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Effective Antimalarial Activities of α-Hydroxy Diynes Isolated from *Ongokea gore*

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ABSTRACT

Three diynes, octadec-17-ene-9,11-diynoate ethyl (**1**), 8-hydroxy-octadeca-13,17-diene-9,11-diynoate ethyl (**2**), and 8-hydroxy-octadec-13-ene-9,11-diynoate ethyl (**3**), were isolated from *Ongokea gore* seed oil. The structure assignment of these three compounds was based according to chemical and spectroscopic data. They were screened against *Plasmodium falciparum*, the parasite that causes malaria. *In vitro* microtest (Mark III, supported by the World Health Organization) was developed to assess the response of *P. falciparum* to the isolated three compounds, and statistical analysis were performed for determination of the concentration that inhibits 50 % of the parasite maturation (IC_{50}). Two of the three diynes (**2** and **3**) showed a very effective *in vitro* antimalarial activity with an IC_{50} of 4.5 and 1.7 µM, respectively. Compound **3** exhibited better activity than quinine (IC_{50} 1.9 µM), the drug reference, while compound **1** had no antimalarial activity (IC_{50} > 125 µM). In the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity screening, all compounds showed no toxicity (mean IC_{50} of 90 µM for each compound).

Introduction

In the world, malaria is the second most common cause of mortality due to infectious diseases, after tuberculosis. It is also the most significant parasitic disease worldwide transmitted to humans by mosquito bites [1, 2]. According to the World Health Organization (WHO) world malaria report of 2016 [3], 100–300 million people contracted malaria worldwide in 2015 and about 429 000 died, mostly young children under the age of five. Pregnant women are more susceptible to malaria than the general population as pregnancy reduces a woman's immunity to malaria, making her more likely to become infected and develop severe complications, and increases the risk of severe anemia, hypoglycemia, and death. Malaria contributes very significantly to maternal and fetal mortality [4, 5].

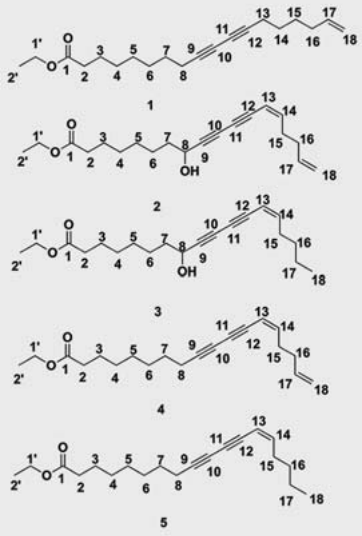
Malaria is caused by several *Plasmodium* species. There is currently no long-term effective vaccine for the prophylaxis of this disease, and the *Plasmodium* vectors (female anopheles mosquitos) have developed resistance to existing insecticides [6, 7]. Moreover, growing resistance to available antimalarial medicines has spread very rapidly, undermining malaria control efforts [8, 9]. The urgent need for effective antimalarial drugs has motivated the search for new compounds, of both natural and synthetic origin, with potential clinical utility [10, 11].

1,3-diynes, abbreviated as diynes, are bioactive compounds with known effects on human physiology and diseases [12]. They constitute a very structurally diverse and useful class of compounds. Indeed, over the past 50 years, hundreds of compounds containing diyne moieties have been isolated from natural sources, and many of these have shown biological activities ranging from antituberculosis [13], antifungal [14], anti-inflammatory [15], anti-HIV [16], antitumor [17], anticancer [18], antiprotozoal [19], insecticidal [20], or antilarvicidal [21] activities. Conjugated diynes play also an important role in the construction of macrocy-

clic annulenes, polymers, and carbon-rich materials [22–24]. Several studies on their isolation and synthesis have been reported. In 2006, Tykwinski et al. [25] reported a review on the artificial synthesis of naturally occurring diynes. Their study showed that the Cadiot-Chodkiewicz coupling is a method of choice for the construction of natural acetylenic compounds. Recently, Shi [26] enriched the Tykwinski study by reporting isolation of natural diynes in plant species, fungi, bacteria, marine sponges, and corals, which were uncovered in previous reviews. This paper also gave an overview of the synthetic route and applications of a 1,3-diyne structure.

In a previous work [27], we reported isolation and identification of five diynes from the *Ongokea gore* (Hua) Pierre (Olacaceae) seeds oil (▶ **Fig. 1**). Our interest on the underutilized *O. gore* seeds for possible development and application is motivated by the high demand and economic importance of seeds oil in the chemical industry.

We herein report on isolation of polyacetylenes **1**–**3** from the *O. gore* seeds oil (also named isano seeds oil) and the study of their antimalarial and cytotoxicity activities (▶ **Fig. 1**).



▶ **Fig. 1** Fatty Acids (as ethyl esters) isolated from isano oil.

Results and Discussion

In order to determine its fatty acid composition, the yellowish extracted isano seed oil (55 %), was transesterified with EtOH to yield 80 % of fatty acid ethyl esters (FAEE). Flash column chromatography on silica gel of these latter gave four fractions (A, B, C, D). Further purification of fraction A by semipreparative HPLC gave three polyacetylenes: **1** (25 mg), **4** (13 mg), and **5** (10 mg). Fraction B under similar conditions gave polyacetylenes **2** (15 mg) and **3** (11 mg) (▶ **Fig. 1**).

Elemental analysis of compound **1**, a yellowish syrup, gave, by HRMS the molecular formula of $C_{20}H_{30}O_2$ (*m/z* 303.23188, $[M + H]^+$). The IR spectra showed absorption bands characteristic to saturated hydrocarbon moieties (2954, 2933, 1446, and 1373 cm^{-1}), vinylic bonds (1641 cm^{-1}), acetylenic bonds (2148 cm^{-1}), and carbonyl esters (1731 cm^{-1}). The ^{13}C NMR spectrum (see ▶ **Table 1**) shows 20 resonances corresponding to the molecular formula derived from HRMS-APCI. The carbon atom at δ 174.0 ppm corresponds to an ester carbonyl group. The presence of a double bond is supported by the observation of signals at 114.7 (C-18) and 138.6 (C-17) ppm. The conjugated diyne moiety is evidenced by signals at 65.4, 65.5, 77.5, and 77.6 ppm. The placement of the two conjugated acetylenic bonds at the 9 and 11 positions, respectively, is further supported by the peaks in MS/MS at *m/z* 257, 215, 145, and 131 corresponding to the loss of 46, 88, 158, and 172 mass units from the molecular ion $[M + H]^+$. The suggestion of a diyne structure is in agreement with the acetylene bands in the IR spectrum and comparison with literature data [28]. Information from the heteronuclear multiple-quantum correlation (HMQC) cross-peaks and the corresponding ^1H NMR integrals indicate one vinyl and one methyl groups. The ^1H NMR spectrum (▶ **Table 1**) shows one triplet for one methyl resonance, two methylenes (one quadruplet and one multiplet), and 2 broad multiplets at δ 1.31–1.26 ppm corresponding to 14 protons reminiscent of an aliphatic chain. The presence of a terminal double bond is supported by the observation of one doublet of doublet at 4.97 ppm (H-17), one doublet of doublet at 5.02 ppm, and one doublet of doublet of triplet at 5.79 ppm (2 H-18). The COSY spectrum (▶ **Fig. 2**) reveals two partial structures, a C-1′ to C-2′ residue and a C-16 to C-18 chain containing one vinyl moiety (C-17 and C-18) and one methylene group (C-16). The ^{13}C NMR resonances were correlated with the corresponding ^1H resonances by an HMQC experiment, and the structural fragments are linked using long-range heteronuclear correlations (HMBC experiment, see ▶ **Fig. 2**). A key correlation is observed between the carbonyl resonance at δ_C 174.0 (C-18) and the methylene proton resonance at δ_H 4.12 (2 H-1′) over three bonds, indicating an ethyl ester. Thus, the most likely structure of **1** is octadec-17-ene-9,11-diynoate ethyl (**1**).

Similarly, spectroscopy analysis on the two other polyacetylenes led to identification of, 8-hydroxy-octadeca-13,17-diene-9,11-diynoate ethyl (**2**) and 8-hydroxy-octadec-13-ene-9,11-diynoate ethyl (**3**). Contrary to compound **1**, compounds **2** and **3** are chiral, with one stereogenic center in each. However, polarized light did not rotate for both compounds, indicating that they were present as racemate.

In vitro antimalarial activity of diynes **1**, **2**, and **3** was assessed against *Plasmodium falciparum* Welch (Plasmodiidae). The study took place in Kinshasa, Democratic Republic of Congo (DRC), where the prevalence of malaria is hyperendemic. The method used for the evaluation of antimalarial activity is the schizont maturation test (supported by the WHO) [29, 30]. The results are presented as inhibition percentage of trophozoites maturation into schizonts (▶ **Table 2**). Blood containing parasite without treatment and quinine was used as positive control and reference drug, respectively.

The concentration that inhibited 50 % of parasite growth (IC_{50}) was estimated from these results and calculated by interpolation between the two adjacent drug concentrations above and below the 50 % incorporation line via Origin 6.1 software [19]. Obtained

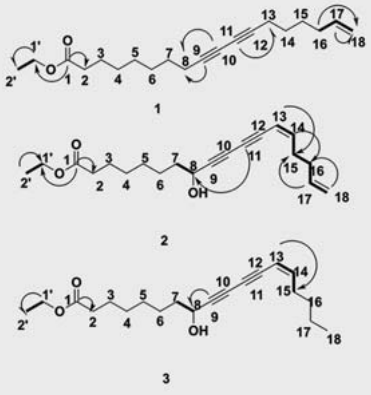
▶ **Table 1** ^{1}H and ^{13}C NMR data for **1**, **2**, and **3**.

Position	1		2		3	
	δ_H (*J* in Hz)	δ_C (mult.)	δ_H (*J* in Hz)	δ_C (mult.)	δ_H (*J* in Hz)	δ_C (mult.)
1		174.0 (C)		173.9 (C)		173.7 (C)
2	2.22–2.29 m	34.4 (CH_2)	2.29 t 7.5	34.2 (CH_2)	2.26–2.38 m	34.4 (CH_2)
3	1.30–1.52 m	25.0 (CH_2)	1.33–1.73 m	24.9–29.8 (CH_2)	1.33–1.75 m	22.3–31.0 (CH_2)
4	1.30–1.52 m	27.8–29.1 (CH_2)	1.33–1.73 m	24.9–29.8 (CH_2)	1.33–1.75 m	22.3–31.0 (CH_2)
5	1.30–1.52 m	27.8–29.1 (CH_2)	1.33–1.73 m	24.9–29.8 (CH_2)	1.33–1.75 m	22.3–31.0 (CH_2)
6	1.30–1.52 m	27.8–29.1 (CH_2)	1.33–1.73 m	24.9–29.8 (CH_2)	1.33–1.75 m	22.3–31.0 (CH_2)
7	1.30–1.52 m	27.8–29.1 (CH_2)	1.33–1.73 m	37.6 (CH_2)	1.33–1.75 m	37.6 (CH_2)
8	2.22–2.29 m	19.2 (CH_2)	4.48 t (6.5)	63.2 (CH)	4.48 t (7.1)	63.1 (CH)
9		65.4 (C)		83.7 (C)		83.4 (C)
10		65.5 (C)		69.3 (C)		67.6 (C)
11		77.6 (C)		77.4 (C)		77.4 (C)
12		77.5 (C)		75.9 (C)		76.2 (C)
13	2.22–2.29 m	19.3 (CH_2)	5.51 m	108.4 (CH)	5.48 m	107.8 (CH)
14	1.30–1.52 m	27.8–29.1 (CH_2)	6.10 m	147.9 (CH)	6.05 dt (10.9, 7.6)	149.2 (CH)
15	1.30–1.52 m	27.8–29.1 (CH_2)	2.19 m	30.1 (CH_2)	2.26–2.38 m	32.0 (CH_2)
16	2.06 m	33.3 (CH_2)	2.44 m	32.9 (CH_2)	1.33–1.75 m	22.3–31.0 (CH_2)
17	5.9 ddt (6.7, 10.2, 16.9)	138.6 (CH)	5.81 m	137.6 (CH)	1.33–1.75 m	22.3–31.0 (CH_2)
18	5.00 dd (1.2, 2.2, 10.2)	114.7 (CH_2)	5.03 m	115.3 (CH_2)	0.91 t (7.1)	14.0 (CH_3)
1′	4.12 q (7,1)	60.2 (CH_2)	4.12 q (7.1)	60.4 (CH_2)	4.13 q (7.1)	60.1 (CH_2)
2′	1.25 t (7.1)	14.4 (CH_3)	1.25 t (7.1)	14.4 (CH_3)	1.25 t (7.1)	14.4 (CH_3)

values are shown in ▶ **Table 3**. IC_{50} values above 5 µg/mL were considered as inactive according to the Research Initiative on Traditional Antimalarial Methods [31]. IC_{50} determinations as dose-response curves as well as the mathematical method of determination of the values are available as Supporting Information.

Observed IC_{50} show that the two compounds **2** and **3** display a very effective antiplasmodial activity with IC_{50} values of 4.5 and 1.7 µM (respectively 1.33 and 0.51 µg/mL); compound **3** exhibit a lower IC_{50} than that of standard quinine (1.9 µM). In contrast, compound **1** is the least active against *P. falciparum* (IC_{50} > 125 µg/mL).

These results suggest that the hydroxyl group at C-8 is an important functional group for the antimalarial activity of compounds **2** and **3**, probably because it increases the polarity and solubility of these compounds as indicated by respective molar refractivity and LogP (▶ **Table 4**). Another contributing factor may be the nonlinear shape of compounds **2** and **3** due to the presence of a cis double bond in the chain; compound **1**, which does not possess an internal double bond, shows a linear structure (see ▶ **Fig. 3**). This analysis is also in agreement with results obtained by Senn et al. in their study of antiprotozoal activities of some conjugated diynols from Tanzanian medicinal plant *Cussonia zimmermannii* Harms (Araliaceae) [19]. *In vitro* data showed that hydroxyl group in these isano oils compounds would increase hydrophilic properties of them and facilitate their absorption into parasite cell



▶ **Fig. 2** Key ^{1}H-^{1}H COSY (–) and ^{13}C-^{1}H HMBC (→) correlations for compounds **1**, **2**, and **3**.

given the amphiphilic nature of plasmic membrane of the latter. In response, it increases their respective antiplasmodial activity after reaching target proteins [32,33]. This may explain the high antiplasmodial activity of compound **2** and **3** compared to compound **1**.

▶ **Table 2** Diynes inhibition of trophozoites maturation into schizonts (in percentage).

Concentration µg/mL	1	2	3	Quinine
125	4.67 ± 4.51	96.33 ± 2.89	95.67 ± 4.51	100.00 ± 0.00
62.5	1.33 ± 1.53	91.00 ± 4.58	87.33 ± 2.52	99.67 ± 0.58
31.25	0.67 ± 1.41	86.67 ± 5.68	82.00 ± 6.71	96.67 ± 4.16
15.625	0.65 ± 0.02	78.00 ± 3.61	74.67 ± 5.13	95.00 ± 3.00
7.81	0.33 ± 0.58	70.67 ± 2.78	70.33 ± 4.04	88.00 ± 1.00
3.9	0.00	64.67 ± 3.18	67.00 ± 7.07	73.00 ± 2.55
1.95	0.00	52.33 ± 2.52	62.33 ± 2.52	69.00 ± 3.61
0.98	0.00	46.67 ± 8.04	57.00 ± 4.64	59.00 ± 2.65
0.49	0.00	37.00 ± 3.46	48.00 ± 2.00	46.33 ± 3.05

▶ **Table 3** Half maximal inhibitory concentration (IC_{50}) of compounds **1**, **2**, and **3**.

Compounds	IC_{50} µM (µg/mL)
1	– (> 125)
2	4.5 (1.33)
3	1.7 (0.51)
Quinine	1.9 (0.62)

▶ **Table 4** Predicted physicochemical properties of diynes **1**–**3**[a].

Molecule	Formula	log P	Molar refractivity (cm^3/mol)
1	$C_{20}H_{30}O_2$	5.47	90.00
2	$C_{20}H_{28}O_3$	4.39	97.62
3	$C_{20}H_{30}O_3$	4.66	97.57

[a] These predictions were made using ChemDraw Ultra 12.0 software

The cytotoxic activity of compounds **1**–**3** was evaluated and expressed as an IC_{50} (▶ **Table 5**). Since this test takes place in an aqueous medium, the three compounds were first saponified because most esters are insoluble in water. The saponification was carried out according to the method proposed by Fontanel [34]. Cytotoxic assay was assessed, as previously described [35, 36], against the proliferation of a limited panel of cancer cell lines displaying sensitivity to proapoptotic stimuli, including the mouse B16F10 melanoma, the human prostate PC-3, the breast MCF-7, and the colon LoVo carcinoma cells and against a panel of cancer cell lines displaying resistance to proapoptotic stimuli, including the human U373 and T98G glioma, the SKMEL-28 melanoma, and the A549 non-small-cell lung carcinoma cells. The cells were cultured for 3 d in the absence (control) or the presence of the isolated compounds.

Interestingly, all the compounds show no or very low growth inhibition activity (mean IC_{50} > 90 µM) from the above eight cancer cell lines. These results indicate that antiparasitic activity observed for compounds **1**–**3** might be specific to parasites of interest. Given the excellent *in vitro* antimalarial activities observed (IC_{50} 4.5 and 1.7 µM, respectively) for diynols **2** and **3** isolated from *O. gore* seed oil, these two compounds could be used as lead for the development of novel antimalarial drugs. Indeed, the absence of cytotoxicity confirms the relevant use of these two compounds as potential antimalarial drugs. Docking study indicated that hydroxyl group at C-8 is essential for high antimalarial activity. Compound **1**, without hydroxyl group, is inactive (IC_{50} > 125 µg/mL). To the best of our knowledge, this is the first report on the evaluation of antimalarial activities of these diynes against *P. falciparum*. Further work is being done to identify the mechanism of action of these active diynes. Our results show that *O. gore* plant could be considered as a potential asset in the seed oil for pharmaceutical application.

Materials and Methods

Materials

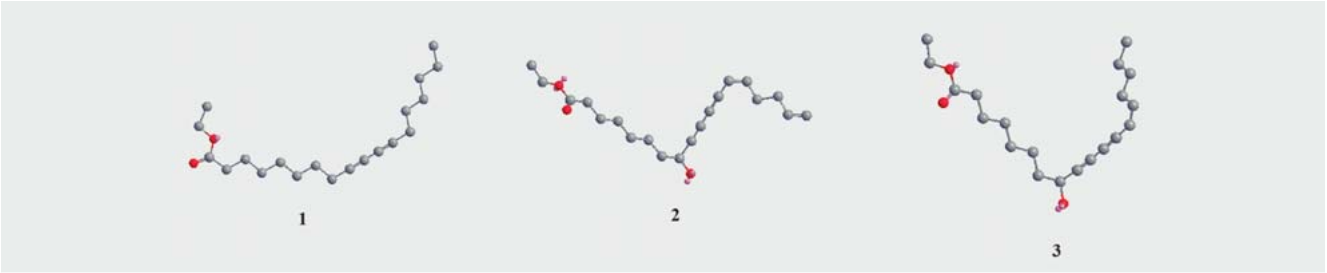
O. gore fruits were collected from the Eala botanical garden in Mbandaka, DRC. A voucher specimen was deposited in the herbarium of the Institut National de Recherches Agronomiques, Departement of Biology, Faculty of Sciences, University of Kinshasa, DRC, and the material was authenticated by Anthony Kikufi (n° MM/AK/215).

Analytical grade chemicals and solvents were used without further purification.

Methods

Extraction of the oil

The seeds were removed from the fruits, dried at room temperature, and then de-husked and ground into powder in a blender. The oil was obtained from the milled seeds by a solvent extraction process (maceration), using cyclohexane at room temperature for 48 h, followed by filtration through Whatman Filter Paper No. 4. At the end of filtration, the oil was recovered by evaporation of the solvent under reduced pressure. The oil was expressed in



▶ **Fig. 3** 3D structures of compounds **1**–**3**.

▶ **Table 5** IC_{50} (μM) *in vitro* growth inhibitory concentrations (MTT test).

Compounds	Glioma		Carcinoma				Melanoma	
	U373	T98G	A549	LoVo	MCF-7	PC-3	SKMEL-28	B16F10
1	> 100	> 100	> 100	95	> 100	> 100	> 100	75
2	> 100	> 100	> 100	83	> 100	> 100	> 100	70
3	> 100	> 100	> 100	97	> 100	> 100	> 100	69

terms of mass percentage of the samples and calculated as follows:

Total yield % (w/w) = [(Mass of oil extracted (g)/Mass of seeds (g)] × 100

Fatty acid composition

The chromatographic techniques revolutionized the study of lipids by making it possible to determine the complete fatty acid composition of a lipid in a very short time [37, 38]. In our study, we used HPLC because of the presence UV chromophores.

In order to improve resolution, detection, and recovery, natural lipids may be subjected to derivatization, such transesterification, prior to HPLC [38, 39].

Transesterification of oil to FAEE [40, 41]. The oil was transesterified with EtOH to FAEE according to a modified Christie's method [37]: into a 100-mL round bottom flask equipped with a thermostat, a mechanical stirring, a sampling outlet, and a condensation system, 1 g of sample was dissolved in dry EtOH (50 mL) and 0.45 g of sodium ethoxide (approximately six equivalents to oil) was added. The mixture was stirred at 70 °C for 1 h and then cooled to room temperature. The solvent was evaporated under reduced pressure, and 30 mL of ethyl acetate and 40 mL of water were added to the residue. Neutralization of the FAEE mixture was then performed with a few drops of aqueous HCl 1 N, followed by extraction with ethyl acetate (three times). The organic extract was dried over anhydrous magnesium sulfate, and the solvent was removed under reduced pressure.

Isolation. Fractionation using a combination of chromatography on silica gel and semipreparative HPLC [42] was realized as follows: the obtained mixture (FAEE) was subjected to fractionation by flash column chromatography using a gradient of cyclohexane/ethyl acetate (98 : 2 → 0 : 100) as mobile phase and 60–200-μm silica gel as stationary phase (crude/silica w/w ratio of ca. 50). Fractions obtained (A, B, C, D) were further purified using semipreparative HPLC separation with an isocratic reversed phase HPLC method. The chromatographic system (Waters 600) consisted of a quaternary solvent delivery pump, a degasser, an auto-injector, a column, and a UV detector. The chromatographic column (250 mm length and internal diameter of 10 mm) was filled with a 5 μm particle size C18 Alltech Adsorbosphere UHS stationary phase. The flow rate was set at 4 mL/min with an injection volume of 100 μL. The mobile phase consisted of a homogenous mixture of CH_3CN and H_2O (60 : 40–70 : 30, v/v).

Structural determination. Identification of fatty acids ethyl esters [39] was carried out using IR, NMR (1D and 2D), MS, and HRMS analysis. IR spectra were recorded on Shimadzu's FTIR-8 400 S apparatus. The samples were analyzed as films deposited on a ZnSe crystal. Wavenumber values (ν) are given in cm^{-1}. NMR spectra (^1H, ^{13}C, COSY, HMQC, HMBC), were recorded in $CDCl_3$ on a Bruker Avance at 500 MHz spectrometer for ^1H NMR and at 125 MHz for ^{13}C NMR. Chemical shifts (δ) are given in parts per million downfield from internal tetramethylsilane. Mass spectra (MS and HRMS) were obtained on a Finnigan LCQ spectrometer using the APCI ionization mode. Optical rotations [α_D] were measured with an ATAGO Polax 2000 polarimeter.

Ethyl octadec-17-ene-9,11-diynoate (**1**): Yellowish, viscous oil. Yield 25 mg obtained after semipreparative HPLC from 100 mg of fraction A obtained after column chromatography (Rf 0.8). FT-IR (cm^{-1}): 2954, 2933, 2422, 2363, 2148, 1731, 1641, 1446, 1373, 1182, 1095, 1035, 993, 912, 856, 732. NMR ^1H ($CDCl_3$, 500 MHz): see ▶ **Table 1**. NMR ^{13}C ($CDCl_3$, 125 MHz): see ▶ **Table 1**. MS (APCI): m/z = 335 $[M + CH_3$ and $OH]^+$, 319 $[M + OH]^+$, 303 $[M + H]^+$, 257 $[M - C_2H_5O]^+$, 171 $[M - C_2H_{11}]^+$, 131 $[M - C_{10}H_{19}O_2]^+$. HRMS (APCI) m/z found 303.23188; calcd for $C_{20}H_{31}O_2$ 303.23186.

Ethyl 8-hydroxy-octadeca-13,17-diene-9,11-diynoate (**2**): Yellowish, viscous oil. Yield 15 mg obtained after semipreparative HPLC

from 100 mg of fraction B obtained after column chromatography (Rf 0.4). FT-IR (cm^{-1}): 3627, 3500, 2923, 2607, 2352, 1791, 1708, 1703, 1642, 1463, 1253, 1184, 1031, 912, 734. NMR ^1H ($CDCl_3$, 500 MHz): see ▶ **Table 1**. NMR ^{13}C ($CDCl_3$, 125 MHz): see ▶ **Table 1**. MS (APCI): $m/z = 360$ $[M + H_2O]^+$, 333 $[M + OH]^+$, 331 $[M + CH_3]^+$, 299 $[M - OH]^+$, 170 $[M - C_{10}H_9]^+$, 129 $[M - C_{10}H_{18}O_2]^+$, 105 $[M - C_{12}H_{18}O_2]^+$, 81 $[M - C_{14}H_{18}O_2]^+$. HRMS (APCI): m/z found 299.20064; calcd for $C_{20}H_{27}O_2$ 299.20056.

Ethyl 8-hydroxy-octadec-13-ene-9,11-diynoate (**3**): Yellowish, viscous oil. Yield 11 mg obtained after semipreparative HPLC from 100 mg of fraction B obtained after column chromatography (Rf 0.4). FT-IR (cm^{-1}): 3665, 2925, 2858, 2583, 2357, 2080, 1712, 1638, 1456, 1353, 1217, 1199, 1054, 1013, 727, 686. NMR ^1H ($CDCl_3$, 500 MHz): see ▶ **Table 1**. NMR ^{13}C ($CDCl_3$, 125 MHz): see ▶ **Table 1**. MS (APCI): $m/z = 333$ $[M + CH_3]^+$, 301 $[M - OH]^+$, 131 $[M - C_{10}H_{18}O_2]^+$, 107 $[M - C_{12}H_{18}O_2]^+$. HRMS (APCI): m/z found 301.21625; calcd for $C_{20}H_{29}O_2$ 301.21621.

Antimalarial evaluation

Micro *in vitro* test [29,30] was used to assess the response of *P. falciparum* to compounds of interest. This method evaluates baseline sensitivity by monitoring the drug response of *P. falciparum* to drugs of interest. This is an *in vitro* method known as *ex vivo* by the use of human malarial blood instead of an isolated strain of *P. falciparum*. This technique consists of measuring the ability to inhibit the transformation of young trophozoites (first stage of *P. falciparum* in red blood) into schizonts (trophozoites having undergone multiple asexual division) at increasing doses of a drug by counting healthy schizonts for about 200 trophozoites after incubation of malarial blood in the presence of drugs in microtitration plates. The patient (a male child of four years old) included in the study had a monoinfection with *P. falciparum* with a parasitemia of 4000 asexual parasites per microliter of blood. Before collection the blood sample, oral consent was obtained from his parents. These latter denied having given him any antimalarial medication during the preceding 4 wk. From the patient, 0.2 mL of blood was collected by finger prick using sterile heparinized capillary tubes and mixed with 1.8 mL Roswell Park Memorial Institute 1640 medium previously buffered with 25 mM Hepes and 25 mM $NaHCO_3$. Aliquots (50 µL) of the blood mixture were distributed to each well of the microtitration plates and pre-dosed with various several drug quantities dilutions in EtOH: 125, 62.5, 31.25, 15.625, 7.81, 3.9, 1.95, 0.98, and 0.49 µg/mL drugs/well. Controls wells without drug were included in the assay. The test plates were placed in a candle jar and incubated at 37 °C for 24–32 h. At the end of incubation, thick blood smears were prepared and stained with Giemsa (1%) for 10 min. For each sample drug concentration tested, the number of schizonts per 200 asexual parasites was determined under the microscope. Isolates with a schizont maturation of less than 10% in the control wells were not used for evaluation. In the tests, only schizonts with a normal morphology and at least eight nuclei were counted. The schizonts maturation percentage in each well was calculated by dividing the schizonts count per 200 trophozoites in the control well and multiplied by 100, and the inhibition of schizont maturation percentage was calculated by subtracting the percentage of maturation from 100. The results were statistically evaluated using log-dose response probit analysis (Origin 6.1) for determining the concentration that inhibited 50% of the parasite maturation. Tests were assessed in triplicate.

Cytotoxicity evaluation

The colorimetric MTT assay was performed to determine the concentration that reduced the whole cell population by 50% after 72 h of exposure to the compound as described in the literature [35,36]. This test allows evaluating the number of living cells that are able to transform the yellow product 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into the blue formazan dye. The amount of formazan obtained at the end of the experiment, measured via spectrophotometry, is directly proportional to the number of living cells. Cytotoxicity assays of compounds **1–3** were conducted against seven human and one mouse cancer cell lines. The cancer cell lines were obtained from the American Type Culture Collection (ATCC) or the European Collection of Cell Culture (ECACC). The glioma cell lines were the U373 cell line (ECACC code 89081403) and the T98G cell line (ATCC code CRL-1690). The carcinoma cell lines were the A549 non-small-cell carcinoma cell line (ATCC code CCL185), the MCF-7 mammary breast carcinoma cell line (ATCC code HTB-22), the LoVo colon cancer cell line (ATCC code CCL-229), and the PC-3 prostate cancer cell line (ATCC code CRL-1435). The melanoma cell lines were the mouse B16F10 cell line (ATCC code CRL-6475) and the human SKMEL-28 cell line (ATCC code HTB-72). To perform the assay, cells were grown in a 96-well, flat-bottomed plate in 100 µL of medium. Each cell line was seeded in its appropriate culture medium. Each experimental condition was conducted in sextuplicate.

Supporting Information

IC_{50} determinations as dose-response curves as well as the mathematical method of determination of the values are available as Supporting Information.

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Conflict of Interest

The authors declare no conflicts of interest.

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