Chapter 4

Antimalarial activity of 10-alkyl/aryl/aroyl esters and -aminoethylethers of artemisinin - Article 2

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Compounds **11**-**18** were synthesised by Krebs, H. J. (See table of consent) and compounds **19**-**29** were synthesised by Cloete, T. T.

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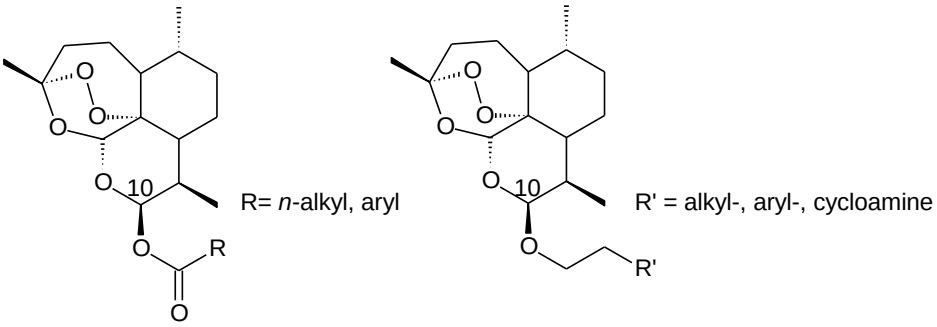
Graphical abstract



10α-n-alkyl/aryl ester **11**-**18** 10β-aminoethylethers **19**-**29**

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Abstract

A series of n-alkyl/aryl/aroyl esters were synthesized and their *in vitro* antiplasmodial activity was measured alongside that of previously synthesized aminoethylethers of artemisinin ozonides against various strains of *Plasmodium falciparum*. The cytotoxicity against human cell lines was also assessed. The esters were synthesized in a one-step reaction by derivatization on carbon C-10 of dihydroartemisinin. Both classes were active against both the 3D7 and K1 strains of *P. falciparum*, with all compounds being significantly more potent than artemether against both strains. The majority of compounds possessed potency either comparable or more than artesunate with a high degree of selectivity towards the parasitic cells. The 10α-n-propyl **11** and 10α-benzyl **18** esters were the most potent of all synthesized ozonides, possessing a moderate (~3-fold) and significant (22- and 12-fold, respectively) potency increases against the 3D7 and K1 strains, respectively, in comparison with artesunate.

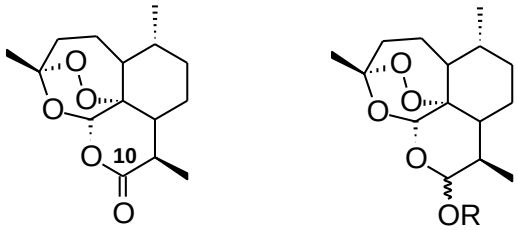
Keywords: Ozonide, artemether, artesunate, *Plasmodium falciparum*, cytotoxicity

4.1 Introduction

Each year roughly 800,000 people die of malaria, with 95% being African children [1]. The development and spread of multidrug resistant (MDR*) Plasmodium falciparum* has led to the adoption of artemisinin-based combination therapies (ACTs) as the first-line treatment for *falciparum* malaria in most malaria-endemic countries of the world [2]. However, the recently confirmed emergence of artemisinin resistance in western Cambodia is a major threat for current initiatives to control and eliminate malaria [3-5]. While artemisinin resistance has not yet spread to other areas [6], the World Health Organization (WHO) is coordinating a large-scale elimination campaign in this region aiming to contain the spread of resistance [7,8].

The ACTs combine fast-acting artemisinin (ART) derived drugs with other antimalarials possessing longer half-lives such as mefloquine. Because the utility of artemisinin is limited by its solubility in both oil and water, this sesquiterpene has been structurally modified by derivatization into short-chain oil soluble ether derivatives of dihydroartemisinin (DHA, **2**), such as artemether (AM, **2a**) and arteether (AE, **2b**), and the water soluble sodium artesunate (AS, **2c**) (Figure 1).

Thus, all derivatives currently in use are either alkyl acetals or an ester acetal derivative of dihydroartemisinin (DHA, **2**). The problem with these semi-synthetic compounds, however, is their short pharmacological half-lives, a reflection of their acid lability, and facile metabolism to DHA [9-12]. In particular, AS is hydrolytically unstable, even at neutral pH, and has a half-life of only a few minutes [13].



Artemisinin 1

2: R = H
2a: R = CH_3
2b: R = C_2H_5
2c: R = $C(O)CH_2CH_2COONa$

Figure 1: Structures of artemisinin **1** and its clinically used derivatives; dihydroartemisinin **2**, artemether **2a**, arteether **2b** and sodium artesunate **2c**.

A large amount of work has been carried out with the aim of generating new derivatives [14-16]. Artemisone (Figure 2) is thus far the only second-generation semi-synthetic artemisinin derivative that has been found suitable for further clinical development, progressing through Phase II but showing no major benefits over existing derivatives [17]. Arterolane [18], a synthetic ozonide (Figure 2, OZ277) has a longer half-life, is fast acting, well tolerated, and effective. It is now in Phase III

clinical trials in combination with piperaquine [19,20]. OZ439 (Figure 2), a purely synthetic ozonide, is a drug candidate designed to provide a single-dose oral cure in humans. It has successfully completed Phase I clinical trials, where it was shown to be safe at doses up to 1.6 g, and is currently undergoing Phase IIa trials in malaria patients [21]. In *in vitro* and *vivo* animal studies, OZ439 has been reported to have a better antimalarial drug profile than OZ277 and all other semi-synthetic artemisinin derivatives. On the basis of their structural differences, it is believed that these synthetic ozonides may replace the standard artemisinins if they become obsolete [17]. Nevertheless, the shortcomings of the current drugs, the emergence of *P. falciparum* resistance to the artemisinin class of compounds and the fact that the mechanism by which the parasite acquires resistance is still largely unknown, warrant the search for new classes or derivatives.

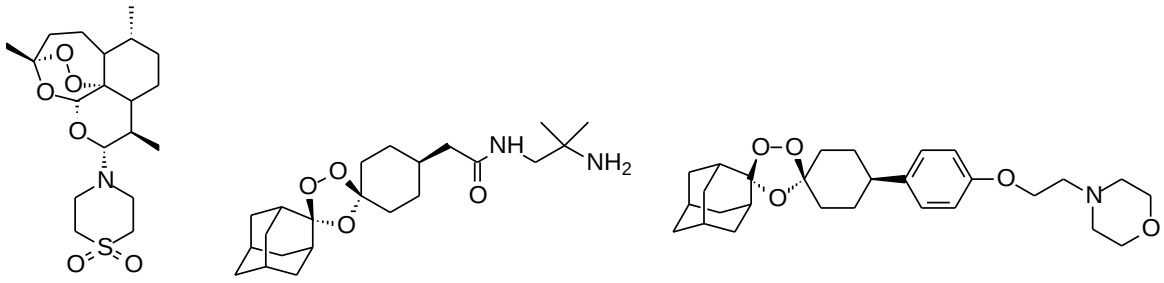


Figure 2: Structures of the semi-synthetic artemisinin derivative artemisone and the synthetic ozonide OZ277 and OZ439.

In this communication, we report the synthesis of a series of alkyl/aryl/aroyl esters and their antimalarial activity as well as cytotoxicity. Previously synthesized aminoethylethers of artemisinin were also screened alongside the esters to highlight their inhibitory potential against a variety of *P. falciparum* strains.

4.2 Materials and methods

4.2.1 *Materials*

All the acyl chlorides used in the synthesis were purchased from Fluka (Johannesburg, South Africa). Anhydrous dichloromethane was purchased from Sigma-Aldrich (Johannesburg, South Africa). Dihydroartemisinin (α and β racemates) was purchased from HuBei Enshi TianRanYuan Science and Technology Herbal Co., Ltd (Hangzhou, China). HPLC grade methanol was obtained from Labchem Ltd.

(Johannesburg, South Africa). All the reagents and chemicals were of analytical grade.

4.2.2 *General procedures*

Thin-layer chromatography (TLC) was performed using silica gel plates ($60F_{254}$ Merck). Preparative flash column chromatography was carried out on silica gel (230-240 mesh, G60 Merck) and silica gel 60 (70-230 mesh ASTM, Fluka).

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 spectrometer (at a frequency of 600.17 and 150.913 MHz, respectively) in deuterated chloroform ($CDCl_3$). Chemical shifts are reported in parts per million (δ ppm) using tetramethylsilane (TMS) as internal standard. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets), t (triplet), td (triplet of doublets), tt (triplet of triplet) and m (multiplet).

Low resolution mass spectra (LRESI) were collected on the Thermo Finnigan LXQ ion trap mass spectrometer in ESI mode. NaCl was added as a modifier to stabilize the compounds in ESI mode. The high resolution ESI (HRESI) mass spectra (MS) were recorded on a Thermo Electron DFS LXQ ion mass spectrometer in ESI mode and $[M+ Na]^+$ were recorded.

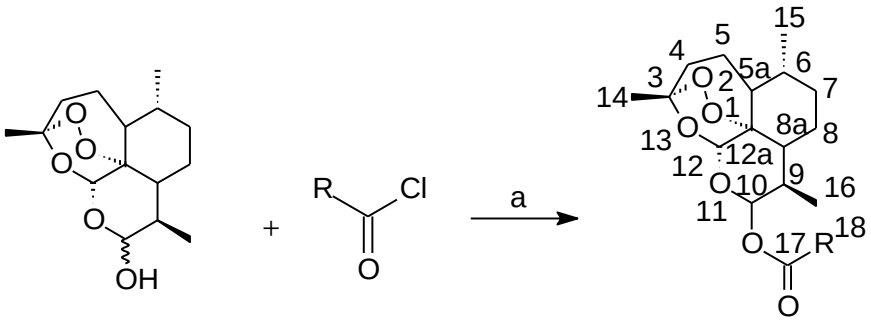
4.2.3 *High performance liquid chromatography (HPLC)*

The HPLC system consisted of an Agilent 1100 series auto sampler, Agilent 1100 series variable wave detector (VWD) and Agilent 1100 series isocratic pump. A Zorbax Eclipse XDB C18, 5 µm (150 x 4.60 mm) column was used and the Agilent Chemstation reverse A08.03 for LC systems software package for data analysis. The compounds were quantified using a gradient method (A = 0.2 % triethylamine in H_2O, pH 7.0, B = acetonitrile) at a flow rate of 1 ml/min with 20 µl standard sample injections. The gradient consisted of 25 % of solvent B (ACN) until 1 min, then increased linearly to 95 % of B after 8 min, and held for 15 min, where after the instrument was re-equilibrated to the starting conditions for 5 min. A calibration plot of peak area versus drug concentration for each compound showed excellent linearity ($0.993 < r^2 \leq 1$ over the concentration range (0-2000 µg/ml) employed for the assays. The absorption maximum for dihydroartemisinin and all its derivatives was at 205 nm; this wavelength was consequently used for the HPLC detection.

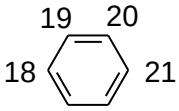
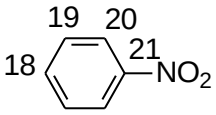
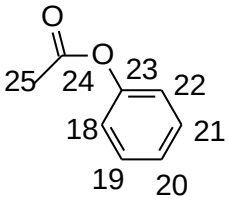
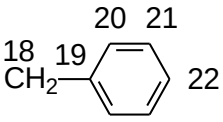
4.3 Experimental procedures

4.3.1 *Synthesis of esters 11-18*

The synthesis of artemisinin esters is depicted in the scheme 1 and was achieved as follows: dihydroartemisinin **2** (7 mmol) and acyl/aryl chlorides **3**-**10** (14 mmol, 2.0 equiv.) were dissolved in anhydrous dichloromethane (65 mL) together with triethylamine (14 mmol, 2.0 equiv.) and was stirred at room temperature for 24 h. The progress of the reaction was monitored by thin layer chromatography (TLC).



2 **3** - **10** **11** - **18**

Comp.	R	Comp.	R
11	CH_2CH_3	**15**	
12	$CH_2CH_2CH_3$	**16**	
13	$CH_2CH_2(CH_2)_4CH_3$	**17**	
14	$CH_2CH_2(CH_2)_6CH_3$	**18**	

Scheme 1: A general reaction scheme to illustrate the synthesis of 10-alkyl/aryl/aroyl esters **11-18** of artemisinin from dihydroartemisinin and various acyl/aryl/aroyl chlorides. Reagents and conditions: (a) dihydroartemisinin **2**, acyl/aryl/aroyl chlorides **3**-**10**, dichloromethane, room temperature, 24 h.

After completion, the reaction mixture was evaporated to dryness *in vacuo*. The resulting residue was purified by flash chromatography using dichloromethane. All the synthesized compounds were solids, except **13** and **14**, which were oils. ^{1}H and ^{13}C NMR chemical shifts as well as low and high resolution MS data of compounds **11**-**18** are reported.

4.3.1.1 10α-Dihydroartemisinyl-propanoate 11

Yield: 47.2%, off-white powder; mp: 70-72 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.16 (s, 1H, H-10), 5.51 (s, 1H, H-12), 2.09-1.95 (m, 2H, H-18), 2.59-1.74 (m, 5H, H-5a, -9, -8a and H-8), 2.16-1.96 (m, 2H, H-7), 1.88 (ddd, *J* = 25.2, 14.5, 10.9 Hz, 1H, H-6), 1.74-1.10 (m, 10H, H-14, -4, -5 and H-19), 0.98 (d, *J* = 10.5 Hz, 3H, H-16), 0.88 (d, *J* = 10 Hz, 3H, H-15); ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.5 (C-17), 134.94 (C-3), 108.07 (C-10), 104.48 (C-12), 68.10 (C-12a), 51.39 (C-5a), 44.39 (C-8a), 38.68 (C-4), 37.43 (C-9), 36.18 (C-7), 34.06 (C-6), 30.31 (C-18), 29.95 (C-14), 28.88 (C-8), 25.82 (C-5), 20.25 (C-15), 16.15 (C-16), 10.92 (C-19). COSY & HSQC (See annexure B). LRESI *m/z*: 363 (M+23, 50%); HRESI *m/z*: 363.1700 (M+23) (M+23, $C_{18}H_{28}O_6Na$, 363.1784 calc.).

4.3.1.2 10α-Dihydroartemisinyl butanoate 12

Yield: 18.7%, off-white powder; mp: 60-63 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.78 (d, *J* = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 2.64-2.24 (m, 3H, H-5a and H-18), 2.09-1.92 (m, 1H, H-4a), 1.90-1.81 (m, 1H, H-9), 1.8-1.15 (m, 14H, H-8a, -8, -5, 4b, -6, -7, -14 and H-19), 1.06-0.88 (m, 6H, H-16 and H-20), 0.82 (d, *J* = 7.1 Hz, H-15); ^{13}C NMR (151 MHz, $CDCl_3$): δ 172.41 (C-17), 104.42 (C-3), 91.63 (C-10), 91.47 (C-12), 80.14 (C-12a), 77.22 (C-5a), 77.00 (C-8a), 77.00 (C-4), 37.25 (C-9), 36.19 (C-7), 34.08 (C-6), 31.81 (C-18), 25.96 (C-14), 24.56 (C-8), 21.99 (C-5), 20.21 (C-15), 18.15 (C-19), 13.63 (C-16), 12.1 (C-20). COSY & HSQC (See annexure B). LRESI *m/z*: 377 (M+23, 30%); HRESI *m/z*: 377.1935 (M+23) (M+23, $C_{19}H_{30}O_6Na$, 377.1940 calc.).

4.3.1.3 10α-Dihydroartemisinyl octanoate 13

Yield: 65.7%, clear oil, ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.77 (d, J = 9.9 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 2.62–2.46 (m, 1H, H-5a), 2.42-2.27 (m, 3H, H-18 and H-4a), 2.01 (m, 1H, H-9), 1.92-1.81 (m, 1H, H-4b), 1.79-1.66 (m, 2H, H-19), 1.66-1.51 (m,

4H, H-8 and H-7), 1.49-1.36 (m, 4H, H-8a, H-6 and H-14), 1.35–1.19 (m, 10H, H-5 and H-20), 1.1-0.95 (m, 3H, H-16), 0.9-0.74 (m, 6H, H-15 and H-21); ^{13}C NMR (151 MHz, $CDCl_3$): δ 172.60 (C-17), 104.43 (C-3), 91.63 (C-10), 91.48 (C-12), 80.15 (C-12a), 77.22 (C-5a), 77.00 (C-8a), 77.00 (C-4), 37.26 (C-9), 36.22 (C-7), 34.33 (C-6), 34.09 (C-18), 31.82 (C-6), 31.63 (C-19), 29.03 (C-14), 25.96 (C-8), 24.62 (C-5), 22.57 (C-15), 22.00 (C-20), 14.05 (C-16), 12.11 (C-21); LRESI *m/z*: 433 (M+23, 100%). COSY & HSQC (See annexure B). HRESI *m/z*: 433.2662 (M+23) (M+23, $C_{23}H_{38}O_6Na$, 433.2566 calc.).

4.3.1.4 10α-Dihydroartemisinyl decanoate 14

Yield: 78.45%, clear oil, ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.77 (d, J = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 2.62-2.45 (m, 3H, H-5a), 2.42-2.27 (m, 3H, H-18 and H-4a), 2.01 (d, J = 14.5 Hz, 1H, H-4b), 1.93 – 1.83 (m, 1H, H-9), 1.79-1.66 (m, 2H, H-3'), 1.66-1.51 (m, 5H, H-6, -7 and H-7), 1.50-1.34 (m, 5H, H-8a, H-19 and H-14), 1.34-1.15 (m, 14H, H-5 and H-20), 1.05-0.90 (m, 3H, H-16), 0.9- 0.76 (m, 6H, H-15 and H-21); ^{13}C NMR (151 MHz, $CDCl_3$): δ 172.51 (C-17), 104.25 (C-3), 91.45 (C-10), 91.18 (C-12), 80.05 (C-12a), 77.96 (C-5a), 77.05 (C-8a), 77.02 (C-4), 37.96 (C-9), 36.37 (C-7), 34.48 (C-6), 34.19 (C-18), 31.92 (C-6), 31.79 (C-20), 29.25 (C-14), 25.56 (C-8), 24.54 (C-5), 21.57 (C-15), 21.00 (C-19), 14.85 (C-16), 10.11 (C-21); LRESI *m/z*: 461 (M+23, 20%). COSY & HSQC (See annexure B). HRESI *m/z*: 461.2875 (M+23) (M+23, $C_{25}H_{42}O_6Na$, 461.2879 calc.).

4.3.1.5 10α-Dihydroartemisinyl benzoate 15

Yield: 70.5%, clear crystal; mp: 115-18 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.09 (dd, J = 8.2 and 1.0 Hz, 2H, H-19), 7.60-7.48 (m, 1H, H-21), 7.42 (t, J = 7.8 Hz, 2H, H-20), 5.98 (d, J = 9.8 Hz, 1H, H-10), 5.50 (s, 1H, H-12), 2.84-2.60 (m, 1H, H-9), 2.36 (ddd, J = 17.7, 13.9 and 4.0 Hz, 1H, H-8a), 2.02 (ddd, J = 14.6, 4.6 and 3.1 Hz, 1H, H-5a), 1.94-1.57 (m, 2H, H-4), 1.57-1.16 (m, 8H, H-6, -7, -8 and H-14), 1.14-0.7 (m, 8H, H-5, -15 and H-16); ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.27 (C-17), 133.27 (C-21), 130.09 (C-18), 129.58 (C-19), 128.26 (C-20), 104.40 (C-3), 92.49 (C-10), 91.56 (C-12), 80.17 (C-12a), 51.62 (C-5a), 45.31 (C-8a), 37.25 (C-4), 36.22 (C-9), 34.09 (C-7), 31.96 (C-6), 25.93 (C-14), 24.55 (C-8), 22.03 (C-5), 20.21 (C-15), 12.21 (C-16); LRESI *m/z*: 411 (M+23, 20%). COSY & HSQC (See annexure B). HRESI *m/z*: 411.1773 (M+23) (M+23, $C_{22}H_{28}O_6Na$, 411.1784 calc.).

4.3.1.6 10α-Dihydroartemisinyl 4-nitrobenzoate 16

Yield: 94%, yellow solid; mp: 94-98 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.27 (s, 4H, H-19 and H-20), 6.00 (d, J = 9.8 Hz, 1H, H-10), 5.51 (s, 1H, H-12), 2.84-2.64 (m, 1H, H-9), 2.37 (dt, J = 14.1 and 3.7 Hz, 1H, H-5a), 1.94-1.60 (m, 2H, H-4), 1.57-1.24 (m, 8H, H-6, -7, -8 and H-14), 1.11-0.81 (m, 8H, H-5, -15 and H-16); ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.49 (C-17), 150.76 (C-21), 135.03 (C-18), 131.21 (C-19), 123.5 (C-20), 104.55 (C-3), 93.37 (C-10), 91.69 (C-12), 80.10 (C-12a), 51.58 (C-5a), 45.27 (C-8a), 37.28 (C-4), 36.20 (C-9), 34.06 (C-7), 31.89 (C-6), 25.90 (C-14), 24.56 (C-8), 22.03 (C-5), 20.20 (C-15), 12.22 (C-16); LRESI *m/z*: 456 (M+23, 40%). COSY & HSQC (See annexure B). HRESI *m/z*: 456.1808 (M+23) (M+23, $C_{22}H_{27}NNaO_8$, 456.1634 calc.).

4.3.1.7 10α-Dihydroartemisinyl 2-(acetyloxy) benzoate 17

Yield: 84.5%, off-white solid; mp: 80-83 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.09 (dd, J = 17.7 and 1.6 Hz, 1H, H-19), 7.86 (ddd, J = 17.7, 7.9 and 1.6 Hz, 1H, H-21), 7.55 (td, J = 7.9 and 1.7 Hz, 1H, H-22), 7.30 (td, J = 7.7 and 0.9 Hz, 1H, H-20), 5.98 (d, J = 9.8 Hz, 1H, H-10), 5.48 (s, 1H, H-12), 2.73-2.56 (m, 1H, H-9), 2.41-2.22 (m, 4H, H-8a and H-25), 2.09-1.96 (m, 1H, H-5a), 1.92 – 1.84 (m, 1H, H-4a), 1.80-1.54 (m, 4H, H-7 and H-8), 1.54-1.20 (m, 3H, H-6 and H-14), 0.99-0.92 (m, 3H, H-15), 0.86 (d, J = 7.1 Hz, H-3); ^{13}C NMR (151 MHz, $CDCl_3$): δ 169.93 (C-24), 163.10 (C-17), 151.01 (C-23), 134.21(C-21), 132.29 (C-19), 125.95 (C-20), 124.44 (C-22), 122.80 (C-18), 104.4 (C-3), 92.54 (C-10), 91.51 (C-12), 80.07 (C-12a), 53.42 (C-5a), 51.56 (C-8a), 45.33 (C-4), 37.29 (C-9), 36.23 (C-7), 34.09 (C-6), 32.01 (C-14), 25.56 (C-8), 21.07 (C-5), 20.22 (C-15), 12.20 (C-16). COSY & HSQC (See annexure B). LRESI *m/z*: 469 (M+23, 100%); HRESI *m/z*: 469.1833 (M+23) (M+23, $C_{24}H_{30}O_8Na$, 469.1838 calc.).

4.3.1.8 10α-Dihydroartemisinyl 2-phenylacetate 18

Yield: 40.1%, off-white solid; mp: 101-105 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.36-7.19 (m, 5H, H-20, 21 and H-22), 5.76 (d, J = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 3.77-3.55 (m, 1H, H-18), 2.64-2.43 (m, 1H, H-5a), 2.36 (td, J = 14.1 and 3.8 Hz, 2H, H-4), 1.77-1.62 (m, 1H, H-9), 1.62-1.53 (m, 8H, H-6, -7, -8 and H-14), 1.5-1.15 (m, 3H, H-16), 0.89 (d, J = 7.1 Hz, 1H, H-15); ^{13}C NMR (151 MHz, $CDCl_3$): δ 170.35 (C-17), 133.58 (C-19), 129.39 (C-20), 128.50 (C-21), 127.13 (C-22), 104.46 (C-3), 92.20

(C-10), 91.53 (C-12), 80.12 (C-12a), 51.54 (C-5a), 45.23 (C-8a), 41.28 (C-18), 37.23 (C-4), 36.20 (C-9), 34.05 (C-7), 31.85 (C-6), 25.96 (C-14), 24.56 (C-8), 21.94 (C-5), 20.19 (C-15), 11.88 (C-16); LRESI *m/z*: 425 (M+23, 40%). COSY & HSQC (See annexure B). HRESI *m/z*: 425.1938 (M+23) (M+23, $C_{23}H_{30}O_6Na$, 425.1940 calc.).

4.3.2 *Synthesis of 10-aminoethylethers of artemisinin 19-29*

The 10-aminoethylethers of artemisinin **19**-**29** of artemisinin were previously synthesized using a two-step process using a method reported by Li *et al*. [20]. The synthesis and characterization of these compounds have been reported elsewhere [22] and their structures are delineated in the Figure 3.

4.3.3 *Physicochemical properties*

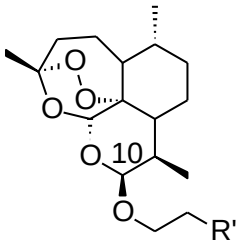
4.3.3.1 Aqueous solubility (S_W)

The aqueous solubility (S_w) of crystalline compounds **11**, **12**, and **15**-**18** as well as DHA **2** were obtained by preparing solutions in PBS (pH 5.5 & pH 7.4). The slurries were stirred in a water bath at 37 °C for 24 h. An excess of solute was present at all times to provide saturated solutions. After 24 h, the solutions were filtered and analyzed directly by HPLC to determine the concentration of solute dissolved in the solvent. For the oils **13** and **14**, after 24 h stirring, the mixtures were allowed to stand at room temperature then the aqueous layers were carefully separated and analyzed by HPLC [23]. The experiment was performed in triplicate for each compound. The average results are reported in Table 1. Furthermore, solubility in *n*-octanol was used to mimic the solubility in a lipophilic environment. The lipid solubility (SL) values were deduced from the equation $\log S_L = \log D + \log S_W$, where S_W and log D are experimental.

4.3.3.2 Distribution coefficient (log D)

Equal volumes of *n*-octanol and PBS (pH 5.5 & 7.4) were mixed with vigorous stirring for at least 24 h. 2 mg of each derivative was dissolved in 0.75 mL of this solution, the solution was then stoppered and agitated for 10 min in 2 mL graduated tubes (0.5 mL division). Subsequently 0.75 mL of pre-saturated buffer was transferred to the tubes containing the before mentioned solutions. The tubes were stoppered and agitated for 45 min after which they were centrifuged at 4 000 rpm (1503 g) for 30 min. The *n*-octanol and aqueous phases were allowed to separate at

room temperature for 5 min, where after their volume ratio (v/v; *n*-octanol:buffer) was determined. The volume ratio was found in all cases to be 1. The *n*-octanol and aqueous phases were then analyzed by HPLC. From this data, the concentrations of the derivative in both phases were determined. The log D values were calculated as logarithmic ratios of the concentrations in the *n*-octanol phase compared to the concentrations in the buffer [23]. The experiment was performed in triplicate and the results, expressed as means, are listed in Table 1. As oxalate salts, the aminoethylethers **19**-**29** were not subjected to experimental aqueous solubility and distribution coefficient determinations.



10-aminoethylethers of artemisinin **19** - **29**

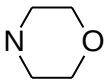
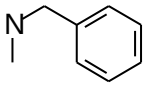
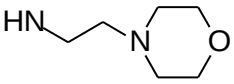
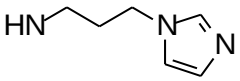
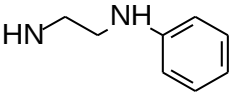
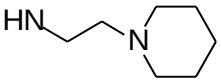
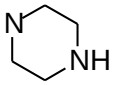
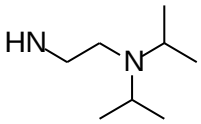
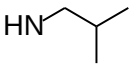
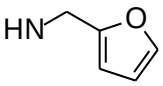
Comp.	R’	Comp.	R’
19		**25**	
20		**26**	
21		**27**	
22		**28**	
23		**29**	
24			

Figure 3: 10-aminoethylethers **19**-**29** of artemisinin obtained from dihydroartemisinin **2** and various amines.

4.3.4 *Methodology for* in vitro *biological evaluation*

4.3.4.1 Determination of antimalarial effective concentration (EC)

The esters and aminoethylethers of artemisinin were screened against the chloroquine-sensitive (CQS, 3D7) and chloroquine-resistant (CQR, K1) strains of *P. falciparum*. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen [24]. Asynchronous parasites were grown in the presence of fresh group O-positive erythrocytes (Lifeblood, Memphis, TN) in Petri dishes at a hematocrit of 4-6% in RPMI-based medium consisting of RPMI 1640 supplemented with 0.5% AlbuMAX II, 25 mM HEPES, 25 mM $NaHCO_3$ (pH 7.3), 100 µg/mL hypoxanthine, and 5 µg/mL gentamicin. Cultures were incubated at 37 °C in a gas mixture of 90% N_2, 5% O_2, and 5% CO_2. For EC_{50} determinations, 20 µL of RPMI 1640 with 5 µg/mL gentamicin were dispensed per well in an assay plate (384-well microtiter plate, clear-bottom, tissue-treated). Next, 40 nL of each compound, previously serial diluted in a separate 384-well white polypropylene plate, were dispensed in the assay plate, and then 20 µL of a synchronized culture suspension (1% rings, 10% hematocrit) were added per well to make a final hematocrit and parasitemia of 5% and 1%, respectively. Assay plates were incubated for 72 h, and the parasitemia was determined by a method previously described [25]. Briefly, 10 µL of 10X Sybr Green I, 0.5% v/v Triton, and 0.5 mg/mL saponin solution in RPMI were added per well. Assay plates were shaken for 30 s, incubated in the dark for 4 h, and then read with the Envision spectrofluorometer at Ex/Em 485 nm/535 nm. EC_{50}s were calculated using proprietary software developed in house (RISE) in the Pipeline Pilot environment that pools data from all replicates of the experiment and fits a consensus model.

4.3.4.2 In vitro *cytotoxicity against HEK293 and HEP G2 cells*

HEK 293 (human embryonic kidney) and Hep G2 (hepatocellular carcinoma) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to recommendations. Cell culture media were purchased from ATCC. Cells were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). Exponentially growing cells were plated in Corning 384 well white custom assay plates, and incubated overnight at 37 °C in a humidified, 5% CO_2 incubator. DMSO inhibitor stock solutions were added the following day to a top final concentration of 25 µM, 0.25% DMSO and then diluted 1/3 for a total of ten testing concentrations. Cytotoxicity was determined

following a 72 hour incubation using Promega Cell Titer Glo Reagent according to the manufacturer's recommendation. Luminescence was measured on an Envision plate reader (Perkin Elmer). EC_{50}s were calculated using proprietary software developed in house (RISE) in the Pipeline Pilot environment that pools data from all replicates of the experiment and fits a consensus model.

4.3.4.3 Dose-response curve fitting

Dose-response curves were calculated from percent activity values and log10-transformed concentrations, the proprietary robust interpretation of screening experiments (RISE) application written in Pipeline Pilot (Accelrys, v. 8.5) and the R program [26]. Briefly, non-linear regression was performed using the R *drc* package with the four-parameter log-logistic function (LL2.4) [27]. The data for all replicates for each compound was fit three separate times by varying the parameters that were fixed during regression: (1) all parameters free, (2) high response fixed to 100, (3) low response fixed to 0. The best fit from these three nested models was selected using the *anova.drc* function. 95% confidence intervals were produced based upon this fit.

Dose-response curves were assigned a quality score according to the following heuristic. Compounds which failed to fit to any curve, or with curves having efficacy <25% or >150% or hill slope <0.5 or >25 were designated class 'D1'. Compounds passing this first criteria with curves having efficacy <50%, calculated EC_{50} > the highest concentration tested, lower and upper EC_{50} confidence limits > 10-fold EC_{50}, or slope at the highest concentration tested >75% (non-saturating) were designated class 'C1'. Compounds passing previous criteria with curves having lower and upper EC_{50} confidence limits > 5-fold EC_{50} or slope at the highest concentration tested >25% (not completely saturating) were designated class 'B1'. All remaining curves were designated 'A1', which is indicative of ideal, well-behaved sigmoidal response. Curves that were inverted (activity decreased as concentration increased) were prefixed with the letter 'N', such as 'NA1' In tabulating data, EC_{50} values are only called for A1 and B1 class curves with a single value and error being provided for A1 curves and a range provided for B1 curves. C1 and D1 curves were assigned an arbitrary value of either greater than the highest concentration tested or lower than the lowest concentration tested, when appropriate. All the biological results are summarized in Table 2.

4.4 Results and discussion

4.4.1 *Chemistry*

The esters were obtained by acylation of C-10 of dihydroartemisinin. The configuration at that stereocenter was assigned based on the vicinal coupling constant $J_{H\text{-}9:H\text{-}10}$ [28]. A large coupling constant (9-10 Hz) is generally found for the 10α-isomer [29], indicating the relative *trans* configuration. The 10β-isomer, on the other hand has a smaller coupling constant (3.6-5 Hz) [30]. Thus, the esters **12**-**18**, all with $J_{H\text{-}9:H\text{-}10}$ values of 9.8 Hz were the 10α-isomers. Compound **11** showed no detectable coupling constant thus, the stereochemistry at the C-10 could not be deduced on the basis of that parameter.

In the ^{1}H spectra of these 10α-esters, the doublet due to resonance of H-10 appeared downfield compared to the singlet of H-12. This is as a result of the electron-withdrawing effect of the ester carbonyl. In the ^{13}C spectra, the presence of carbonyl carbon C-17 is noticeable at 170-160 ppm. The C-10, which usually resonates at ~99 ppm for DHA, was further shielded for these compounds resulting in the signal shifting up field to 92-90 ppm. All signals due to DHA's carbons were also found in the spectra of the esters confirming the presence of that moiety in their structures.

The chemical structures of the title esters **11**-**18** were further confirmed by MS analysis. LRESI data showed the molecular ions M+23 *m/z* of compounds **11**, **12**, **13**, **14**, **15**, **16**, **17** and **18** at 363, 377, 433, 461, 411, 455, 425 and 469, respectively. The HRESI-MS confirmed the exact mass of **11** [363.1700], which corresponds to the formula $C_{18}H_{28}O_6Na$, of **12** [377.1935] for $C_{19}H_{30}O_6Na$, of **13** [433.2662] for $C_{23}H_{38}O_6Na$, of **14** [461.2875] for $C_{25}H_{42}O_6Na$, of **15** [411.1773] for $C_{22}H_{28}O_6Na$, of **16** [456.1808] for $C_{22}H_{27}O_8Na$, of **17** [469.1838] for $C_{24}H_{30}O_8Na$, and of **18** [425.1938] for the formula $C_{23}H_{30}O_6Na$.

The 10-aminoethylethers **19**-**29** were all 10β-isomers [22].

4.4.2 *Aqueous solubility and log D*

Aqueous solubility and lipophilicity influence the way a molecule passes through biological membranes and barriers to ultimately enter the systemic circulation. Drugs must thus possess balanced lipophilic/hydrophilic properties to both permeate biological membranes and be taken up for systemic circulation. The logarithm of the ratio of octanol solubility *vs*. water solubility (log P) is a good measure of this balance, with values between 0 and 5 being targeted and 0-3 being ideal [31,32]. The

distribution coefficient (log D), a pH dependent version of the partition coefficient (log P), was determined experimentally in a phosphate buffer at two distinctive pH values (7.4 & 5.5) chosen to mimic the cytosolic and parasite digestive vacuole environments, respectively.

Overall the esters had aqueous solubility slightly lower than that of DHA, regardless of the medium. While this may not be significant enough to influence their relative absorption through biological membranes, it can be attributed to the fact that the hydrogen bonding polar site of DHA, is masked by a lipophilic group.

Comparison of S_W, log D, and S_L values in both media also revealed no significant differences among the esters. They all displayed comparable aqueous and lipid solubility as well as distribution coefficients in both environments. No significant structure-physiochemical property relationship could thus be deduced from this study.

Table 1: Aqueous solubility, distribution coefficients and lipid solubility of esters **11**-**18**.

The aqueous solubility (S_W) was determined in PBS (pH 5.5, 7.4) at 37 °C after incubation in a water bath with stirring for 24 h; distribution coefficient (log D) was obtained after *n*-octanol/PBS (pH 5.5; 7.4) partition, and lipid solubility (S_L) was deduced from experimental S_W and S_L

Comp.	***J* (Hz)** [a]	**Isomer**	**S_W 7.4 (µM)** [b]	**SD**	**$logD_{7.4}$** [c]	**S_L 7.4 (µM)** [d]	**S_W 5.5 (µM)** [a]	**SD**	**$logD_{5.5}$** [a]	**S_L 5.5 (µM)** [b]
DHA **2**	ND		1.2	0.09	0.1	1.5	3.2	0.08	0.3	6.4
11	ND		0.3	0.01	0.8	1.9	0.4	0.04	0.3	0.8
12	9.8	α	0.3	0.01	0.7	1.5	0.9	0.01	0.7	2.3
13	9.9	α	0.2	0.04	0.9	1.6	0.5	0.05	0.2	0.8
14	9.8	α	0.5	0.01	0.9	4.0	0.5	0.03	0.9	4.0
15	9.8	α	0.3	0.07	0.5	1.0	0.6	0.07	0.2	1.0
16	9.8	α	1.0	0.08	0.4	2.5	1.4	0.05	0.6	5.6
17	9.8	α	0.7	0.02	0.3	1.4	0.9	0.03	0.2	1.4
18	9.8	α	0.7	0.01	0.7	3.5	0.9	0.01	0.3	1.8

[a] Coupling constant (*J*) between H-10 and H-9; [b] Experimental aqueous solubility (S_W), each value represents the mean of 3 independent measurements; [c] Experimental distribution coefficient, log D (*n*-octanol: PBS, pH 5.5 & 7.4), each value represents the mean of 3 independent measurements; [d] Lipid solubility (S_L) deduced using $\log S_L = \log D + \log S_W$ where logD and S_W are experimental data; standard deviation (SD).

4.4.3 In vitro *antimalarial activity and cytotoxicity*

The esters **11**-**18** and aminoethers **19**-**29** were tested *in vitro* against both the chloroquine sensitive (CQS) 3D7, and chloroquine resistant (CQR) K1 strains of *P. falciparum* (Table 2). Both compound-types were active against both the 3D7 and K1 strains. The esters were all significantly more potent than AM, up to 60- and 5000-fold against 3D7 and K1, respectively. The aminoethylethers also displayed significantly better potency than AM but to a lesser extent, 20- and 700-fold.

In comparison with AS, however, the behaviour was quite different. Ester **14** and ethers **26** and **27** were 3-fold less potent than AS, while all other derivatives had comparable activity to AS against the 3D7 strain. This trend was also observed against the K1 strain. Ester **14** and ethers **26** and **27** were still less potent than AS but the remaining derivatives were either as active or more potent than the reference drug. Thus, against this strain, the esters were found still more active than the ethers. Compounds **11**, **15**, **16** and **18**, all esters, were distinctively the most active of all artemisinin derivatives synthesized during this study. They were significantly more potent than AS against the K1 strain with **11** and **18** possessing 22- and 12-fold improvements, respectively. No SAR could be deduced with the esters due to limited structural diversity.

In the 10-aminoethylether series, ether **29** was the most active of all. It was as potent as AS against 3D7 and possessed a moderate potency improvement (~3-fold) against K1. Ether **26**, on the other hand was the least potent. It was 3-times less potent than AS against both the 3D7 and K1 strains. The series may be divided into two sub-series *viz*. the monoamine series comprising ethers **19**, **21**, **24**, **25**, **28** and **29**, and the polyamine one including **20**, **22**, **23**, **26** and **27**. Cloete *et al*. [22] reported the monoamine sub-series to be more active than the polyamine in screening against CQS D10 and CQR Dd2 strains. This was also the case against the 3D7 and K1 strains in this study. It was found that these ethers appear more active against D10 than 3D7, and K1 than Dd2. Average 3- to 6-fold and 5- to 10-fold variances were found between activity against D10 and 3D7, and K1 and Dd2, respectively, which

shows that these strains are still different cell lines and have different behaviours in the presence of the compounds. It is, thus, noteworthy that these 10-aminoethylethers of artemisinin are active against four different strains of *P. falciparum*.

Table 2: *In vitro* antimalarial activity of esters **11**-**18** and aminoethylethers **19**-**29** of artemisinin against 3D7 and K1 strains of *Plasmodium falciparum*, and their cytotoxicity against HEK 293 and Hep G2 cells.

Cells were incubated with compounds at various concentrations for 72 h; antimalarial activity and cytotoxicity were determined using SYBR Green I-based fluorescence and CellTiter-Glo luminescent cell viability assays, respectively.

	Activity EC_{50} (nM)[a]			Cytotoxicity EC_{50} (μM)[c]			
Comp.	**3D7**	**K1**	**RI**[b]	**HEK293**	**HepG2**	10^3.**SI_1**[d]	10^3.**SI_2**[e]
11	3.1	0.3	0.1	>26.0	>26.0	>8.4	>8.4
12	13.3	6.2	0.5	>26.0	>26.0	>2.0	>2.0
13	9.4	5.0	0.5	>26.0	>26.0	>2.8	>2.8
14	21.3	17	0.8	>26.0	>26.0	>1.2	>1.2
15	3	0.9	0.3	>26.0	>26.0	>8.7	>8.7
16	3.1	1.1	0.4	>23.8	>23.8	>7.7	>7.7
17	5.1	3.3	0.7	>26.0	>26.0	>5.1	>5.1
18	2.4	0.5	0.2	>26.0	>26.0	>10.9	>10.9
19	8.7	3.1	0.4	>27.5	>27.5	>3.2	>3.2
20	14.8	6.3	0.4	>21.4	>21.4	>1.5	>1.5
21	10.1	5.5	0.6	>31.7	>31.7	>3.1	>3.1
22	11.3	6.6	0.6	>13.3	>13.3	>1.2	>1.2
23	11.3	4.1	0.4	>18.6	>18.6	>1.7	>1.7
24	7.9	3.6	0.5	>29.6	>29.6	>3.8	>3.8
25	9	5.8	0.6	>26.6	>26.6	>3.0	>3.0
26	22.1	26.1	1.2	>22.7	>22.7	>1.0	>1.0
27	23.7	10.8	0.5	>30.1	>30.1	>1.3	>1.3
28	7.9	4.0	0.5	>28.9	>28.9	>3.7	>3.7
29	6.8	2.4	0.4	>16.0	>16.0	>2.4	>2.4
AM **2a**	166	1723.3	10.4	>26.0	>26.0	>0.2	>0.2
AS **2c**	6.6	6.6	1	>26.0	>26.0	>3.9	>3.9

[a] Effective concentration of compound inducing 50% reduction in parasitic cells count; [b] Effective concentration of compound selectively inducing 50% reduction in parasitic cells count in the presence of mammalian cells; [c]Resistance index (RI) = EC_{50} K1 / EC_{50} 3D7; [d] Selectivity index (SI_1) = EC_{50} HEK293/EC_{50} 3D7; [e] Selectivity index (SI_2) = EC_{50} Hep G2/EC_{50} 3D7.

Furthermore, the aminoethylethers are hydrophilic and weak bases able to undergo protonation at lower pH value such as that occurring in the parasitic digestive vacuole (pH~5.5), and thus accumulate in higher concentration by pH-trapping mechanism. One would then expect a synergism between the action inherent to the DHA endoperoxide and the pH-trapping mechanism to render these aminoethers more active than the esters, which lack the amine(s) for pH-trapping mechanism. However, the EC_{50} data against both strains showed the opposite, confirming that the drug's absorption is dependent upon multiple physicochemical parameters [33].

To determine whether the synthesized compounds were selective in their action against the plasmodia cells, relative to host cells, a selectivity index (SI) was determined for multiple mammalian cell types. All compounds **11**-**29** proved to be non-toxic to both HEK 293 and Hep G2 cells as can be seen from the high SI_1 and SI_2 values, all above 1000.

4.5 Conclusions

A series of 10α-n-alkyl/aryl/aroyl esters were synthesized through derivatization at C-10 of dihydroartemisinin. The structures of the compounds were verified and confirmed by NMR and MS analyses. The esters were subjected to distribution coefficient and aqueous solubility experimental determinations in both weakly acid (pH 5.5) and physiological (pH 7.4) media. The esters were found moderately less hydrophilic than DHA with comparable lipophilicity, and displayed similar distribution coefficients and aqueous solubility in both media. The esters were screened for antimalarial activity alongside 10–aminoethylethers of artemisinin against the CQS 3D7 and CQR K1 strains of *P. falciparum*. Both compound types were active with the esters having a moderate upper edge over the ethers. They possessed significant potency improvement over artemether against both strains, up to 500-fold on average. In comparison to AS, the majority of compounds possessed either similar or a higher degree of potency against both strains. The 10α-n-propyl **11** and 10α-benzyl **18** esters were the most potent of all synthesized derivatives with activity comparable to that of AS against 3D7 but more importantly by their significant potency (22-and 12-fold, respectively) over AS against the resistant strain K1. Short biological half-life is a feature common to ozonides. Thus, these two esters stand as good candidates to undergo pharmacokinetic study to ascertain whether they possess half-lives longer than those of the current clinically available artemisinin derivatives.

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Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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