Antiparasitic Activity of Some Xanthones and Biflavonoids from the Root Bark of *Garcinia livingstonei*[#]

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A new biflavanoid, *ent*-naringeninyl-(I-3α,II-8)-4′-*O*-methylnaringenin (**6**), along with five known xanthones and two known biflavonoids, was isolated from the root bark of *Garcinia livingstonei* collected in Tanzania. The absolute configuration of **6** was established by CD spectroscopy. This compound showed moderate activity against *P. falciparum* (IC_{50} 6.7 μM). Antitrypanosomal activity (IC_{50} 0.87 μM) was observed for 1,4,5-trihydroxy-3-(3-methylbut-2-enyl)-9*H*-xanthen-9-one (**3**). The dimeric xanthone garcilivin A (**4**) showed a higher and nonselective antiparasitic activity and cytotoxicity (IC_{50} 2.0 μM against MRC-5 cells) than its diastereoisomer garcilivin C (**5**) (IC_{50} 52.3 μM).

Garcinia is a large genus comprising over 200 species confined to the tropics as trees or shrubs, and rarely subshrubs, exuding drops of yellow to red latex when cut.[1,2] *Garcinia livingstonei* T. Anderson (Clusiaceae) is a distinctive evergreen tree or shrub, 2−10 m tall, widely distributed in Tanzania, where it is commonly known as "mpekechu" or "mutumbi". The plant thrives in riverine forests and in open woodland at low altitude (0−800 m), often under larger trees.[2] In certain parts of Tanzania, fruits of *G. livingstonei* have been reported as edible.[3] Previous phytochemical analysis of *G. livingstonei* resulted in the isolation of a series of xanthones, for which no biological activity was reported,[4,5] and a HIV-inhibitory prenylated benzophenone, guttiferone A.[6] This paper describes the isolation, antiparasitic activity against *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma brucei brucei*, and *T. cruzi*, and cytotoxicity against MRC-5 cells of the five known xanthones and one new and two known biflavonoids.

Results and Discussion

Root bark of *Garcinia livingstonei* was extracted with MeOH−CH_2Cl_2 (1:1), and after defatting with petroleum ether, a $CHCl_3$-soluble and an EtOAc-soluble extract were prepared. The $CHCl_3$-soluble portion yielded the xanthones **1**−**4**, which were identified by ^{1}H, ^{13}C, and two-dimensional NMR and MS data as 6,11-dihydroxy-3-methyl-3-(4-methylpent-3-enyl)pyrano[2,3-*c*]xanthen-7(3*H*)-one (**1**), 4[(*E*)-3,7-dimethylocta-2,6-dienyl]-1,3,5-trihydroxy-9*H*-xanthen-9-one (**2**), 1,4,5-trihydroxy-3-(3-methylbut-2-enyl)-9*H*-xanthen-9-one (**3**), and garcilivin A (**4**), a dimeric xanthone, reported by Sordat-Diserens et al. from the same plant.[4,5] The EtOAc-soluble portion yielded compounds **5**−**8**. Compound **5** was identified as the xanthone garcivilin C, also reported by Sordat-Diserens et al. from *G. livingstonei*.[4,5] Compounds **6**−**8** are biflavonoids, with (+)-volkensiflavone (**7**) and (+)-morelloflavone (**8**)[7−9] reported from other *Garcinia* spp., but not from *G. livingstonei*. Compound **6** was obtained as a new biflavonoid.

For xanthones **1**, **4**, and **5** the absolute configuration is not known, and their optical rotations have not been reported. The dimeric xanthones garcilivin A (**4**) and garcilivin C (**5**), which are diastereoisomers, both had a negative optical rotation ($[\alpha]^{30}_D$ −8 and −6, respectively), whereas compound **1** was not optically active and, hence, must be a racemic mixture. Therefore this compound may be an artifact, formed during processing of the plant material or extraction and isolation, by oxidative ring closure of compound **2**.

Compounds **7** and **8** were identified as (+)-volkensiflavone and (+)-morelloflavone, respectively, by ^{1}H, ^{13}C, and two-dimensional NMR and MS data and optical rotation measurements. Compound **6** showed similar NMR spectra and a molecular weight identical to that of (+)-morelloflavone (**8**). Whereas (+)-volkensiflavone (**7**) and (+)-morelloflavone (**8**) consist of (I-3, II-8) linked flavanone and flavone units, the ^{13}C NMR and DEPT-135 spectra of compound **6** showed two sets of aliphatic resonance signals due to C-2 and C-3 of the heterocyclic C-ring of a flavanone. Similar to (+)-volkensiflavone (**7**) and (+)-morelloflavone (**8**), duplication of most ^{1}H and ^{13}C NMR signals was observed due to atropisomerism, complicating interpretation of the spectra. In addition to the ^{13}C NMR signals of C-I-2 and C-I-3 at δ 81.8/81.2 (CH) and δ 47.5/47.3 (CH), respectively, due to the substituted flavanone (sf) unit, resonances were observed at δ 78.0 (CH) and 42.9/42.6 (CH_2) (C-II-2 and C-II-3, respectively, of the lower flavanone (f) unit). Assignments were confirmed by COSY, HSQC, and HMBC experiments. In the same way, the typical flavanone carbonyl resonances around 195 ppm could be attributed to C-4 (δ 196.8/196.5) of the upper and lower unit (δ 195.8/195.6), respectively. The *trans*-configuration of the upper flavanone unit could be deduced from the coupling constant between H-I-2 (δ 5.69/5.35) and H-I-3 (δ 4.65/4.50) in the ^{1}H NMR spectrum, the signal at δ 5.69 being coupled with the one at δ 4.50 (d, J = 11.6 Hz), and the signal at δ 5.35 with the one at δ 4.65 (d, J = 12.1 Hz).[10] The ^{1}H as well as the ^{13}C NMR spectra suggested that this biflavonoid consisted of two naringenin units with a I-3,II-8 linkage, as also observed in (+)-morelloflavone (**7**) and (+)-volkensiflavone (**8**). In addition, the presence of an *O*-methyl group (δ_C 55.1/55.1, δ_H 3.77, s/3.71, s) was observed, the position of which was established by two-dimensional NMR methods. Indeed, the ^{13}C NMR signal of C-I-2 (δ 81.8/81.2) showed an HMBC correlation with a doublet

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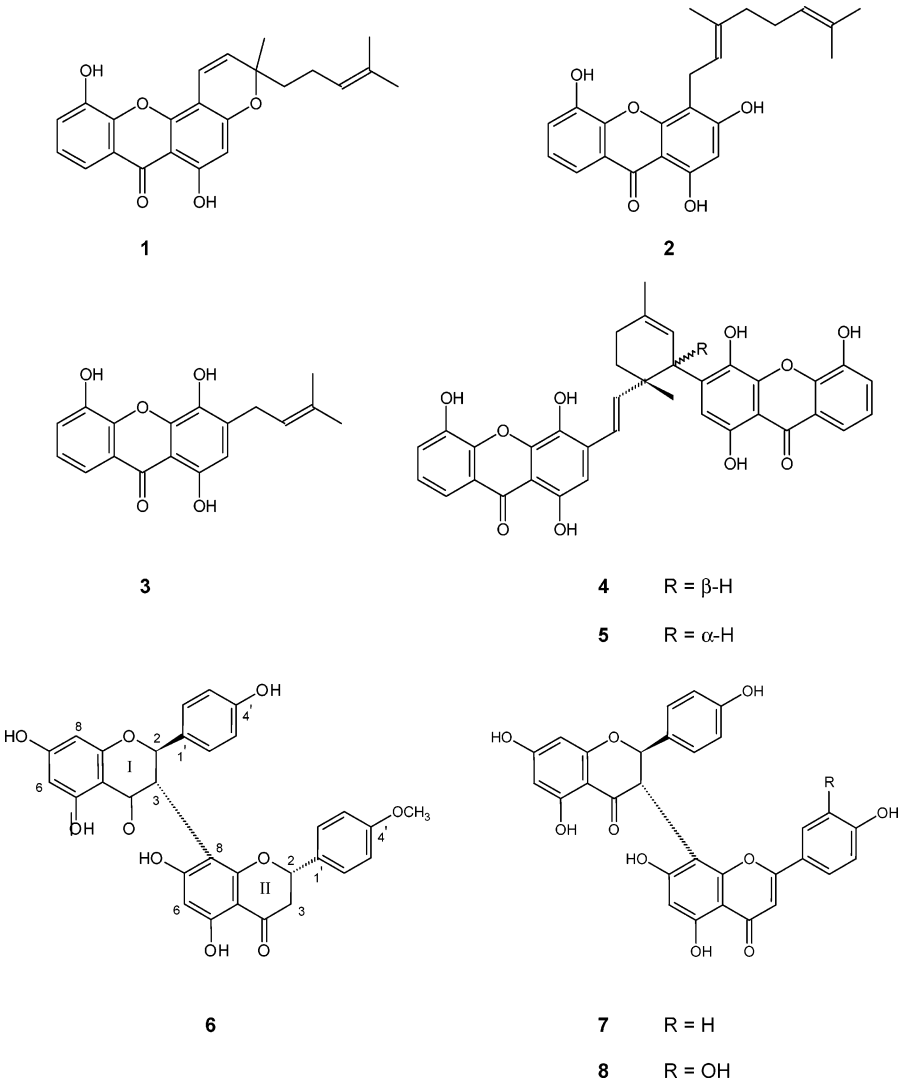
Chart 1



at δ 7.10 (J = 8.6 Hz), assigned to H-I-2′, and C-II-2 (δ 78.0) with a multiplet (due to atropisomerism) at δ 7.3, assigned to H-II-2′. Since the *O*-methyl signals in the ^{1}H NMR spectrum (δ 3.77, s/3.71, s), as well as the multiplet assigned to H-II-2′ at δ 7.26, showed HMBC correlations with a quaternary carbon at δ_C 159.3/158.9, assigned to C-II-4′, the *O*-methyl group could be located at position C-II-4′ (lower unit). In the same way, the ^{13}C NMR signals at δ 157.6/157.5 were assigned to C-I-4′. Complete ^{1}H and ^{13}C NMR assignments were based on two-dimensional NMR experiments (COSY, HSQC, HMBC) and were in good agreement with those reported for I-3,II-8-binaringenin or GB-1a (*Garcinia* biflavonoid 1a), the demethyl analogue of compound **6**, isolated from several *Garcinia* spp.[8] For C-II-1′, an upfield shift of about 1 ppm was observed (δ 128.9 in GB-1a to 131.0/130.8 in **6**), which is in agreement with a *para*-methoxy substitution and which is close to the chemical shift for C-II-1′ of GB-1a hexamethyl ether (δ 130.9).[8] The (I-3,II-8)-biflavanoids are mainly found in plants of the genera *Garcinia*, *Rheedia*, and *Allanblackia* (Clusiaceae). However, in contrast to compound **6**, most of these compounds consist of flavanone and 3-hydroxyflavanone (dihydroflavonol) units.[10]

The determination of the absolute configuration of (I-3,II-8)-linked biflavonoids, including the revision of absolute configurations, has recently been the subject of detailed analysis.[9,10] The CD spectrum of compound **6** reveals the Cotton effects of the electronic n→p* and π→p* transitions of the two constituent structural moieties, i.e., the 3-substituted flavanone (sf) upper unit and the flavanone (f) lower unit (Figure 1). Since the electronic transitions of a C-3-substituted flavanone unit are blue-shifted,[9,10] the observed Cotton effects may be assigned as follows: The positive Cotton effect near 340 nm for the n→p* transition and the negative Cotton effect near 300 nm for the π→p* transition may be assigned to the lower f-unit. Similarly, the negative Cotton effect (shoulder) near 320 nm for the n→p* transition and the positive Cotton effect near 250 nm for the π→p* transition may be assigned to the upper sf-unit. Application of Gaffield's rule then permits assignment of (2*R*,3*S*) and (2*S*) absolute configurations for the sf- and f-moieties, respectively.[11] Therefore, the structure of compound **6** could be established unambiguously as *ent*-naringeninyl-(I-3α,II-8)-4′-*O*-

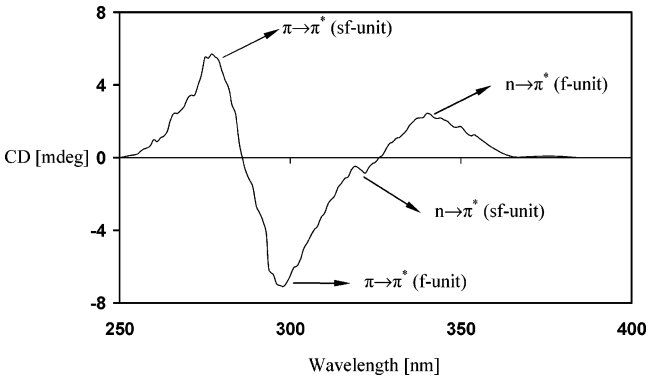


Figure 1. CD spectrum of *ent*-naringeninyl-(I-3α,II-8)-4′-*O*-methylnaringenin (**6**).

methylnaringenin, which is a new biflavonoid. It should be noted that the absolute configuration of the (II-4′)-demethyl analogue, GB-1a, was not established.[8]

The antiparasitic activity and cytotoxicity of compounds **1**−**8** were evaluated against *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma brucei brucei*, and *T. cruzi*, and the cytotoxicity against MRC-5 cells. The three monomeric trioxygenated prenylated xanthones showed no cytotoxicity in the concentration range tested; whereas compounds **1** and **2** were inactive against *L. infantum* and *P. falciparum*, compound **3** showed a marginal activity (IC_{50} 27 and 10 μM, respectively). However, all three compounds were active against *Trypanosoma*, especially compound **3** against *T. brucei* (IC_{50} 0.87 μM). Antitrypanosomal xanthones (against *T. cruzi*) were reported previously from *Garcinia subelliptica*. The most active compound was garciniaxanthone B, showing an MC_{100} against trypomastigotes (defined as the minimum concentration at which all the trypomastigotes become immobilized after 48 h incubation) of 8 μM.[12]

With regard to the dimeric xanthones there was a remarkable difference in activity between garcilivins A (**4**) and C (**5**), which are diastereoisomers. Garcilivin A (**4**) is a relatively cytotoxic compound (IC_{50} 2.0 μM) and displayed a nonselective activity against all parasites tested (and a cytotoxic effect on the infected macrophages in the *Leishmania* assay). Garcilivin C (**5**) on the other hand was about 25 times less cytotoxic and showed activity only against *T. brucei* (IC_{50} 7.7 μM), being about 20 times less active than garcilivin A (**4**).

The biflavonoids (+)-volkensiflavone (**7**) and (+)-morelloflavone (**8**) were found to be almost inactive. For the new biflavanoid (**6**), noteworthy activity only against *P. falciparum* (IC_{50} 6.0 μM) was observed. Antihepatotoxic and bactericidal properties have been demonstrated for some 3,8″-biflavanoids.[10]

Experimental Section

General Experimental Procedures. Column chromatography was performed on silica gel (Merck) and Sephadex LH-20 (Pharmacia). TLC was carried out on silica gel (Merck). Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. UV−vis spectra were recorded on a Uvikon 931 instrument (Kontron Instruments). CD analyses were done on a JASCO J-715 spectrometer in MeOH. NMR spectra were recorded at 30 °C on a Bruker DRX-400 instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts (δ) are reported in ppm downfield from TMS, using TMS or the solvent signal as the internal standard. The HMBC spectra were optimized for a long-range coupling constant of 8.3 Hz. Two-dimensional NMR experiments were carried out using pulsed field gradients. LC/ESIMS were recorded in the negative-ion and/or the positive-ion mode on an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltronics) coupled to an HP 1100 (DAD) liquid system (Agilent) equipped with an automatic sampler. Accurate mass data were acquired on a quadrupole-time-of-flight mass spectrometer (Q-Tof-II, Micromass), equipped with a standard electrospray ionization (ESI) interface.

Plant Material. Root bark of *G. livingstonei* T. Anderson was collected by Mr. B. Muhoro, Institute of Traditional Medicine, MUCHS, in August 2003 and identified by Mr. F. Mbago, Botany Department, University of Dar es Salaam, Tanzania. A voucher specimen (BM11116) has been deposited at the Herbarium of the Institute of Traditional Medicine, MUCHS, Dar es Salaam, Tanzania.

Extraction and Isolation. Air-dried and powdered root bark (2 kg) was extracted with MeOH−CH_2Cl_2 (1:1) (3 × 2.5 L) by maceration. The resultant extracts were combined and concentrated to dryness under reduced pressure at 40 °C to afford 280 g of a black gum residue. Of this residue, 200 g was extracted with petroleum ether to give an oily petroleum ether-soluble extract (32 g). The residue was then suspended in distilled H_2O and partitioned with $CHCl_3$ and EtOAc, to afford a $CHCl_3$-soluble (50 g) and an EtOAc-soluble (80.5 g) extract, respectively.

The $CHCl_3$ extract (50 g) was subjected to column chromatography on silica gel (250 g) and eluted with mixtures of petroleum ether−$CHCl_3$ and $CHCl_3$−MeOH of increasing polarity to give 102 fractions of 50 mL each. Fractions 9−16 were pooled and, on standing at room temperature, gave a yellow precipitate. The precipitate was filtered and purified on Sephadex LH-20 (3.50 × 40 cm), eluted with MeOH−$CHCl_3$ (1:1), to afford **1** (500 mg, 0.025% yield). Fractions 41 and 42 eluted with petroleum ether−$CHCl_3$ (1:4) and $CHCl_3$ (100%) gave a yellow precipitate, which on recrystallization from $CHCl_3$ afforded **2** (400 mg, 0.020% yield). Fractions 43−48 gave yellow crystals of **3** (260 mg, 0.013% yield). Fractions 70−77 eluted with $CHCl_3$−MeOH (98:2) gave a yellow precipitate, which was repeatedly washed and recrystallized in $CHCl_3$ to afford **4** (410 mg, 0.021% yield).

The EtOAc-soluble extract (70.0 g) was adsorbed on 60 g of silica gel and applied to a normal column packed with 360 g of silica gel slurry in CH_2Cl_2. The column was eluted with CH_2Cl_2 and then with CH_2Cl_2−MeOH mixtures of increasing polarity. A total of 66 fractions of 100 mL were collected. Elution with CH_2Cl_2 gave fractions 1−5; 5% MeOH−CH_2Cl_2 gave fractions 6−15; 10% MeOH−CH_2Cl_2 gave fractions 16−27; 15% MeOH−CH_2Cl_2 gave fractions 28−40; 25% MeOH−CH_2Cl_2 gave fractions 41−56; and 50% MeOH−CH_2Cl_2 gave fractions 57−74. Fractions 9−23 (580 mg) were subjected to Sephadex LH-20 (3.50 × 40 cm), eluted with MeOH−$CHCl_3$ (9:1). Altogether, 30 subfractions of 10 mL each were collected and developed by TLC using 5% MeOH−$CHCl_3$. Subfractions 3−6 afforded **5** (67 mg, 0.00335% yield). Subfractions 10−15 were pooled, dried under reduced pressure, and left to crystallize in acetone to afford **6** (33 mg, 0.00165% yield). Fractions 27−48 (2.5 g) were repeatedly subjected to silica gel column chromatography eluted with CH_2Cl_2−MeOH mixtures of increasing polarity and passed through Sephadex LH-20 (3.50 × 40 cm), eluted with MeOH−$CHCl_3$ (9:1), to afford **7** (29 mg, 0.00145% yield) and **8** (72 mg, 0.0036% yield).

6,11-Dihydroxy-3-methyl-3-(4-methylpent-3-enyl)pyrano[2,3-*c*]-xanthen-7(3*H*)-one (1): $[\alpha]^{30}_D$ 0 (*c* 0.26, MeOH); ^{1}H NMR, ^{13}C NMR, and MS as reported.[4]

4[(*E*)-3,7-Dimethylocta-2,6-dienyl]-1,3,5-trihydroxy-9*H*-xanthen-9-one (2): ^{1}H NMR, ^{13}C NMR, and MS as reported.[4]

1,4,5-Trihydroxy-3-(3-methylbut-2-enyl)-9*H*-xanthen-9-one (3): ^{1}H NMR, ^{13}C NMR, and MS as reported.[4]

Garcilivin A (4): $[\alpha]^{30}_D$ −8 (*c* 0.26, DMSO); ^{1}H NMR, ^{13}C NMR, and MS as reported.[5]

Garcivilin C (5): $[\alpha]^{30}_D$ −6 (*c* 0.16, DMSO); ^{1}H NMR, ^{13}C NMR, and MS as reported.[5]

***ent*-Naringeninyl-(I-3α,II-8)-4′-*O*-methylnaringenin (6):** $[\alpha]^{30}_D$ −44 (*c* 0.23, MeOH); ^{1}H NMR (30 °C, CD_3OD, 400 MHz) (most signals are duplicated due to atropisomerism) δ 7.26 (2H, m, H-II-2′), 7.10 (2H, d, J = 8.6 Hz, H-I-2′), 6.99/6.79 (2H, d, J = 7.3 Hz/7.6 Hz, H-II-3′), 6.70/6.63 (2H, d, J = 8.6 Hz, H-I-3′), 5.89 (1H, br d, H-I-6), 5.85 (1H, br d, H-I-8), 5.78 (1H, s, H-II-6), 5.69/5.35 (1H, d, J = 11.6 Hz/12.1 Hz, H-I-2), 5.58/5.42 (1H, d, J = 13.1 Hz/11.7 Hz, H−II-2), 4.65/4.50 (1H, d, J = 12.1 Hz/11.6 Hz, H-I-3), 3.77/3.71 (3H, s, −OCH_3), 2.98/2.67 (2H, dd, 16.1 Hz, 13.1 Hz/18.4 Hz, 11.7 Hz, H-II-3); ^{13}C NMR (30 °C, CD_3OD, 100 MHz) (most signals are duplicated due to atropisomerism) δ 196.8/196.5 (C, C-I-4), 195.8/195.6 (C, C-II-4), 166.3 (C, C-I-7), 165.1 (C, C-II-7), 163.7, 163.6, 162.7, 162.6, 162.4, 162.0, 160.5, 159.6 (C, C-I-5, C-II-5, C-I-9, C-II-9), 131.0/130.8 (C, C-II-1′), 128.9/128.7 (CH, C-I-2′), 128.1/128.0 (C, C-I-1′), 127.6/126.8 (CH, C-II-2′), 114.6 (CH, C-I-3′), 113.9/113.6 (CH, C-II-3′), 101.4 (C, C-I-10, C-II-8, C-II-10), 96.0 (CH, C-I-6), 95.6/95.2 (CH, C-II-6), 95.0 (CH, C-I-8), 81.8/81.2 (CH, C-I-2), 78.0 (CH, C-II-2), 55.1/55.1 (CH_3, −OCH_3), 47.5/47.3 (CH, C-I-3), 43.0/42.6 (CH_2, C-II-3); HRESIMS *m*/*z* 557.1459 ([M + H]$^+$) (calcd for $C_{31}H_{25}O_{10}$, 557.1448); CD (MeOH) $\{\theta\}_{341}$ +2.44, $\{\theta\}_{322}$ −0.85, $\{\theta\}_{297.5}$ −7.11, $\{\theta\}_{277.4}$ 5.69.

(+)-Volkensiflavone (7): $[\alpha]^{30}_D$ 18 (*c* 0.22, MeOH); ^{1}H NMR, ^{13}C NMR, and MS as reported.[7,13]

(+)-Morelloflavone (8): $[\alpha]^{30}_D$ 202 (*c* 0.26, MeOH); ^{1}H NMR, ^{13}C NMR, and MS as reported.[10,14]

In Vitro Activity against *Plasmodium falciparum*. For the determination of the antiplasmodial activity, the parasite lactate dehydrogenase assay was used, as previously described by Makler et al.,[15] with slight modifications. The assay is based on the observation that the lactate dehydrogenase (LDH) enzyme of *P. falciparum* has the ability to rapidly use 3-acetyl pyridine NAD (APAD) as a coenzyme in the reaction leading to the formation of pyruvate from lactate. Test compounds were added in 4-fold serial dilutions in 96-well multiwell plates to *P. falciparum* (chloroquine-sensitive Ghana strain) cultures (1% parasitemia, 2% HCT). After 48 h at 37 °C, 20 μL of the lysed

Table 1. Antiprotozoal Activity and Cytotoxicity of Compounds **1**−**8**[a] (IC_{50} ± SD, μM) ($n = 3$ unless indicated otherwise)

compound	*Trypanosoma brucei brucei*	*Trypanosoma cruzi*	*Leishmania infantum* ($n = 1$)	*Plasmodium falciparum*	cytotoxicity (MRC-5 cells)
1	5.0 ± 3.0	8.0 ± 3.0	>64	52 ± 17 ($n = 2$)	>64
2	2.0 ± 0.1 ($n = 2$)	5.7 ± 6.4	>64	59.0 ± 8.7	>64
3	0.87 ± 0.23	7.0 ± 0.1 ($n = 2$)	27.0	10.0 ± 0.1 ($n = 2$)	>32
4	0.4 ± 0.1	4.0 ± 2.8 ($n = 2$)	32.0 (T)[b]	6.7 ± 1.5	2.0 ± 0.1
5	7.7 ± 1.1	39.2 ± 23.2	>64	>64	52.3 ± 5.5
6	28.3 ± 2.9	34.7 ± 4.6	>64	6.0 ± 1.7	38.0 ± 2.6
7	37.0 ± 1.0	56.0 ± 13.9	>64	48.0 ± 14.2	40.0 ± 4.4

[a] (+)-Morelloflavone (**8**) was inactive (IC_{50} > 64 μM). [b] T: toxic effect on infected macrophages.

culture was added to 100 μL of Malstat reagent (Flow Inc.). Adding 40 μg of nitroblue tetrazolium (NBT) and 2 μg of phenazine ethosulfate (PES) to the Malstat reagent enabled spectrophotometric (650 nm) assessment of LDH levels, and IC_{50} values were calculated.

In Vitro Activity against *Trypanosoma brucei* Trypomastigotes. IC_{50} values against *T. brucei* were determined as described before.[16] Briefly, bloodstream forms of *T. brucei* were cultivated in HMI-9 medium. In a 96-well microplate, 10 000 haemoflagellates were incubated at different concentrations of the test compound for 4 days. Parasite multiplication was measured fluorimetrically after addition of resazurin (excitation 530 nm, emission 590 nm).[17]

In Vitro Activity against Intracellular *Trypanosoma cruzi* Amastigotes. IC_{50} values against *T. cruzi* were determined as described by Buckner et al.[18] Briefly, primary mouse peritoneal macrophages were seeded in 96-well microplates at 30 000 cells/well. After 24 h, about 10 000 trypomastigotes of *T. cruzi* were added per well together with 4-fold dilutions of the drug. The cultures were incubated at 37 °C in 5% CO_2−95% air for 7 days, and parasite growth is assessed after adding 50 μL/well CPRG (chlorophenol red β-D-galactopyranoside) as substrate. Color change was measured spectrophotometrically at 580 nm after 4 h incubation at 37 °C. The results were expressed as % reduction in parasite burden compared to control wells, and an IC_{50} was calculated.

In Vitro Activity against Intracellular *Leishmania infantum* Amastigotes. *L. infantum* MHOM/MA(BE)/67 was maintained in the Syrian hamster by serial passage to obtain amastigotes for infection. Murine peritoneal macrophages were induced by intraperitoneal administration of 2 mL of 2% starch (aq). The peritoneal cells were harvested about 24−48 h later with cold RPMI 1640 supplemented with antibiotics (penicillin−streptomycin). Starting from the 20 mM stock solution, compounds were 4-fold serially diluted in sterile 96-well microtiter tissue culture plates, with each well containing 10 μL of the watery compound dilution together with 190 μL of macrophage/parasite inoculum (1.5×10^5 macrophages/mL and 1.5×10^6 parasites/mL in RPMI-1640 medium, supplemented with 20 mM L-glutamine, 16.5 mM $NaHC0_3$, 5% fetal calf serum, and 2% P/S-solution). After 5 days of incubation, parasite growth was assessed microscopically after Giemsa staining. The results are expressed as % reduction of parasite burden compared to control wells, and an IC_{50} (50% inhibitory concentration) was determined.

Cytotoxicty on MRC-5 Cells. A human diploid embryonic lung cell line (MRC-5) was used to assess the cytotoxicity of the test compounds. MRC-5 cells were seeded at 5000 cells/well in 96-well microtiter plates. After 24 h, the cells were washed and 4-fold dilutions of the drug were added in 200 μL of standard culture medium (RPMI + 5% FCS). The final DMSO concentration in the culture remained below 0.5%. The cultures were incubated with different concentrations of test compounds at 37 °C in 5% CO_2−95% air for 7 days. Untreated cultures were included as controls. Cell viability was assessed after addition of Alamar-Blue (5 μL of a 1/10 solution/well), and fluorescence was measured (550 nm excitation, 590 nm emission) after 4 h incubation at 37 °C.[19] The results are expressed as % reduction in cell viability compared to untreated control wells.

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