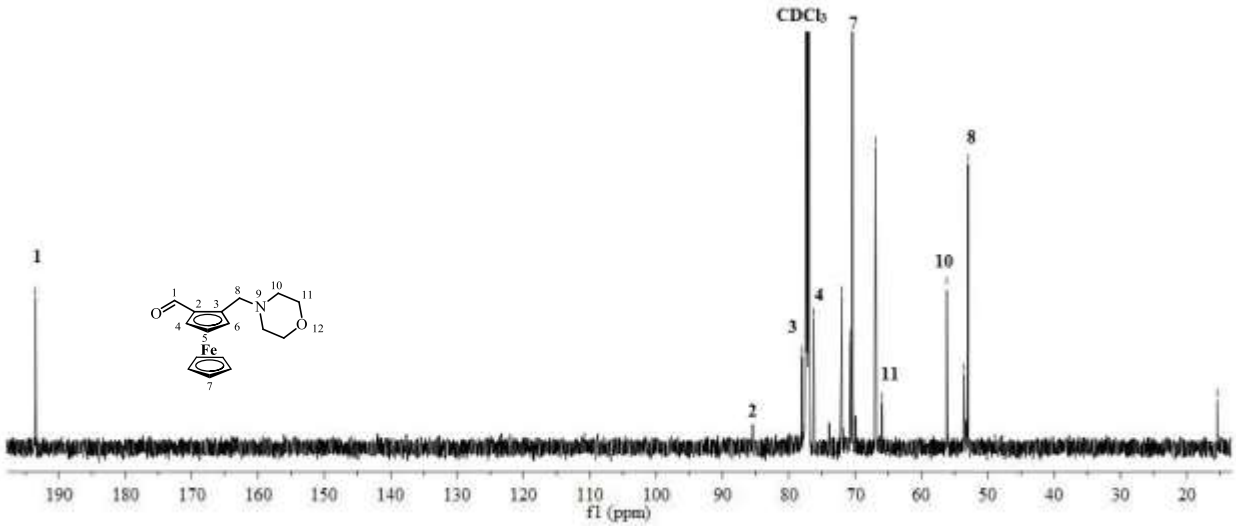
Red oil, 3.32 g (32%).



IR (ATR) ν_{max}/cm^{-1}: 3083, 2957, 2921, 2795 (H–C=O), 1665, 1442, 1393, 1346, 1328, 1276, 1258, 1112, 1068, 1000, 973, 913, 859, 815, 744, 634, 551, 523, 484, 458.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): .10.08 (s, 1H, H-1), 5.29 (s, 1H,H-4), 4.77 (s, 1H,), 4.61-4.55 (d, *J* = 4.5, 2H, H-8), 4.22 (s, 5H, H-7), 3.94-3.92 (d, *J* = 3.9, 1H, H-11), 3.61 (s, 4H, H-5), 3.43 (s, 1H, H-6), 2.48-2.41 (d, *J* = 2.4, 4H, H-10), 1.20 (m,).



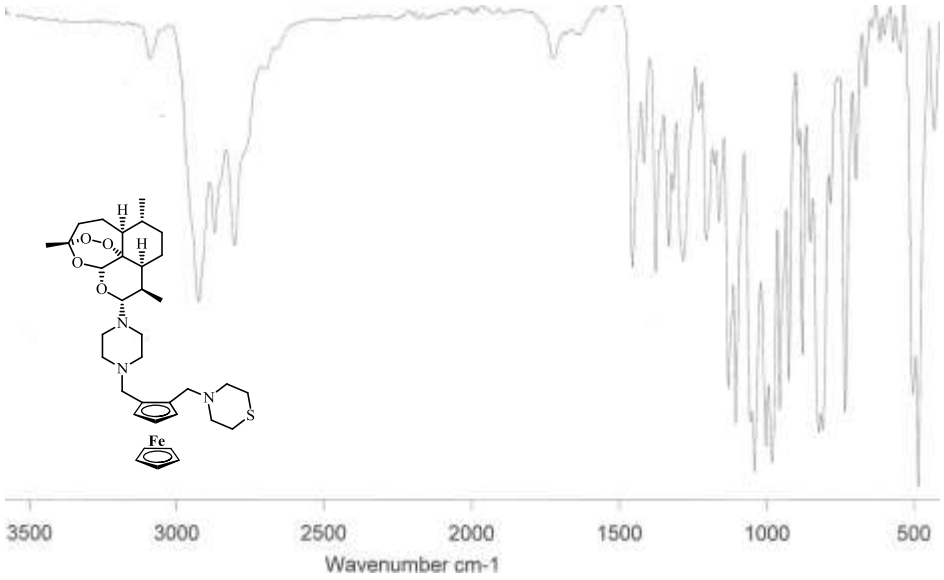
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm):193.50 (C-1), 86.38 (C-2), 78.02 (C-3), 76.22 (C-4), 72.08 (C-6), 70.73 (C-5), 70.39 (C-7), 66.91 (C-11), 65.98, 56.15 (C-10), 53.57, 52.98 (C-8), 15.40.

3.3 Synthesis of amino-artemisinin-1,2-disubstituted ferrocene derivatives

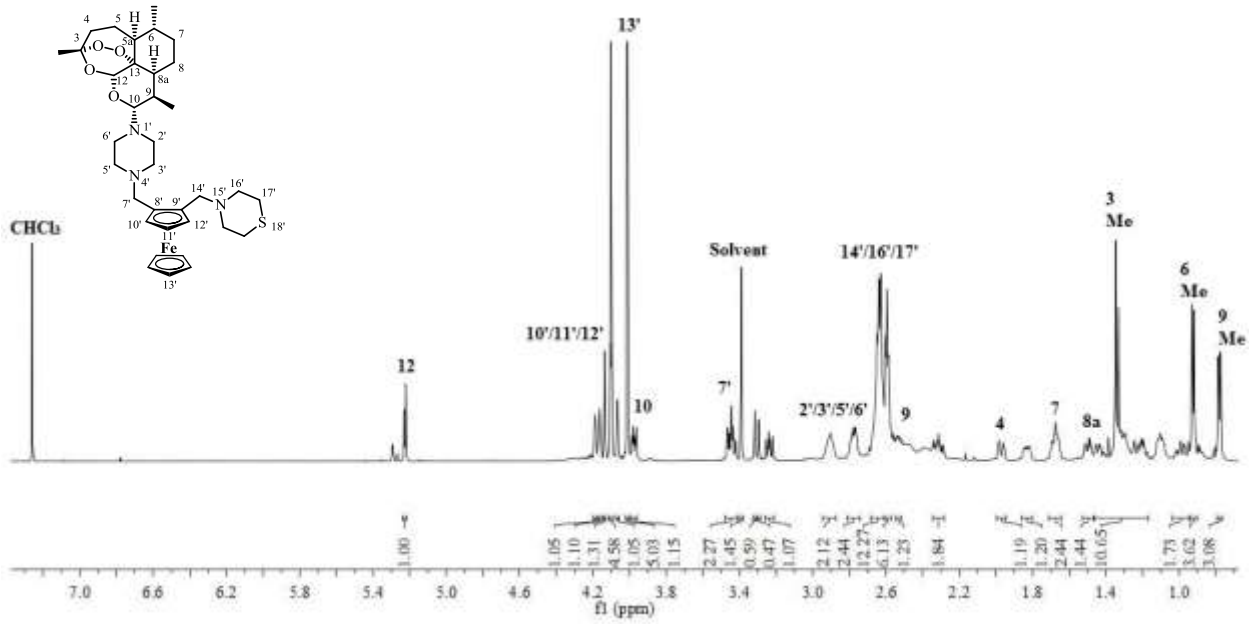
10α-(1'-Piperazino)-10-deoxo-10-dihydroartemisinin **2** (2 mmol, 3 equiv.) and the corresponding aminoferrocenealdehyde (0.6 mmol, 1 equiv.) were dissolved in anhydrous THF (22 mL) under nitrogen at room temperature. The resulting solution was stirred and treated portionwise with sodium triacetoxyborohydride (2 mmol, 3 equiv.). The reaction mixture was left to stir overnight at room temperature under nitrogen. The reaction mixture was basified to pH 10 by treatment with aqueous NaOH (1M, 5-10 mL), and extracted with diethyl ether (4 x 50 mL). The extracts were combined and dried ($MgSO_4$), and then filtered. The filtrate was evaporated under reduced pressure and the residue was purified by column chromatography over silica gel with dichloromethane-MeOH-triethylamine (9:1:1) to give the derivatives.

10α-[1'-Piperazino-4'-(8'-thiomorpholinomethyl ferrocenemethyl)]-10-deoxo-10-dihydro-artemisinin **3**

Red gum, 0.07 g (18%), R_f 0.47 (dichloromethane-MeOH 9:1).

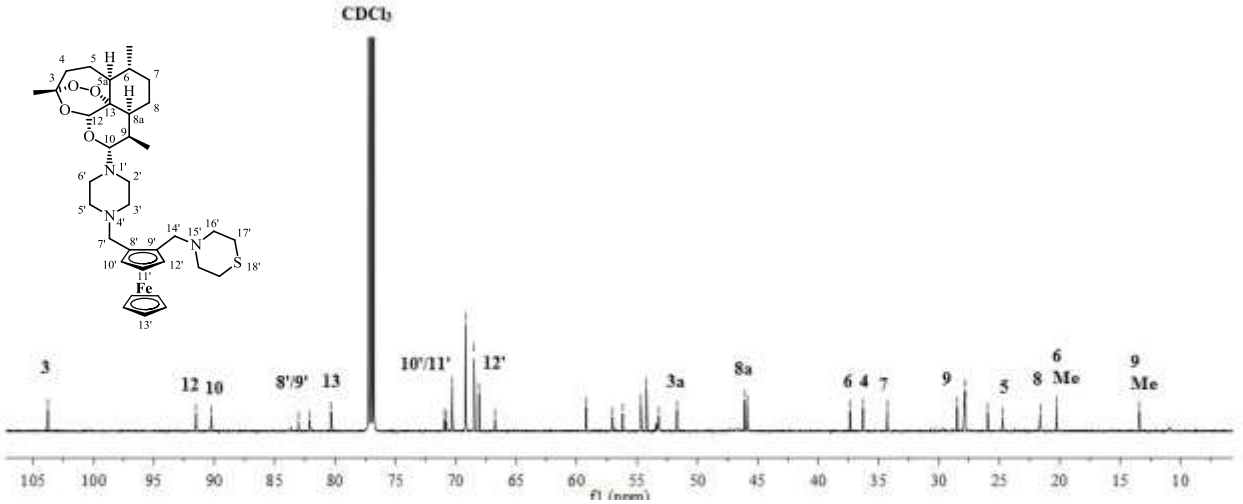


IR (ATR) ν_{max}/cm^{-1}: 3091, 2923, 2869, 2801, 1723, 1453, 1374, 1161, 1053, 979, 784, 733, 503, 483.

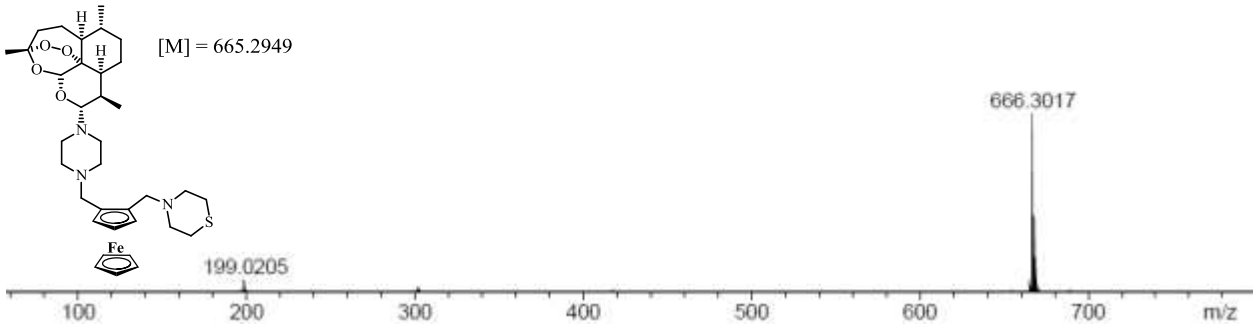


^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.23 (d, *J* = 4.8 Hz, 1H, H-12), 4.19 (m, 1H, H-10'/H-11'/H-12'), 4.16 (m, 1H, H-10'/H-11'/H-12'), 4.10 (s, 5H), 4.07-4.06 (m, 1H, H-10'/H-11'/H-12'), 4.01 (s, 5H, H-13'), 3.98-3.97 (dd, *J* = 10.1, 3.7 Hz, 1H, H-10), 3.47-3.43 (m, 2H, H-7'), 2.91-2.90 (m, 2H, H-2'/H-3'/H-5'/H-6'), 2.79-2.77 (m, 2H, H-2'/H-3'/H-5'/H-6'), 2.65-2.62 (m, 12H, H-14'/H-16'/H-17'), 2.55-2.51 (m, 1H, H-9), 2.34-2.28 (td, *J* = 9.8, 4.4 Hz, 1H, H-4), 1.98-1.96 (d, *J* = 14.5 Hz, 1H, H-4), 1.85-1.81 (m, 1H, H-5), 1.70-1.66 (m, 2H, H-7), 1.52-1.47 (m, 1H, H-8a), 1.46-1.43

(m, 1H, H-5), 1.35 (d, *J* = 9.5 Hz, 3H, H-3Me), 1.32-1.30 (m, 1H, H-6), 0.93-0.92 (d, *J* = 6.3 Hz, 3H, H-6Me), 0.79-0.77 (dd, *J* = 7.1, 2.6 Hz, 3H, H-9Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.77 (C-3), 91.51 (C-12), 90.23 (C-10), 83.02 (C-6'/C-7'), 82.11 (C-6'/C-7'), 80.32 (C-13), 70.91 (C-8'/C-9'), 70.80 (C-8'/C-9'), 70.28, 69.15 (C-10'), 68.47, 68.06 (C-10'), 66.74 (C-8'/C-9'), 59.22, 57.04 (C-12'/C-16'/C-17'), 56.18 (C-12'/C-16'/C-17'), 54.67 (C-2'/C-3'), 54.21 (C-2'/C-3'), 53.21 (C-5'/C-11'), 51.69 (C-5a), 46.08 (C-8a), 45.85 (C-6), 37.35, 36.29 (C-4), 34.28 (C-7), 28.50 (C-9), 27.84, 25.95 (C-3Me), 24.75 (C-5), 21.62 (C-8), 20.28 (C-6Me), 13.40 (C-9Me).

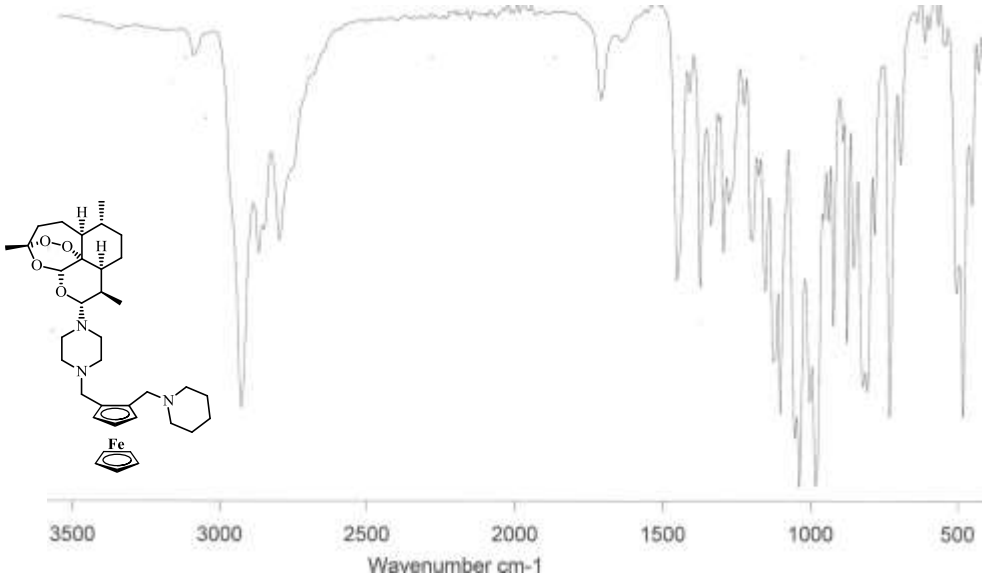


HRMS ESI: m/z $[M+H]^+$ 666.3017 (calculated for $C_{35}H_{52}FeN_3O_4S^+$: 666.3027)

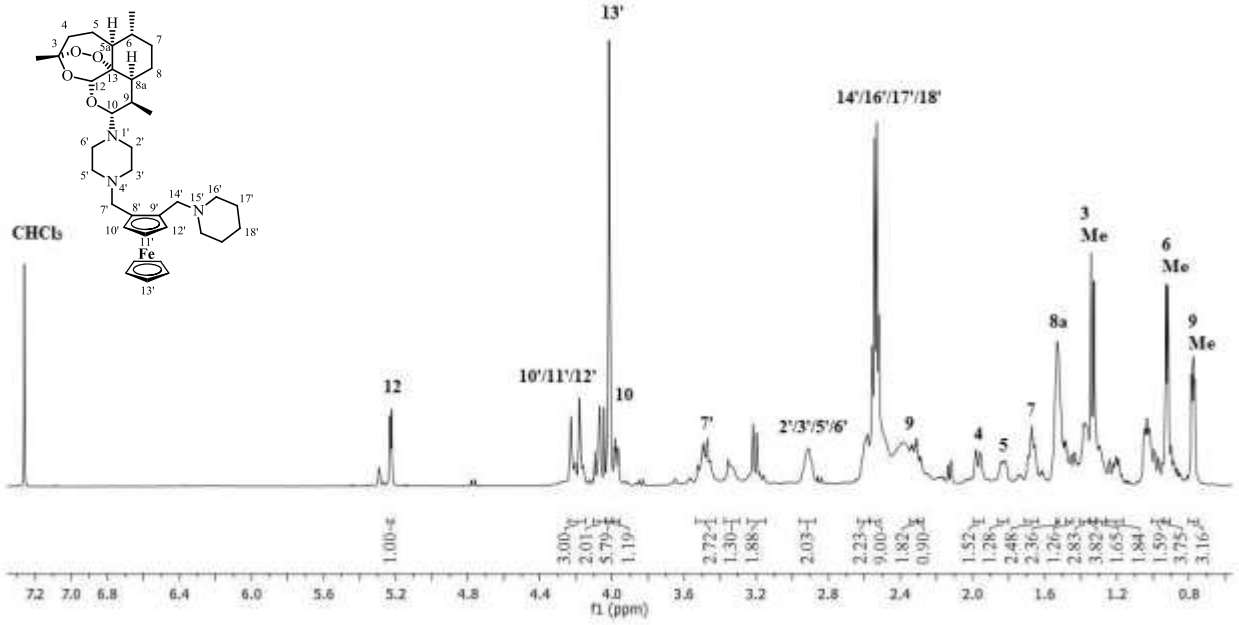
10α-[1'-Piperazino-4'-(8'-piperidinomethyl ferrocenemethyl)]-10-deoxo-10-dihydroartemisinin

4

Red gum, 0.08 g (23%), R_f 0.53 (dichloromethane-MeOH 9:1).

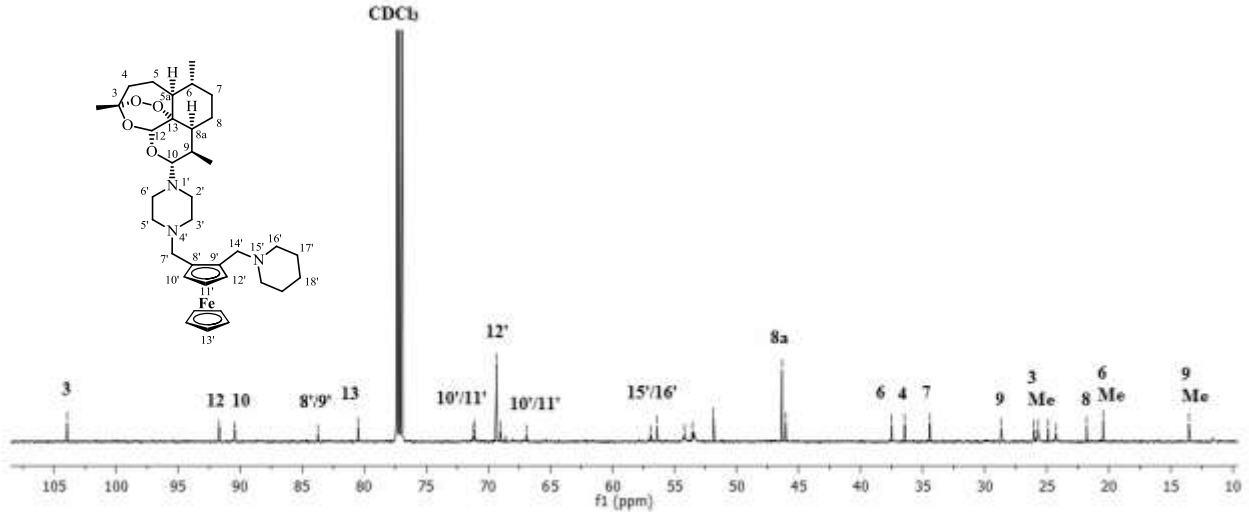


IR (ATR) ν_{max}/cm^{-1}: 3092, 2928, 2870, 2798, 1712, 1453, 1375, 1296, 1103, 1039, 984, 734, 485.

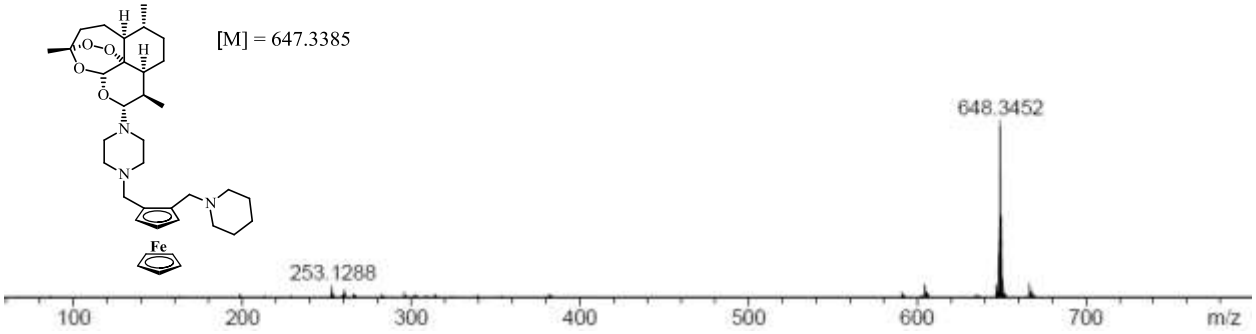


^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.23 (d, *J* = 4.4 Hz, 1H, H-12), 4.23-4.16 (m, 3H, H-10'/H-11'/H-12'), 4.07 (m, 2H, H-10'/H-11'/H-12'), 4.02 (s, 5H, H-13'), 3.98-3.97 (m, 1H, H-10), 3.52-3.46 (s, 2H, H-7'), 2.91 (s, 2H, H-2'/H-3'/H-5'/H-6'), 2.60-2.58 (m, 12H, H-14'/H-16'/H-17'/H-18'), 2.56-2.51 (m, 1H, H-9), 2.34-2.29 (m, 1H, H-4), 1.98-1.96 (d, 1H, H-4), 1.85-1.81 (m, 1H, H-5), 1.69-1.65 (m, 2H, H-7), 1.53 (s, 2H, H-8a), 1.48-1.45 (m, 1H, H-5), 1.34-1.33 (d, *J* = 9.6

Hz, 3H, H-3Me), 1.30-1.26 (m, 1H, H-6), 1.24-1.19 (m, 1H, H-5a), 0.99-0.97 (m, 1H, H-7), 0.93-0.92 (d, *J* = 6.2 Hz, 3H, H-6Me), 0.79-0.77 (dd, *J* = 10.8 Hz, 3H, H-9Me).



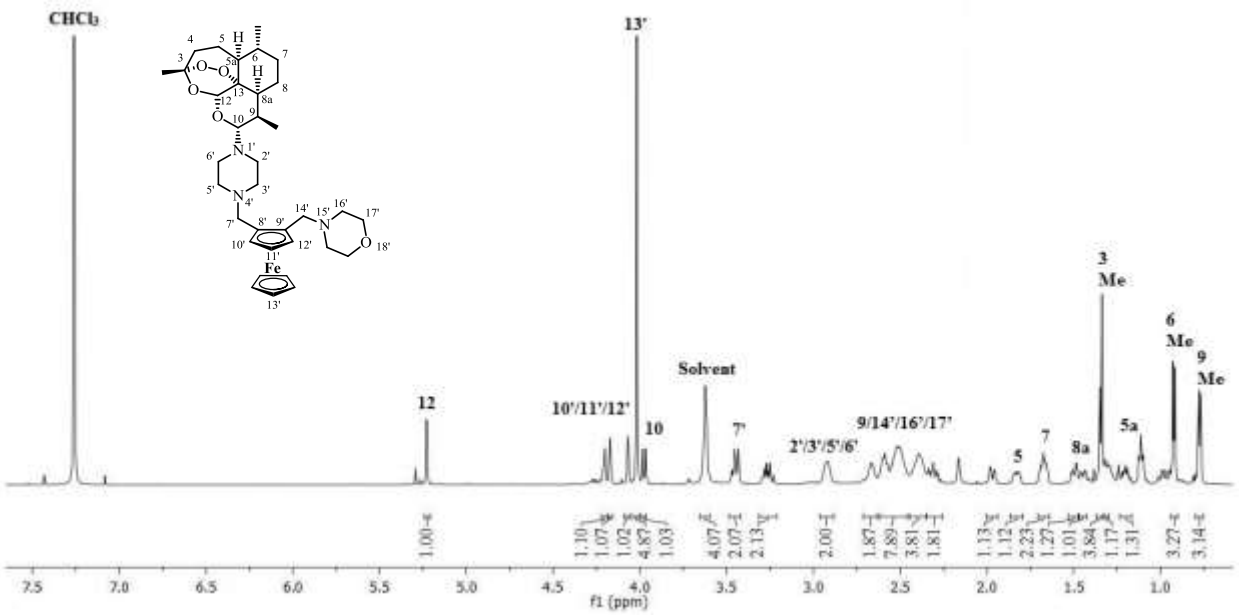
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.92 (C-3), 91.70 (C-12), 90.44 (C-10), 83.72 (C-6'/C-7'), 80.48 (C-13), 71.21 (C-8'/C-9'), 69.37 (C-10'), 69.01, 66.91 (C-8'/C9'), 56.91 (C-16'/C-17'), 56,41 (C-16'/C-17'), 53.56, 53.44, 51.85 (C-5a), 46.02 (C-8a), 37.51 (C-6), 36.45 (C-4), 34.44 (C-7), 28.67 (C-9), 26.05 (C-3Me), 25.69, 24.90 (C-5), 24.27, 21.78 (C-8), 20.44 (C-6Me), 13.54 (C-9Me).



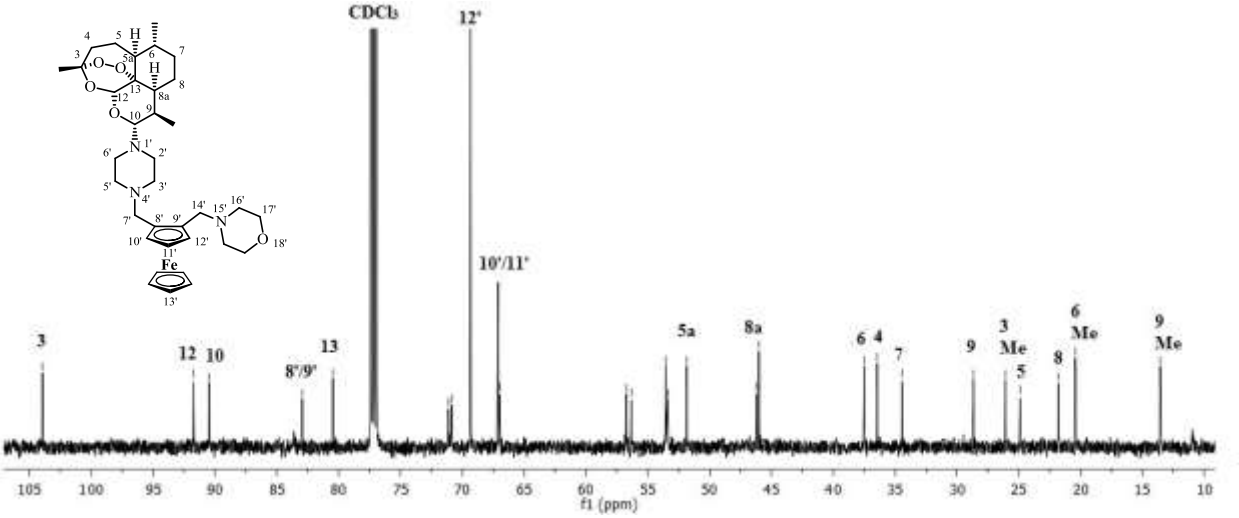
HRMS ESI: m/z $[M+H]^+$ 648.3452 (calculated for $C_{36}H_{54}FeN_3O_4^+$: 648.3460).

10α-[1'-Piperazino-4'-(8'-morpholinomethyl ferrocenemethyl)]-10-deoxo-10-dihydroartemisinin, **5**

Red gum, 0.10 g (26%), R_f 0.43 (dichloromethane-MeOH 9:1).

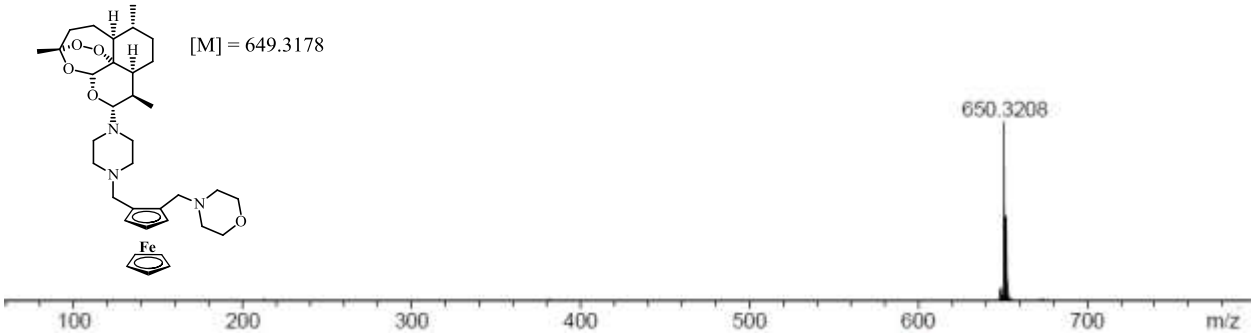


^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.23-5.22 (d, *J* = 2.8 Hz, 1H, H-12), 4.20 (s, 1H, H-10'/H-11'/H-12'), 4.17 (s, 1H, H-10'/H-11'/H-12'), 4.07 (s, 1H, H-10'/H-11'/H-12'), 4.02 (s, 5H, H-13'), 3.98-3.97 (d, *J* = 10.1 Hz, 1H, H-10), 3.47-3.43 (m, 2H, H-7'), 2.92 (s, 2H, H-2'/H-3'/H-5'/H-6'), 2.71-2.66 (m, 2H, H-2'/H-3'/H-5'/H-6'), 2.60-2.50 (m, 8H, H-9/H-14'/H-16'/H-17'), 2.33-2.28 (m, 2H, H-4), 1.98-1.96 (d, *J* = 14.4 Hz, 1H, H-4), 1.85-1.81 (m, 1H, H-5), 1.69-1.65 (m, 2H, H-7), 1.51-1.48 (m, 1H, H-8a),1.46-1.41 (m, 1H, H-5), 1.35-1.34 (d, *J* = 6.9 Hz, 3H, H-3Me), 1.32-1.31 (m, 1H, H-8), 1.31-1.30 (m, 1H, H-6), 1.22-1.17 (m, 1H, H-5a), 0.93-0.92 (d, *J* = 6.3 Hz, 3H, H-6Me), 0.78-0.77 (dd, *J* = 7.1, 1.9 Hz, 3H, H-9Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.94 (C-3), 91.71 (C-12), 90.45 (C-10), 82.95 (C-6'/C-

7’), 80.47 (C-13), 71.21 (C-8’/C-9’), 70.86 (C-8’/C-9’), 69.33 (C-10’), 66.97 (C-8’/C9’), 56.74, 56,34, 53.55, 53.39, 51.86 (C-5a), 46.02 (C-8a), 37.51 (C-6), 36.46 (C-4), 34.44 (C-7), 28.67 (C-9), 26.09 (C-3Me), 24.89 (C-5), 21.79 (C-8), 20.44 (C-6Me), 13.56 (C-9Me).



HRMS ESI: m/z $[M+H]^+$ 650.3208 (calculated for $C_{35}H_{52}FeN_3O_5^+$: 650.3256).

4. References:

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