Anti-plasmodial and Cholinesterase Inhibiting Activities of some Constituents of *Psorospermum glaberrimum*

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Glaberianthrone (1), a new bianthrone was isolated from the hexane extract of the stem bark of *Psorospermum glaberrimum* together with thirteen known compounds: 3-geranyloxyemodin anthrone (2), friedelan-3-one (3), 3-prenyloxyemodin anthrone (4), 3-geranyloxyemodin (5), 3-prenyloxyemodin (6), friedelan-3-ol (7), acetylvismione D (8), betulinic acid (9), 2-geranylemodin (10), bianthrone A2b (11), bianthrone 1a (12), emodin (13) and 2-prenylemodin (14). The structures of the isolated compounds were established by means of spectroscopic methods. The extracts and the isolated compounds were tested *in vitro* for their anti-plasmodial activity against *Plasmodium falciparum* (chloroquine resistant strain W2) and for their acetyl- and butyrylcholinesterase inhibitory properties. The *n*-hexane extract showed good anti-plasmodial activity against *P. falciparum* W2 strain, with IC_{50} of 0.87 μg/ml. It also exhibited 65.5% and 98.2% of acetyl- and butyrylcholinesterase inhibition at 0.2 mg/ml, respectively. Compounds 2 and 8 showed the best potencies against *P. falciparum* W2 strain with IC_{50} of 1.68 μM and 0.12 μM, (0.66 μg/ml and 0.054 μg/ml) respectively. All tested compounds showed good butyrylcholinesterase inhibition activities with compound 12 displaying the best potency (IC_{50} 9.25±0.25 μM). All the tested compounds showed weak inhibitory activity against acetylcholinesterase.

Key words *Psorospermum glaberrimum*; Hypericaceae; anthranoid; anti-plasmodial; cholinesterase inhibition

Malaria is still the most devastating parasitic disease in the world, causing 2—3 million deaths every year, mostly among children under five years old and pregnant women. Africa accounts for over 90% of malaria mortality.[1)] In the last decades resistance of *Plasmodium falciparum*, the causative agent of the most severe form of the disease, to several antimalarials, especially chloroquine and antifolates, became widely disseminated, while the cost of effective treatment is prohibitive for the large majority of the populations. For these reasons, new effective and affordable antimalarials are urgently needed.[2)] In Africa and elsewhere, the use of indigenous plants plays an important role in malaria treatment.[3)] These plants are indeed an interesting source of new anti-plasmodial compounds perhaps with new mechanisms of action.

Psorospermum glaberrimum HOCHR. (Hypericaceae) is a small- to medium-sized shrub (up to 3 m high) distributed in tropical regions. Leaves and bark extracts of this plant are used in traditional medicine for the treatment of several diseases across Africa.[4)] The decoctions of leaves and bark are used to treat epilepsy, respiratory affections and external uses for skin diseases and leprosy. The extracts of the root bark are used for the treatment of severe cases of malaria. The pulped roots and bark extracts and the bark red resin powdered with dried leaves are remedies used externally for skin diseases and leprosy. The boiled bark gives a soapy product that is mixed with oil to rub on the skin or applied to sores for animals to keep away flies that cause scabies.[4)] Previous phytochemical investigations of *Psorospermum* species reported the presence of bioactive xanthones, anthraquinones, vismiones and psorolactones.[5—16)] Although some phytochemical studies have been carried out on *P. glaberrimum*, to the best of our knowledge, no investigation has been reported on its anti-plasmodial activity and inhibitory properties towards acetyl- and butyrylcholinesterase (AChE and BChE). AChE and BChE have been identified as attractive targets in the treatment of Alzheimer's dementia, myasthenia gravis, glaucoma, and in the recovery of victims of nerve agent exposure. Any chemical that inhibits cholinesterase activity increases the availability of acetylcholine to sustain nerve cell communications.[17)] Hence, cholinesterase inhibitors act as potential leads in drug discovery for nervous-system disorders.

In our continuing interest in new bioactive metabolites from Cameroonian medicinal plants, we investigated the hexane extract of stem bark of *P. glaberrimum* which showed potent anti-plasmodial activity against the W2 strain of *P. falciparum*, and good AChE and BChE inhibition *in vitro*. In this paper, we report on the isolation and structure elucidation of a new bianthrone derivative **1**, the ^{13}C-NMR data of the known bianthrone A2b (**11**) together with the anti-plasmodial and cholinesterase inhibiting activities of the isolated compounds.

Results and Discussion

The air dried stem bark of *P. glaberrimum* was extracted at room temperature with *n*-hexane, EtOAc and MeOH successively. The residues obtained after concentration of different

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extracts under vacuum were tested for their anti-plasmodial activities *in vitro* against the chloroquine-resistant strain W2. *n*-Hexane and EtOAc extracts with similar TLC profiles showed good anti-plasmodial activity with 50% inhibitory concentration (IC_{50}) of 0.87 μg/ml and 0.95 μg/ml respectively, while the MeOH extract was inactive (Table 2). The hexane extract was also tested for AChE and BChE inhibitory properties and exhibited 65.5% and 98.2% of AChE and BChE inhibition at a concentration of 0.2 mg/ml, respectively (Table 3).

The *n*-hexane extract residue was subjected to flash chromatography and repeated column chromatography to yield glaberianthrone (**1**), 3-geranyloxyemodin anthrone (**2**), friedelan-3-one (**3**), 3-prenyloxyemodin anthrone (**4**), 3-geranyloxyemodin (**5**), 3-prenyloxyemodin (**6**), friedelan-3-ol (**7**), acetylvismione D (**8**), betulinic acid (**9**), 2-geranylemodin (**10**), bianthrone A2b (**11**), bianthrone 1a (**12**), emodin (**13**) and 2-prenylemodin (**14**).[5—7,18—20)]

Compound **1** was obtained as yellow powder. It gave a positive ferric chloride test, indicating its phenolic nature. The molecular formula $C_{45}H_{46}O_8$ was deduced from its HR-ESI-MS which showed a pseudo molecular ion $[M+H]^+$ peak at *m/z* 715.32654, (Calcd 715.327095 for $C_{45}H_{47}O_8$) corresponding to 23 degrees of unsaturation. The IR spectrum exhibited strong absorption bands due to carbonyl groups at 1716 and 1619 cm^{-1} and chelated hydroxyl groups at 3397 cm^{-1}. The UV spectrum showed absorption bands at λ_{max} 230, 278 and 369 nm, which were very close to those of bianthrone derivatives.[21)] The broad band decoupled ^{13}C-NMR spectrum of compound **1** (Table 1) displayed 45 carbon signals which were sorted by distortionless enhancement by polarization transfer (DEPT) and heteronuclear single quantum coherence (HSQC) techniques into seven methyl groups, four methylene groups including one oxymethylene group at δ 65.2, twelve methine groups and twenty-two quaternary carbons, including two carbonyl groups of bianthrone at δ 190.2 and 190.4.[22)] The ^1H-NMR (Table 1) of compound **1** contained signals of four chelated hydroxyl groups at δ 12.56, 12.19, 11.80 and 11.79 and one proton, exchangeable with D_2O, at δ 6.17, seven aromatic protons between δ 5.90 and 6.62 and two aromatic methyl groups at δ 2.22 and 2.23. The ^1H-NMR spectrum of **1** also showed signals of two vicinal methine protons at δ 4.27 (1H, d, J=3.0 Hz) and 4.28 (1H, d, J=3.0 Hz), one 3,3-dimethylallyloxy moiety [δ 5.48 (1H, t, J=7.2 Hz, H-12′), 4.53 (2H, d, J=7.0 Hz, H-11′), 1.77 (3H, s, H-14′) and 1.83 (3H, s, H-15′) and one geranyl moiety [δ 5.28 (1H, t, J=7.2 Hz, H-12), 5.06 (1H, t, J=7.2 Hz, H-17), 3.44, (2H, dd, J=15.5, 7.0 Hz, H-11), 2.09—2.14 (4H, m, H-15, H-16), 1.60 (3H, s, H-19), 1.68 (3H, s, H-20) and 1.82 (3H, s, H-14)]. The presence of 3,3-dimethylallyloxy and geranyl moieties was further confirmed by the signals and different homonuclear couplings observed respectively in the ^{13}C-NMR and correlation spectroscopy (COSY) spectra.

The heteronuclear multiple bond correlation (HMBC) spectrum of compound **1** (Fig. 1) showed long-range couplings between the methylene group of the geranyl moiety at δ 3.44 (H-11) and the carbon signals at δ 161.6 (C-1), 161.3 (C-3) and 112.9 (C-2), indicating its location on the C-2 position of the anthronyl nucleus. This was further confirmed by the correlations observed between the C-1 chelated hydroxyl group (δ 12.56) and carbons C-2 (δ 112.9) and C-9a (δ 110.2) on one side and between the exchangeable hydroxyl proton at δ 6.17 and carbons C-2 and C-4 (δ 108.2) on the other side. The oxymethylene proton of the prenyloxy moiety at δ 4.53 (H-11′) gave a HMBC cross peak with the carbons at δ 164.5 (C-3′), 118.5 (C-12′) and 139.7 (C-13′) clearly indicating its attachment to C-3′. This was confirmed by long range CH correlations observed between H-2′ (δ

Table 1. ^{13}C- (125 MHz) and ^1H-NMR (500 MHz) of Glaberianthrone **1** and Bianthrone A2b (**11**) in $CDCl_3$

No.	Compound **1**		Compound **11**
	$\delta^{13}C$	δ^1H (J Hz)	$\delta^{13}C$
1, 1′	161.6, 164.8	—	164.6
2, 2′	112.9, 100.6	—, 6.37, d (1.9)	100.2
3, 3′	161.3, 164.5	—	164.7
4, 4′	108.2, 108.2	6.02, s, 6.12, d (1.9)	108.2
4a, 4a′	140.2, 139.5	—	139.9
5, 5′	120.7, 120.7	5.90, 5.92, s	120.8
6, 6′	146.8, 146.8	—	146.6
7, 7′	116.9, 116.9	6.62, 6.62, s	117.0
8, 8′	161.9, 161.8	—	161.9
8a, 8a′	114.0, 114.0		114.0
9, 9′	190.4, 190.2	—	190.2
9a, 9a′	110.2, 111.2	—	
10, 10′	56.5, 56.3	4.27, d (3.0), 4.28, d (3.0)	56.5
10a, 10a′	141.5, 143.0		143.4
11, 11′	21.9, 65.2	3.44, dd (15.5, 7.0), 4.53, d (7.0)	65.2
12, 12′	120.8, 118.5	5.28, t (7.2), 5.48, t (7.2)	118.5
13, 13′	140.2, 139.7	—	139.5
14, 14′	16.3, 18.3	1.82, 1.77, s	18.2
15, 15′	39.7, 25.8	2.09—2.14, m, 1.83, s	25.9
16	26.3	2.09—2.14, m	
17	123.6	5.06, t (7.2)	—
18	132.2	—	—
19	17.7	1.60, s	—
20	25.7	1.68, s	—
21, 21′	21.5, 21.5	2.22, 2.23, s	21.9
1-OH, 1′-OH		12.56, 12.19, s	—
3-OH		6.17, s	—
8-OH, 8′-OH		11.80, 11.79, s	—

All chemical shift assignments were done on the basis of ^1H–^1H COSY, HSQC, HMBC and DEPT NMR techniques.

Table 2. Anti-plasmodial Activity of Compounds **1**—**10** and **12** against *P. falciparum* W2 Strain

Extracts and compounds	IC_{50}	
	μg/ml	μM
n-Hexane extract	0.87	
EtOAc extract	0.95	
MeOH extract	n.a.	
1	2.10	2.94
2	0.66	1.68
3	3.28	7.70
4	0.64	1.98
5	n.a.	n.a.
6	n.a.	n.a.
7	n.a.	n.a.
8	0.054	0.12
9	2.33	5.10
10	2.17	5.34
12	1.98	2.53
Chloroquine[a)]	0.03	0.11

a) Reference drug used in this assay. n.a.: not active.

6.37), H-4′ (δ 6.12) and C-3′ (δ 164.5). The signal attributed to H-4 (δ 6.02) showed correlations with the carbons at δ 161.3 (C-3), 112.9 (C-2), 110.2 (C-9a) and a non aromatic methine carbon at δ 56.5 (C-10). The two methyl groups resonating at δ 2.22 (H-21) and 2.23 (H-21′) showed correlations with C-5 (δ 120.7), C-6 (δ 146.8), C-7 (δ 116.9) and C-5′ (δ 120.7), C-6′ (δ 146.8), C-7′ (δ 116.9) respectively, supporting their location at C-6 and C-6′ of the bianthrone skeleton. The C-10/C-10′ junction of the two anthronyl moieties was deduced from the HMBC correlations observed between the proton at δ 4.27 (H-10) and carbons C-4 (δ 108.2), C-5 (δ 120.7), C-10′ (δ 56.3) in one hand and the proton at 4.28 (H-10′) and carbons C-4′ (δ 108.2), C-5′ (δ 120.7), C-10 (δ 56.5) in other hand. A 3.0 Hz coupling between H-10 and H-10′ required these protons to have a *syn* relationship.[23,24] All these data suggested that compound **1** was an isomer of the well-known bianthrone A2a (**1a**) isolated from the root bark of *P. tenuifolium* by Delle Monache *et al.* in 1987.[20] As it was isolated from *P. glaberrimum*, we gave the trivial name glaberianthrone.

The ^{13}C-NMR data of bianthrone A2b (**11**), previously isolated from *P. tenuifolium*[20] are reported here for the first time (Table 1). This compound has no specific rotation and the signals of the two anthronyl moieties were superimposable suggesting that bianthrone A2b (**11**) was the *cis*- (*meso*-) stereoisomer.

Table 3. *In Vitro* Acetyl- and Butyrylcholinesterase Inhibiting Activities of Extracts and Compounds

Extract and compounds	% inhibition[a]		IC_{50} (μM)	
	AChE	BChE	AChE±S.E.M.[b]	BChE±S.E.M.[b]
n-Hexane extract	65.5	98.2	—	—
2	5.4	—	—	11.6±0.20
4	13.8	—	—	10.1±0.50
6	35.0	—	—	13.3±1.10
8	45.7	—	—	10.1±0.20
10	12.9	—	—	11.30±0.23
12	60.9	—	63.0±0.46	9.25±0.25
Galanthamine[c]			0.5±0.001	8.5±0.001

a) Tested at 0.2 mg/ml for extract and 0.1 mM for pure compound. *b*) Standard error of mean of five assays. *c*) Positive control used in the assays.

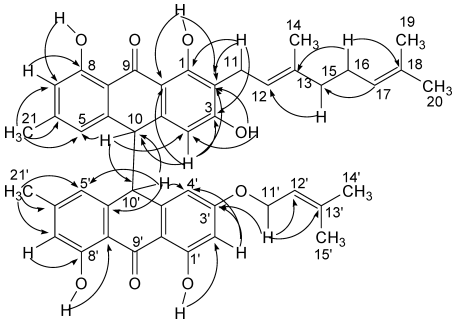

Fig. 1. Selected HMBC Correlations in Compound **1**

Compounds **1**—**10** and **12** were tested for their anti-plasmodial activity against the W2 strain of *P. falciparum*, which is resistant to chloroquine and other antimalarial agents (Table 2). They showed toxicity to erythrocytes at concentrations above 20 mM, about three orders of magnitude above concentrations with antimalarial activity. Acetylvismione D (**8**) (IC_{50} of 0.12 μM) showed the best anti-plasmodial potency. The anti-plasmodial activity of acetylvismione D against the same resistant *Plasmodium* strain K1 was previously reported.[25] Vismione H, a closely related anthranoid isolated from *Vismia guineensis* was found to be very active *in vitro* against *P. falciparum* (NF14, clone A1A9) with an IC_{50}=0.23 μM. Several prenylated anthranoids have also been reported with respect to their anti-plasmodial activity.[26]

The isolated anthranoids are derivatives of emodin **13**. Compounds **5**, **6**, and **10** with anthracene-9,10-dione skeleton were less active than the anthrones **2**, **4** or bianthrones **1** and **12**. The oxidation at C-10 seems to decrease the activity. The activities of compounds **2** and **4** were almost similar and indicated that the contribution of the C-3 *O*-prenyl or *O*-geranyl substituents for the anti-plasmodial activity was nearly equal. This was also the case for bianthrones **1** and **12** with the same basic skeleton and for which the difference of activity should be due to the position of geranyl and prenyl groups. Previously, related bianthrones were isolated from the roots of *Kniphofia foliosa* and showed *in vitro* activity comparable to that of chloroquine.[27]

Compounds **2**, **4**, **6**, **8**, **10** and **12** were also tested against the cholinesterase family of enzymes consisting of AChE and BChE which represent the most attractive target for drug design and discovery of mechanism-based inhibitors for the

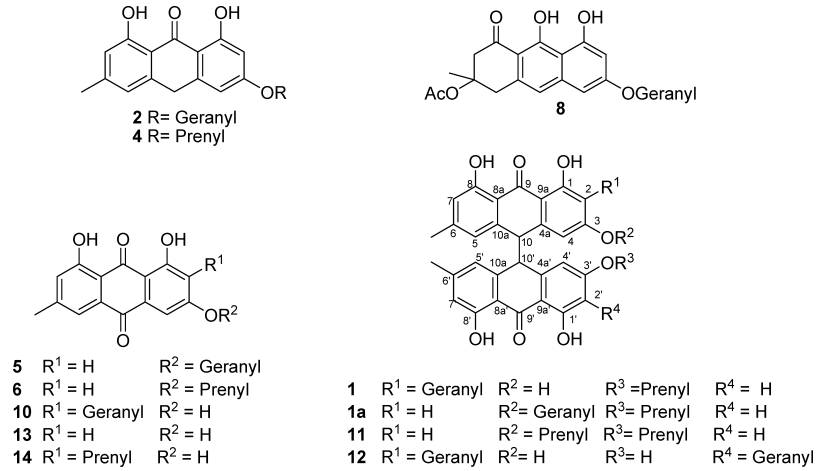

Fig. 2. Structures of Isolated Phenolic Compounds

treatment of neurodegenerative disorders such as Alzheimer's disease.[28] All the tested compounds showed good activity against BChE (IC_{50} 9.2—13.3 μM) with bianthrone **12** showing the best potency. The *n*-hexane extract as well as tested compounds exhibited weak activity against AChE. This is the first time the cholinesterase inhibiting properties of anthranoids are reported.

All these results highlight the bioactive potency of this class of secondary metabolites and partially validate the use of *P. glaberrimum* in traditional medicine in Cameroon. Furthermore, this plant might be a rich source of bioactive metabolites that can be investigated in the search for new drugs against Alzheimer's disease and other related diseases.

Experimental

General Experimental Procedure Melting points were determined on a Büchi-540 melting point apparatus. Optical rotations were measured in $CHCl_3$ solution on a Jasco digital polarimeter (model DIP-3600). IR spectra were determined on Jasco Fourier Transform IR spectrometer. UV spectra were determined on a Spectronic Unicam spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were run on a Bruker spectrometer equipped with 5 mm ^{1}H and ^{13}C probes operating at 500 and 125 MHz respectively, with TMS as internal standard. Silica gel 230—400 mesh (Merck) and silica gel 70—230 mesh (Merck) were used for flash and column chromatography, while percolated aluminium silica gel 60 F_{254} sheets were used for TLC with different mixtures of petrol ether, cyclohexane, ethyl acetate, and acetone as eluents; spots were visualised under UV lamps (254 nm) and (365 nm) or by MeOH–H_2SO_4 reagent.

Acetylcholinesterase (Electric-eel EC 3.1.1.7), butyrylcholinestease (horse-serum EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis[2-nitrobenzoic-acid] (DTNB) and galanthamine were purchased from Sigma (St. Louis, MO, U.S.A.). Buffers and other chemicals were of analytical grade.

Plant Material Stem bark of *P. glaberrimum* was collected in 2005 at Ekombitié in the centre province of Cameroon. The plant was identified by Mr. Nana Victor, botanist at the National Herbarium of Cameroon where a voucher specimen (No. 17257/SRF/Cam) has been deposited.

Extraction and Isolation Stem bark of *P. glaberrimum* (3.5 kg) was extracted successively with *n*-hexane (3×7 l), EtOAc (3×7 l) and MeOH (3×5 l) at room temperature for 24 h each time. The solvent was evaporated under reduced pressure and 93 g, 145 g and 86 g of *n*-hexane, EtOAc and MeOH extracts, respectively, were obtained. *n*-Hexane and EtOAc extracts showed identical profiles in TLC. All these extracts were screened for their anti-plasmodial activity and both the *n*-hexane and the EtOAc extract showed the best potency.

Forty grams of the residue from the *n*-hexane extract were fractionated by flash chromatography over silica gel (70—230 mesh, Merck), eluting with *n*-hexane/EtOAc of increasing polarity to yield five main fractions F1—F5.

Fraction F1 (11 g) was subjected to column chromatography over silica gel (70—230 mesh) eluting with a petrol ether/acetone mixture (90 : 10) resulting in the collection of 78 sub-fractions of 250 ml each which were combined on the basis of TLC analysis. Successive chromatography of these fractions yielded 3-geranyloxyemodin anthrone (**2**, 200 mg), friedelan-3-one (**3**, 13 mg) and 3-prenyloxyemodin anthrone (**4**, 65 mg).

Fraction F2 (6 g) was also subjected to column chromatography over silica gel (70—230 mesh) eluting with a petrol ether/acetone mixture (85 : 15) resulting in 56 sub-fractions of 100 ml each, which were combined on the basis of TLC analysis into 2 sub-fractions F21 and F22. Further chromatography of F21 yielded 3-geranyloxyemodin (**5**, 7 mg) and 3-prenyloxyemodin (**6**, 17 mg). Further chromatography of F22 afforded friedelan-3-ol (**7**, 26 mg).

Fraction F3 (7 g) was subjected to column chromatography over silica gel (70—230 mesh) eluting with a petrol ether/acetone mixture (100 : 0 to 85 : 15) to yield acetylvismione D (**8**, 32 mg) and betulinic acid (**9**, 19 mg).

Fractions F4 and F5 were combined on the basis of their similar TLC and chromatographed on a silica gel column, eluting with petrol ether/acetone (100 : 0 to 70 : 30) to yield three sub-fractions labelled F51, F52 and F53.

Fraction F51 was column chromatographed over silica gel (70—230 mesh), eluting with petrol ether/acetone mixtures of increasing polarity starting from 85 : 15. 35 Fractions of 50 ml were collected and combined on the basis of TLC analysis to give two series F51A and F51B. Fraction F51A was further purified chromatographically with a mixture of petrol ether/acetone (85 : 15) to give 2-geranylemodin (**10**, 22 mg) and glaberianthrone (**1**, 28 mg). Likewise, fraction F51B afforded bianthrone A2b (**11**, 17 mg) when eluted with a mixture of petrol ether/acetone (85 : 15).

Fraction F52 and Fraction F53 (7 g) were combined and subjected to column chromatography over silica gel (70—230 mesh) eluting with a petrol ether/acetone mixture (80 : 20 to 70 : 30). 73 fractions of 50 ml were collected and combined on the basis of TLC analysis in three sub-fractions C, D, E.

Sub-fraction C was chromatographed by column over silica gel (70—230 mesh) and eluted with a petrol ether/acetone (80 : 20) to yield bianthrone 1a (**12**, 40 mg).

Column chromatography of sub-fraction D over silica gel eluting with petrol ether/acetone (80 : 20) mixture yielded emodin (**13**, 21 mg) and 2-prenylemodin (**14**, 6 mg).

Glaberianthrone (**1**): Yellow powder, $[\alpha]_D^{25}$ −22 (c=0.5, $CHCl_3$), IR (KBr) cm^{-1}: 3397, 2920, 1716, 1619, 1486, 1161, 975, 796. UV λ_{max} nm: 230, 278, 369. ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 1. HR-ESI-MS m/z 715.32654 $[M+H]^+$ (Calcd 715.327095 for $C_{45}H_{47}O_8$). EI-MS m/z (rel. int.): 495 (6), 480 (8), 392 (100), 323 (56), 307 (52), 269 (39), 256 (17), 203 (54), 69 (44).

Bianthrone A2b (**11**): Yellow powder, $[\alpha]_D^{25}$ 0 (c=0.5, $CHCl_3$), IR (KBr) cm^{-1}: 3347, 2926, 1614, 1566, 1482, 1258, 1071, 794. UV λ_{max} nm: 232, 278, 365. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 12.23 (s, 1-OH, 1′-OH), 11.84 (8-OH, 8′-OH), 6.68 (s, H-7, H-7′), 6.42 (d, J=1.9 Hz, H-2, H-2′) 6.14 (s, H-4, H-4′), 6.03 (s, H-5, H-5′), 5.52 (t, J=7.2 Hz, H-12, H-12′), 4.56 (d, J=7.2 Hz, H-11, H-11′), 4.36 (s, H5, H-5′), 2.28 (s, H16, H-16′), 1.87 (s, H15, H-15′), 1.82 (s, H14, H14′). ^{13}C-NMR ($CDCl_3$, 125 MHz) see Table 1. EI-MS m/z (rel. int.): 578 (2), 510 (4), 324 (73), 256 (100).

Evaluation of Erythrocyte Susceptibility to Compounds *in Vitro* A preliminary toxicological assessment was carried out to determine the highest drug concentrations that can be incubated with erythrocytes without any significant damage. This was done according to the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide/phenazine methosulfate (MTT/PMS) colorimetric assay described by Cedillo-Rivera *et al.* in 1992 with some modifications.[29] The drugs were serially diluted in 96-well culture plates, and each concentration incubated in triplicate with erythrocytes (2% hematocrit) in a final 100 μl culture volume (at 37 °C, in a 3% O_2, 5% CO_2 and 91% N_2 atmosphere, in the presence of RPMI 1640, 25 mM HEPES, pH 7.4 for 48 h). At the end of the incubation period, the cultures were transferred into polypropylene microcentrifuge tubes and centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. 1.5 ml MTT solution with 250 mg PMS were added to the pellets. Controls contained no erythrocytes. The tubes were thereafter incubated for 45 min at 37 °C, then centrifuged, and the supernatant was discarded. The pellets were re-suspended in 0.75 ml of HCl 0.04 M in isopropanol to extract and dissolve the dye (formazan) from the cells. After 5 min, the tubes were vigorously mixed and centrifuged, and the absorbance of the supernatant was determined at 570 nm.

Evaluation of Anti-plasmodial Activity *P. falciparum* W2 strain, which is resistant to chloroquine and other antimalarials[30] was cultured in sealed flasks at 37 °C, in a 3% O_2, 5% CO_2 and 91% N_2 atmosphere in RPMI 1640, 25 mM HEPES, pH 7.4, supplemented with heat inactivated 10% human serum and human erythrocytes to achieve a 2% hematocrit. Parasites were synchronized in the ring stage by serial treatment with 5% sorbitol (Sigma) and studied at 1% parasitemia.[31] Compounds were prepared as 10 mM stock solutions in DMSO, diluted as needed for individual experiments, and tested in triplicate. The stock solutions were diluted in supplemented RPMI 1640 medium so as to have at most 0.2% DMSO in the final reaction medium. An equal volume of 1% parasitemia, 4% hematocrit culture was thereafter added and gently mixed thoroughly. Negative controls contained equal concentrations of DMSO. Positive controls contained 1 μM chloroquine phosphate (Sigma). Cultures were incubated at 37 °C for 48 h (1 parasite erythrocytic life cycle). Parasites at ring stage were thereafter fixed by replacing the serum medium by an equal volume of 1% formaldehyde in PBS. Aliquots (50 μl) of each culture were then added to 5 ml round-bottom polystyrene tubes containing 0.5 ml 0.1% Triton X-100 and 1 nM YOYO nuclear dye (Molecular Probes) in PBS. Parasitemias of treated and control cultures were compared using a Becton-Dickinson FACSort flow cytometer to count nucleated (parasitized) erythrocytes. Data acquisition was performed using CellQuest software. These data were normalized to percent control activity and IC_{50} calculated using Prism 4.0 software (GraphPad) with data fitted by non linear regression to the variable slope sigmoidal dose–response formula $y=100/[1+10^{(\log IC_{50}-x)H}]$, where H is the hill coefficient or slope factor.[30]

***In Vitro* Cholinesterase Inhibition Assay and Determination of IC_{50}** Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured according to a slightly modified spectrophotometric method.[32] Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay acetylcholinesterase and butyrylcholinesterase, respectively. 5,5′-Dithiobis[2-nitrobenzoic-acid] (DTNB) was used for the measurement of cholinesterase activity. One hundred and forty microliters of (100 mM) sodium phosphate buffer (pH 8.0), 10 μl of DTNB, 20 μl of test compound solution and 20 μl of acetylcholinesterase or butyrylcholinesterase solution were mixed and incubated for 15 min (25 °C). The reaction was then initiated by the addition of 10 μl acetylthiocholine or butyrylthiocholine, respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). Test compounds and control were dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro-plates and monitored in a SpectraMax 340 (Molecular Devices, U.S.A.) spectrometer.

The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

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