Cholinesterase Inhibiting and Antiplasmodial Steroidal Alkaloids from *Sarcococca hookeriana*

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Received April 17, 2007; accepted June 2, 2007

Bioguided phytochemical investigation of *Sarcococca hookeriana* with respect to the cholinesterase enzyme inhibitory assay yielded two new pregnane-type steriodal alkaloids hookerianamide H (1) and hookerianamide I (2), along with three known alkaloids N_a-methylepipachysamine D (3), sarcovagine C (4) and dictyophlebine (5). Their structures were determined with the aid of extensive spectroscopic analysis. All compounds showed good inhibitory activities against the enzymes acetylcholinesterase (IC_{50} 2.9—34.1 μM) and butyrylcholinesterase (IC_{50} 0.3—3.6 μM). These compounds also showed moderate antiplasmodial activity (IC_{50} 2.4—10.3 μM) against the *Plasmodium falciparum* chloroquine resistant W2 strain.

Key words *Sarcococca hookeriana*; steroidal alkaloid; Alzheimer's disease; cholinesterase inhibition; antiplasmodial

Alzheimer's dementia is characterized by a progressive memory loss that leads to a profound emotional disturbance in later stages. The disease is accompanied by dysfunctions in the system of cholinergic neurotransmission of the central nervous system. The nerve impulse in cholinergic synapses is terminated by the enzyme acetylcholinesterase (AChE, EC 3.1.1.7), which catalyses the hydrolysis of the neurotransmitter acetylcholine.[1)] According to the cholinergic hypothesis, the memory impairment in the patients with senile dementia of Alzheimer's type results from a deficiency in cholinergic function in the brain.[2)] Reversible inhibition of this enzyme leads to an increase in neurotransmitter concentration within the synaptic cleft, which positively affects Alzheimer's disease (AD) patients. Butyrylcholinesterase (BChE, EC 3.1.1.8) is produced in the liver and enriched in the circulation. The exact physiological role of BChE is still elusive, but it is generally viewed as a backup for the homologous AChE.[3)] Hence, the search for the new cholinesterase inhibitors seems to be an important strategy to introduce new drug candidates against Alzheimer's disease and related dementia. Several compounds of the genus *Sarcococca* are known for their anticholinesterase properties.[4)]

Sarcococca hookeriana (BAILL.) HOOK. (Buxaceae) is an evergreen shrub, widely distributed from Eastern to Western Nepal, Northern Assam, Southern Tibet and Bhutan.[5)] Rural communities in Nepal have been using the root extracts of this plant against gout.[6)] Previous studies on the plants from the genus *Sarcococca* have reported the isolation as well as chemical and microbial transformation of a number of bioactive steroidal alkaloids.[7—12)] Although a number of studies have reported the biological properties of steroidal alkaloids isolated from the family Buxaceae such as cholinesterase inhibtion[7,8,11—14)] as well as antibacterial,[15)] antitumour[16)] and antiulcer[17)] activities, no investigation has been reported, to the best of our knowledge, on their antiplasmodial activity. Malaria, mainly caused by the parasite *Plasmodium falciparum*, remains one of the most important infectious diseases in the world and constitutes a public health problem in more than 90 countries, inhabited by about 40% of the world's population. The World Health Organization (WHO) estimates that there are 300—500 million malaria cases annually, causing 2—3 million deaths, mostly among children under five years old. Africa accounts for over 90% of malaria mortality.[18)] Most countries where *P. falciparum* malaria, the most severe and dangerous form of this disease, is endemic, face significant parasite resistance to frequently used antimalarial drugs, in particular chloroquine and antifolates. For these reasons, new effective and affordable antimalarial drugs are urgently needed.[19)] In this regard, research on traditional medicinal plants for their antimalarial constituents is important, to facilitate utilization of available botanical resources and to provide potentially active lead antiplasmodial compounds, perhaps with new mechanism of action.

In continuation of our on-going study to search the bioactive constituents of Buxaceae family, we investigated the plant *S. hookeriana* and report here the isolation and structure elucidation of two new steroidal alkaloids (**1**, **2**) and the cholinesterase inhibitory as well as antiplasmodial activity of the isolated compounds (**1**—**5**).

Results and Discussion

The fractionation and purification by column chromatography of the alkaline dichloromethane and neutral dichloromethane fraction of 80% MeOH–H_2O extract of the dried plant of *S. hookeriana* through the bio-assay guided screening based on cholinesterase enzyme-inhibitory assay yielded five steroidal alkaloids **1**—**5**.

Compound **1** was obtained as a white amorphous solid, reacting positively with Dragendorff's reagent, characteristic for alkaloids. The optical rotation of compound **1** was found to be $[\alpha]_D^{25}$ +138 (c=0.3, MeOH), which indicated the chiral nature of the molecule. Its UV spectrum showed absorption

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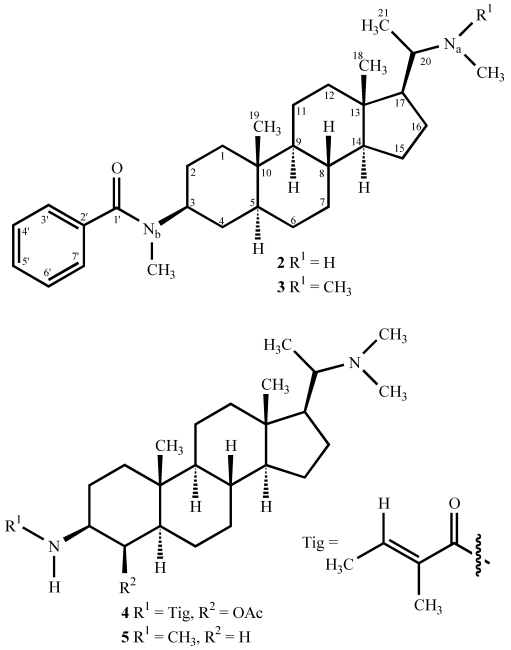

maxima at 224 and 252 nm, characteristic of an α,β-unsaturated carbonyl functionality,[20] while the IR spectrum showed absorptions at 3546, 1724, 1664 and 1622 cm^{-1} indicating the presence of amine, carbonyl, formamide CHO and olefinic functionalities, respectively.[21] The HR-EI-MS displayed the M^+ at *m/z* 386.5687 in agreement with the molecular formula $C_{24}H_{38}N_2O_2$ (Calcd 386.5692) indicating six double-bond equivalents.

The ^{1}H-NMR spectrum of compound **1** (Table 1) indicated a steroidal skeleton, with two methyl singlets resonating in the upfield region at δ 0.65 (3H) and 0.84 (3H), characteristic for C-18 and C-19 methyl groups, respectively. A doublet at δ 1.12 (3H, $J_{21,20}$=6.7 Hz) was due to the C-21 methyl, whereas a singlet resonating at δ 2.30 (6H) was assigned to the dimethylamino group attached to C-20. A proton signal at δ 7.73 (1H, dd, $J_{2,1\alpha}$=6.6 Hz, $J_{2,1\beta}$=2.4 Hz) was assigned to the H-2 olefinic proton. A downfield splitted signal at δ 8.21/8.30 was characteristic of a formamide proton, where such kind of splitting is due to rotational isomerism.[7,12] Another downfield singlet resonating at δ 8.01 was assigned to amide N–H. The broad band decoupled ^{13}C-NMR spectrum of compound **1** (Table 1) displayed 24 carbon signals which were sorted by DEPT, Jmod and HSQC techniques into five methyls, seven methylenes, eight methines and four quaternary carbons including one carbon of formamide group and one keto group at δ_C 162.3 and 196.2, respectively. The ^{1}H- and ^{13}C-NMR data of compound **1** suggested that this compound was similar to sarcovagine D isolated from *Sarcococca vagans*,[22] albeit having a different substituent at the C-3 amino group. In fact, the keto functionality at δ 196.2 was placed at C-4 on the basis of its de-shielding effect on conjugated C-2 vinylic proton.[23] This position was further confirmed by the HMBC correlations observed between H-2, H-5 and C-3 NH/C-4 (Fig. 1). The position of the formamide group at C-3 was confirmed by the ^3J HMBC correlation observed between the proton at δ 8.21/8.30 and C-3. The stereochemistry in compound **1** was largely assigned on biogenetic grounds as all known pregnane-type steroidal

Table 1. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR Data for Hookerianamide H (**1**) and Hookerianamide I (**2**) in $CDCl_3$

No.	Compound **1**		Compound **2**	
	δ ^{1}H (*J* Hz)	δ ^{13}C	δ ^{1}H (*J* Hz)	δ ^{13}C
1	1.15, 1.58, m	37.4	1.21, 1.34, m	36.2
2	7.73, dd, (6.6, 2.4)	125.4	1.14, 1.37, m	28.4
3	—	131.1	3.49, m	52.6
4	—	196.2	1.63, 1.75, m	32.4
5	2.23, m	45.7	1.81, m	44.6
6	1.22, 1.47, m	20.9	1.61, 1.82, m	21.0
7	1.52, 1.76, m	27.1	1.42, 1.81, m	27.6
8	1.17, m	35.3	1.78, m	35.9
9	1.40, m	54.0	1.51, m	53.8
10	—	44.2	—	43.3
11	1.31, 1.57, m	22.2	1.16, 1.22, m	24.3
12	1.11, 1.25, m	39.3	1.72, 1.81, m	39.6
13	—	39.0	—	39.9
14	1.08, m	53.3	1.15, m	51.8
15	0.87, 1.32, m	23.7	1.67, 2.21, m	24.8
16	1.19, 1.32, m	28.7	1.22, 1.43, m	29.7
17	1.14, m	56.6	1.62, m	56.0
18	0.65, s	11.4	0.68, s	12.1
19	0.84, s	12.5	0.77, s	12.7
20	2.84, m	60.7	2.68, m	61.1
21	1.12, d, (6.7)	12.3	1.29, d, (6.6)	12.3
Me_2N	2.30, s	41.7	—	—
MeN_a	—	—	2.46, s	35.8
MeN_b	—	—	2.83, s	35.2
1′	—	—	—	167.9
2′	—	—	—	139.6
3′	—	—	7.34, dd, (7.5, 2.1)	128.4
4′	—	—	7.36, dd, (7.6, 7.5)	129.1
5′	—	—	7.44, dd, (7.5, 2.1)	129.4
6′	—	—	7.36, dd, (7.6, 7.5)	129.1
7′	—	—	7.34, dd, (7.5, 2.1)	128.4
CHO	8.21/8.30, s	162.3	—	—
N–H	8.01, s	—	—	—

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

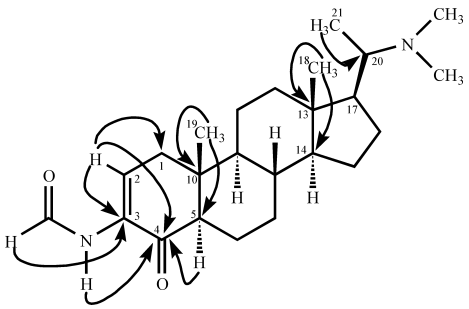

Fig. 1. Selected HMBC Correlations in Compound **1**

alkaloids are being biosynthesized from cholesterol *via* pregenolone.[8,11—16] The assignments were further supported by ROESY experiment and by chemical shift/coupling constant comparison with the reported data.[22] Above mentioned spectroscopic data and comparative literature analysis led us to propose structure **1** to this compound, which was further supported the mass fragmentation pattern [*m/z* (rel. int.): 386 (M^+, 24), 371 (38), 358 (17), 72 (100)] observed in the EI-MS. Hence, compound **1** is 20-(*N*,*N*-dimethylamino)-3-(*N*-formylamino)-5α-pregn-2-ene-4-one to which we give the trivial name hookerianamide H.

Hookerianamide I (**2**) was obtained as a white amorphous

solid also reacts positively with Dragendorff's reagent indicating its alkaloid nature. The optical rotation of compound **2** was found to be $[\alpha]_D^{25}$ +186 (c=0.3, MeOH), which indicated the chirality in the molecule. Its UV spectrum showed absorption maxima at 220 and 266 nm, indicating the presence of unsaturation sites in the molecule.[20] The IR absorptions of compound **2** at 3341, 1614, and 1601—1449 cm^{-1} indicated the presence of a secondary amine, amide carbonyl and aromatic carbons, respectively.[21] The HR-EI-MS supported the formula $C_{30}H_{46}N_2O$ (M^+ m/z 450.3623, Calcd 450.3610) indicating nine double-bond equivalents. The EI-MS showed the M^+ at m/z 450. The base peak at m/z 58 was due to loss of *N*-methyl-*N*-ethyliminium, which is a strong evidence for the attachment of monomethylamino moiety at C-20 of a pregnane-type steroidal skeleton.[11,13]

The study of the ^{1}H-NMR spectrum (Table 1) of compound **2** also supported a pregnane-type steroidal skeleton in compound **2**. A singlet at δ 2.46 (3H) was assigned to the N_a-methyl protons attached to C-20, whereas, another singlet at δ 2.83 (3H) was assigned to N_b-methyl protons attached to C-3. The characteristic downfield resonances of five protons were attributed to aromatic protons. The chemically equivalent *ortho* C-3′/C-7′ protons appeared as a double doublet at δ 7.34 (2H, J=7.5 Hz, J=2.1 Hz), while the *meta* C-4′/C-6′ protons resonated as a double doublet at δ 7.36 (2H, J=7.6 Hz, J=7.5 Hz). Another double doublet at δ 7.44 (1H, J=7.5 Hz, J=2.1 Hz) was due to the *para* C-5′ proton. The broad band decoupled ^{13}C-NMR spectrum of compound **2** (Table 1) displayed 30 carbon signals which were sorted by DEPT, Jmod and HSQC techniques into five methyls, nine methylenes, twelve methines and four quaternary carbons. The carbon signals at δ 128.4, 129.1, 129.4 and 139.6 were characteristic for an aromatic ring, whereas the signal at δ_C 167.9 is consistent to the carbonyl carbon of benzoyl moiety. The ^{1}H- and ^{13}C-NMR data of compound **2** suggested that this compound was similar to N_a-methylepipachysamine D isolated from *Sarcococca saligna*,[15] having a different C-20 *N*-substituent. In the HMBC spectrum, the C-3 N_b-methyl protons showed correlations with C-3, C-1′ and C-2′. The peaks at m/z 105 ($M^+-C_7H_5O$) and 136 ($M^+-C_8H_8NO$) in the EI-MS further supported the presence of an N_b-methylbenzamido moiety at C-3. A multiplet at δ 3.49 ($W_{1/2}$= 16.3 Hz) for the C-3 methine proton suggested its axial orientation.[24] The stereochemistry in compound **2** was also assigned by similar way that explained for compound **1**. In ROESY spectrum, C-3 methine proton showed cross peak with C-5 methine proton indicating its α-orientation. On the basis of above observations, compound **2** was 20-(*N*-methylamino)-3β-(*N*-methylbenzamido)-5α-pregnane given the trivial name hookerianamide I (**2**).

In addition to compound **1** and **2**, three previously known pregnane-type steroidal alkaloids were also isolated and characterized as N_a-methylepipachysamine D (**3**), sarcovagine C (**4**) and dictyophlebine (**5**).[7,15,22]

Compounds **1**—**5** were tested *in vitro* for their inhibitory properties towards AChE and BChE (Table 2). All these steroidal alkaloids displayed potent cholinesterase inhibition. As stated in previous reports,[11,13] the presence of an α,β-unsaturated carbonyl moiety such as in compound **1** increases the inhibitory potential against AChE and BChE. Comparing the activities between compounds **2** and **3** with the C-20 amine substitution pattern being the only difference, compound **2** with N_b-monomethyl substituent was found to show higher inhibitory potential towards BChE.

Table 2. *In Vitro* Anticholinesterase Activities of Compounds **1**—**5**

Compounds	IC_{50} (μM)±S.E.M.[a]	
	AChE	BChE
1	2.9±0.11	1.9±0.06
2	34.1±0.26	0.3±0.0005
3	10.1±1.2	3.2±0.01
4	8.0±0.22	0.3±0.03
5	6.2±0.23	3.6±0.02
Galanthamine[b]	0.5±0.01	8.2±0.02

a) Standard error of the mean of five assays. *b*) Positive control used in the assays.

Table 3. *In Vitro* Antiplasmodial Activities of Compounds **1**—**5** against W2 Strain *P. falciparum*

Compounds	IC_{50} (μM)
1	3.5
2	6.6
3	10.3
4	3.4
5	2.4
Chloroquine[a]	0.11

a) Positive control used in the assays.

The discovery of potent and safe cholinesterase inhibitors has long been an attractive target for the rational drug design and discovery of mechanism based inhibitors for the treatment of the Alzheimer's disease and related dementias. All the known cholinesterase inhibiting drugs used in the treatment of Alzheimer's disease suffer from several drawbacks such as high toxicity, short duration of biological action, low bioavailability and narrow therapeutic windows.[25] Several natural products have shown promising cholinesterase inhibiting activities both *in vivo* and *in vitro*. The alkaloid physostigmine (eserine) obtained from calabar bean *Physostigmine venenosum* and huperzine-A isolated from *Huperzia serrata* have exhibited significant cholinesterase inhibitory activities.[26] Hence, the studies on compounds of *Sarcococca* alkaloids may also be lead candidates in the discovery of clinically useful agents for various nervous system disorders.

Compounds **1**—**5** were also tested *in vitro* for their antiplasmodial activity against the W2 strain of *P. falciparum*, which is resistant to chloroquine and other antimalarials (Table 3). All compounds displayed antiplasmodial activity with compound **5** showing the best potency (IC_{50} 2.4 μM). The contribution of particular functionalities for the activity is not yet known.

These interesting results highlight the antiplasmodial potency of steroidal alkaloids that might be novel lead structures for therapy of malaria or Alzheimer's dementia and related disease.

Experimental

General Experimental Procedure Optical rotations were measured in methanol solution on a Jasco digital polarimeter (model DIP-3600). UV Spectra were recorded in methanol on a Hitachi UV 3200 spectrophotometer. IR Spectra were recorded in $CHCl_3$ on a Jasco A-302 IR spectropho-

tometer. The EI-MS Spectra were recorded on a double focusing mass spectrometer (Varian MAT 311A). HR-EI-MS were recorded on a Jeol HX 110 mass spectrometer. The ^{1}H-NMR Spectra were recorded on a Bruker AM-400 instrument, while ^{13}C-NMR Spectra were recorded on a Bruker AM-400 operating at 100 MHz using $CDCl_3$ as solvent. Methyl, methylene and methine carbons were distinguished by DEPT 90° and 135° experiments. Homonuclear ^{1}H–^{1}H connectivities were determined by using a COSY 45° experiment. One-bond ^{1}H–^{13}C connectivities were determined by HSQC gradient pulse factor selection. Two- and three-bond ^{1}H–^{13}C connectivities were determined by the HMBC experiment. Proton chemical shifts are reported in δ (ppm) with reference to the residual $CDCl_3$ signal at δ 7.26, and ^{13}C-NMR spectra were referenced to the central peak of $CDCl_3$ at δ 77.0. Coupling constants (*J*) were measured in Hz. Column chromatography was carried out on silica gel 60 (70—230 and 240—300 mesh sizes, E. Merck). Pre-coated silica gel TLC plates (E. Merck, F_{254}) were used to check the purity of compounds, and Dragendorff's spray reagent was used for the staining of compounds on TLC.

Acetylcholinesterase (Electric-eel EC 3.1.1.7), butyrylcholinesterase (horse-serum E.C 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 Whole plant of *S. hookeriana* BAILL. was collected from Phulchowki (8000 ft.), Lalitpur District, Nepal in 2002. The plant was identified by Dr. M. K. Adhikari, Senior Scientific Officer, at the National Herbarium and Plant Laboratories Section, Lalitpur, Nepal, where a voucher specimen (no. 101/2002) was deposited.

Extraction and Isolation Air-dried whole plant (31.0 kg) of *S. hookeriana* was extracted with 80% methanol/water (120 l). The concentrated methanolic aqueous extract (2.8 kg) was dissolved in cold distilled water (12 l) and defatted with petrol ether (30 l) (304.3 g). The aqueous layer was extracted with dichloromethane (30 l) to obtain the neutral fraction (107.8 g). The aqueous fraction was then acidified with acetic acid to pH 3—4 and extracted with dichloromethane (30 l) to obtain the acidic fraction (85.0 g). The aqueous acidic fraction was then made alkaline by adding ammonia solution (pH 9—10) and extracted with dichloromethane (30 l) to obtain the alkaline fraction (24.7 g). The cholinesterase inhibitory activity was determined for the crude MeOH/H_2O extract (1.0 mg/ml) amounting 81% and 87% inhibition of AChE and BChE, respectively. Similarly, alkaline and neutral dichloromethane fractions (1.0 mg/ml) showed 88% and 97% inhibition for AChE and 85% and 96% inhibition for BChE, respectively. Based on these results, the alkaline fraction was initially selected for bioassay-guided fractionation through a repeated column chromatography, which was adsorbed on 28.0 g of silica gel and eluted with different gradients of petrol ether, acetone and ethanol. Elution with 15% acetone in petrol ether afforded sub-fraction E (2.1 g), which was adsorbed on 4.0 g of silica gel and chromatographed on a column to afford fifteen sub-fractions E_1—E_{15}. Sub-fractions E_4—E_8 were found to exhibit significant cholinesterase inhibition activity. Compound **1** (11.5 mg) was obtained by column chromatography of sub-fraction E_5 (70.0 mg) upon elution with petrol ether/acetone/diethylamine (80 : 18 : 2). Similarly, compound **2** (18.3 mg) was obtained after elution of sub-fraction E_7 (90.1 mg) with petrol ether/acetone/diethylamine at 72 : 25 : 3. Similarly, compounds **4** (9.4 mg) and **5** (900.0 mg) were isolated from different sub-fractions of the alkaline fraction and compound **3** (6.7 mg) was isolated from the neutral fraction by silica gel column chromatography.

Hookerianamide H [20-(*N*,*N*-Dimethylamino)-3β-(*N*-formylamino)-5α-pregn-2-en-4-one] (**1**): White amorphous solid, $[\alpha]_D^{25}$ +138 (c=0.3, MeOH), UV (MeOH) nm (log ε) λ_{max} 224 (3.3), 252 (2.7); λ_{min} 351 (3.1), 250 (2.9) and 208 (3.2); IR ($CHCl_3$) ν_{max} cm^{-1}: 3546 (NH), 1724 (C=O), 1664 (formamide CHO) and 1622 (C=C); ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$): see Table 1; HR-EI-MS *m/z*: 386.5687 (Calcd for $C_{24}H_{38}N_2O_2$, 386.5692). EI-MS *m/z* (rel. int.): 386 (M^+, 24), 371 (38), 358 (17), 72 (100).

Hookerianamide-I [20-(*N*-Methylamino)-3β-(*N*-methylbenzamido)-5α-pregnane] (**2**): White amorphous solid, $[\alpha]_D^{25}$ +186 (c=0.3, MeOH), UV (MeOH) nm (log ε) λ_{max} 220 (2.1), 266 (3.0); λ_{min} 341 (2.9), 241 (2.7), 215 (3.3); IR ($CHCl_3$) ν_{max} cm^{-1}: 3341 (NH) and 1614 (C=O); ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$): see Table 1. HR-EI-MS *m/z*: 450.3623 (Calcd for $C_{30}H_{46}N_2O$, 450.3610); EI-MS *m/z* (rel. int.): 540 (M^+, 14), 435 (22), 136 (27), 105 (29), 72 (30), 58 (100).

***In Vitro* Cholinesterase Inhibition Assay and Determination of IC_{50}** Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured by a slightly modified spectrophotometric method.[27] Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay acetylcholinesterase and butyrylcholinesterase, respectively. Test compounds and control (galanthamine) were dissolved in EtOH. 5,5′-Dithiobis[2-nitrobenzoic-acid] (DTNB) was used for the measurement of cholinesterase activity. 140 μl 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 or butyrylthiocholine was monitored at a wavelength of 412 nm (15 min) by the formation of the 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. 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.).

Evaluation of Antiplasmodial Activity *P. falciparum* strain W2, which is resistant to chloroquine and other antimalarials[28] 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.[29]

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 with 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 mixed thoroughly. Negative controls contained equal concentration 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 the 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 YOYO nuclear dye (Molecular Probes) in PBS, and 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 50% inhibitory concentrations (IC_{50}) were calculated using Prism 3.0 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.[28]

Acknowledgements We wish to acknowledge Alexander von Humboldt Foundation, Germany and European Commission (Marie Curie IIF, MIF1-CT-2006-021591) for providing post doctoral fellowships to K. P. Devkota and B. N. Lenta, respectively, at Bielefeld University.

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