Antimalarial Activity of Pyrroloiminoquinones from the Australian Marine Sponge *Zyzzya* sp.

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ABSTRACT: A new bispyrroloiminoquinone alkaloid, tsitsikammamine C (**1**), displayed potent *in vitro* antimalarial activity with IC₅₀ values of 13 and 18 nM against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *Plasmodium falciparum*, respectively. Tsitsikammamine C (**1**) displayed selectivity indices > 200 against HEK293 cells and inhibited both ring and trophozoite stages of the malaria parasite lifecycle. Previously reported compounds, makaluvamines J (**2**), G (**3**), L (**4**), K (**5**) and damirones A (**6**) and B (**7**) were also isolated from the same marine sponge (*Zyzya* sp.). Compounds **2-4** displayed potent growth inhibitory activity (IC₅₀ values <100 nM) against both *P. falciparum* lines and only moderate cytotoxicity against HEK293 cells (IC₅₀ value ~1-4 μ M). Makaluvamine G (**3**) was not toxic to mice and suppressed parasite growth in *P. berghei*-infected mice following subcutaneous administration at 8 mg/kg/day.

INTRODUCTION

Malaria is an infectious disease caused by parasites belonging to the genus *Plasmodium*. It is estimated that over 3.2 billion people living in tropical and subtropical regions of the world, such as Sub-Saharan Africa, Central and South America, the Middle East, India and South East Asia are at risk from this disease.¹ Approximately 400 million clinical cases and 800,000 deaths are recorded annually, with more than 90% of malaria cases and the majority of malaria deaths occurring in Sub-Saharan Africa.¹ The frontline defence against malaria is drug prophylaxis or treatment, however, this is under threat due to parasite drug resistance² or decreased clinical efficacy of all antimalarial drugs currently used.^{3, 4} Consequently, new antimalarial drugs are urgently needed in order to combat the global problem of parasite drug resistance.

The basis for the wide ranging biological activity of natural products can be explained by the similarity of the biosynthetic imprint, the molecular recognition between substrate and biosynthetic enzyme, and the natural product binding motif, the molecular recognition

between a ligand and its therapeutic target, that led to the concept of protein fold topology (PFT).⁵ PFT describes cavity recognition points unrelated to fold and sequence similarity, and defines a natural product's ability to recognize biology space.⁶⁻⁸ Natural products have played a key role in antimalarial drug discovery and therapy.⁹⁻¹² Well-known antimalarial natural products include quinine and artemisinin, which were first isolated from the South-American "quinine bark" (*Cinchona succiruba*) and the Chinese "sweet wormwood" (*Artemisia annua*), respectively.^{13, 14} Numerous antimalarial drugs, such as artesunate, artemether, chloroquine, mefloquine, and halofantrine, have all been developed based on the pharmacophore present in quinine or artemisinin.^{14, 15} Artemisinin-based combination therapy (ACT) is currently recommended for first-line treatment of *P. falciparum* malaria worldwide. However, recent reports of decreased efficacy of the ACT artesunate plus mefloquine and artesunate monotherapy in Western Cambodia is of immense concern.⁴ Furthermore, poor safety profiles and undesirable side-effects of many current antimalarials are additional reasons for the need of new small molecule therapies.

In efforts to identify drug-like natural products following high-throughput screening (HTS) campaigns, we have shown it is possible to develop a library of drug-like natural products.¹⁶ In order to increase the diversity, we recently reported a procedure to capture natural products with $\text{LogP} < 5$ in extracts.¹⁷ The extracts of over 18,000 marine and terrestrial biota samples were then fractionated (11 fractions collected per sample). In this study we undertook HTS¹⁸ of the pre-fractionated natural product library in order to identify new antimalarial lead compounds. Analysis of the HTS data identified five fractions derived from the marine sponge *Zyzzya* sp. (Acanthidae) that demonstrated promising growth inhibition activity against *P. falciparum* 3D7 parasites and showed no cytotoxicity against a human embryonic kidney cell line (HEK293). Bioassay-guided fractionation on the large-scale $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ extract of *Zyzzya* sp. (10 g dry wt) resulted in the purification of a new bispyrroloiminoquinone alkaloid, tsitsikammamine C (**1**), along with the previously isolated compounds,

makaluvamines J (**2**) and G (**3**). Subsequent chemical investigations on larger quantities of the CH₂Cl₂/CH₃OH extract derived from *Zyzzya* sp. (145 g dry wt) resulted in the purification of greater quantities of **1-3**, along with the isolation of the known compounds, makaluvamines L (**4**) and K (**5**) and damirones A (**6**) and B (**7**). Herein we report the isolation, and structure elucidation of tsitsikammamine C (**1**) along with *in vitro* antimalarial activity and mammalian cell toxicity studies for compounds **1-7**. DMPK profiling on compounds **2** and **3**, along with *in vivo* tolerability and antimalarial efficacy studies in mice are also described.

RESULTS AND DISCUSSION

The freeze-dried and ground *Zyzzya* sp. (10 g dry wt) was extracted with *n*-hexanes, 4:1 CH₂Cl₂/CH₃OH, and CH₃OH. The CH₂Cl₂/CH₃OH and CH₃OH extracts were combined and chromatography performed using C₁₈ bonded silica HPLC (CH₃OH/H₂O/0.1% TFA) to yield makaluvamine J (**2**, 2.3 mg, 0.023% dry wt) and two other semi-pure bioactive fractions. Both of these fractions were further purified by C₁₈ bonded silica HPLC (CH₃OH/H₂O/0.1% TFA) to afford tsitsikammamine C (**1**, 0.2 mg, 0.002% dry wt), and makaluvamine G (**3**, 0.4 mg, 0.004% dry wt). Due to the promising activity profile for compounds **1-3**, larger quantities of these secondary metabolites, and potentially related analogues were sought. Further large-scale extraction and isolation on the remaining freeze-dried and ground *Zyzzya* sp. (145 g) followed by extensive C₁₈ bonded silica HPLC (CH₃OH/H₂O/0.1% TFA) yielded more of tsitsikammamine C (**1**, 9.0 mg, 0.006% dry wt), makaluvamines J (**2**, 30 mg, 0.021% dry wt) and G (**3**, 24 mg, 0.017% dry wt), along with the additional, and previously isolated compounds, makaluvamines L (**4**, 3.0 mg, 0.002% dry wt) and K (**5**, 6.9 mg, 0.004% dry wt), and damirones A (**6**, 3.3 mg, 0.002% dry wt) and B (**7**, 0.7 mg, 0.0005% dry wt). Compounds **2-7** were all determined to be the TFA salts of makaluvamines J, G, L and K, and damirones A and B, respectively, following spectroscopic data comparison with literature values.¹⁹⁻²¹

The TFA salt of tsitsikammamine C (**1**) was isolated as a stable pink film. The molecular formula of the quaternary ammonium cation of **1** was determined to be $C_{20}H_{18}N_3O_2^+$ by (+)-HRESIMS of the $[M-TFA]^+$ ion at m/z 332.1390. Compound **1** displayed maximum absorptions at 243, 297 and 343 nm in the UV spectrum that were consistent with a pyrroloiminoquinone substructure and a disubstituted aromatic ring.^{22, 23} A bathochromic shift was identified in the UV spectrum of **1** on addition of base suggesting that the new compound contained a phenol. Analysis of the 1H NMR (Table 1) and COSY spectra of **1**, allowed three spin systems to be assigned, these included a *para*-substituted phenyl substructure [δ_H 7.26 (2H, d, $J = 8.4$ Hz) and 6.85 (2H, d, $J = 8.4$ Hz)], two contiguous methylenes [δ_H 2.99 (2H, t, $J = 7.8$ Hz) and 3.98 (2H, t, $J = 7.8$ Hz)] and a $-NH-CH=$ moiety [δ_H 13.46 (1H, s) and 7.31 (1H, s)]. The remaining unassigned proton signals included an isolated aromatic singlet at δ_H 7.16 (1H, s), and two methyl singlets at δ_H 2.98 (s, 3H) and 3.95 (s, 3H). The ^{13}C NMR spectrum of **1** showed 18 unique signals that resonated between δ_C 18 and 167. HSQC data analysis allowed all 16 non-exchangeable protons to be assigned to their directly attached carbons; this identified that **1** contained 10 quaternary carbons. HMBC correlations (Figure 2) from the methine singlet proton (δ_H 7.31), associated with $-NH-CH=$ moiety, to carbons at δ_C 127.6, 113.9 and 135.0 indicated a 2,3,4-trisubstituted pyrrole system.^{24, 25} A second 2,3,4-trisubstituted pyrrole moiety, that was *N*-methylated, was also established on the basis of HMBC correlations from the methyl singlet protons at δ_H 3.95 to carbons at δ_C 125.2 and 127.6, and the methine singlet at δ_H 7.16 to carbons resonating at δ_C 35.8, 125.2, 123.3, and 117.5. Furthermore, the *N*-methyl pyrrolic system was shown to be substituted by the previously identified 1,2-disubstituted ethane unit [δ_H 2.99 / 3.98] via HMBC correlations from the protons at δ_H 2.99 to carbons at δ_C 117.5 and 127.6. A strong ROESY correlation between δ_H 2.99 and 7.16 further supported this substructure assignment. An *N*-methyl iminium moiety was elucidated and attached to the ethane unit following HMBC data analysis for the proton resonance at δ_H 2.98. Although no phenolic signal was observed in the 1H NMR

spectrum of **1**, HMBC correlations from both of the aromatic methine doublet protons associated with the *para*-substituted phenyl ring to an oxygenated quaternary carbon at δ_C 157.3, in combination with the MS data, indicated the presence of a 4-hydroxyphenyl group.²⁶ At this stage all atoms associated with the molecular formula of the quaternary ammonium cation of **1** had been identified, however HMBC data did not show sufficient overlap of correlations to the quaternary carbons associated with **1** to link all the partial fragments. Analysis of the natural product bispyrroloiminoquinone literature, in conjunction with biosynthetic considerations, and the 14 degrees of unsaturation required by the molecular formula of **1**, identified that the new compound had to contain a tetracyclic skeleton, such as that present in tsitsikammamines A (**8**) and B (**9**).^{22, 23} ^1H and ^{13}C NMR data comparison (DMSO- d_6) of **1** with **8** and **9**, showed only minor differences and suggested that **1** was the 18-methyl analog of tsitsikammamine B.^{22, 23} Confirmation of the structure of **1**, was further supported by a $^3J_{\text{CH}}$ correlation from the proton at δ_H 7.26 to the β -pyrrole carbon at δ_C 127.5, as well as a strong ROESY correlation between these protons and the *N*-methyl iminium protons at δ_H 2.98. Therefore, the structure of **1** was assigned to tsitsikammamine C.

Makaluvamines A-F were first isolated from the Fijian sponge, *Zyzzya* c.f. *marsailis*, in 1993 and were tested for mammalian topoisomerase II inhibition and cytotoxicity in HCT-116 (human colon) and xrs-6 (Chinese hamster ovary) cells.²⁷ Using a decatenation inhibition assay only makaluvamines A and F were shown to inhibit mammalian topoisomerase II, with IC_{90} values of 41 and 25 μM , respectively.²⁷ Cytotoxicity data for makaluvamines A-F also varied greatly, with IC_{50} values against HCT-116 cells ranging from 0.17 to >50 μM .²⁷ In a similar manner, cytotoxicity data for makaluvamines A-F against xrs-6 cells had IC_{50} s in the range of 0.08-13.49 μM .²⁸ The pyrroloiminoquinone substructure has not only been isolated from *Zyzzya* spp.^{19, 27, 29-31} but have also routinely been purified from sponges belonging to the genera *Batzella*,³²⁻³⁵ *Damiria*,²¹ *Histodermella*,²⁰ *Latrunculia*,³⁶⁻⁴⁰ *Negombata*,⁴¹ *Prianos*,³⁸

Spongosorites,⁴² *Higginsia*⁴² and *Tsitsikamma*.^{22, 23} Other marine organisms such as ascidians, have also been the sources of this structure class.⁴³ Pyrroloquinone-derived natural products have been shown to display a remarkable array of biological activities, and as a result a number of synthetic methodologies have been developed to prepare either the natural products or analogs.⁴⁴⁻⁴⁶ Tsitskammamines A and B also belong to this important structure class; these metabolites were first isolated from a latrunculid sponge collected in South African waters,²³ and were shown to exhibit cytotoxicity and mammalian topoisomerase I inhibition. Subsequent re-investigation of other South African latrunculid sponges yielded *N*-18 oxime analogs of tsitskammamines A and B.²² These derivatives displayed significantly reduced cytotoxicity against HCT-116 cells compared to the parent alkaloids. Tsitsikammamines A and B have been synthesized and analogs reported.^{47, 48}

Compounds **1-7** were all tested *in vitro* against the chloroquine-sensitive 3D7 and chloroquine-resistant Dd2 *P. falciparum* lines. The Dd2 line is also mefloquine-resistant. Cytotoxicity data was also obtained for **1-7** using a human embryonic kidney cell line (HEK293). In order to assess the drug-like and lead-like properties of alkaloids **1-7**, *in silico* physicochemical properties were calculated using Instant JChem software,⁴⁹ and the data compared to Lipinski's drug-like "rule of five",⁵⁰ and Hann and Oprea's lead-like criteria.⁵¹ All compounds complied with Lipinski's rules (log P <5, HBA < 10, HBD <5, MW <500), and Hann-Oprea guidelines (log P <4, HBA <9, HBD <5, MW <460, rotatable bonds <10). The biological activity, selectivity indices, and *in silico* calculated physicochemical properties of compounds **1-7** are detailed in Table 2. Compounds **1-4** had IC₅₀ values <100 nM against both the 3D7 and Dd2 lines and showed moderate cytotoxicity against the HEK293 cell line, with IC₅₀ values in the range of 1.1-3.6 μ M. The new compound, tsitsikammamine C (**1**) showed the most promising *in vitro* biological profile with IC₅₀ values of 13 nM and 18 nM against the 3D7 and Dd2 lines, respectively. Importantly, of the four most active constituents,

compound **1** also displayed the least toxicity against the HEK293 cell line, with an IC₅₀ of 3.6 μ M.

A number of noteworthy structure-activity relationships (SARs) were identified following analysis of the *in vitro* biological activity for compounds **1-7**. For the makaluvamine series (**2-5**) methylation of the iminium nitrogen translates to better antimalarial activity, while methylation of the pyrrolo moiety appears not to affect the biological output. The *N*-methyl iminium analogs **2-4** all display IC₅₀ values <41 nM (3D7) compared to the protonated iminium makaluvamine K (**5**), with an IC₅₀ value of 396 nM (3D7). *N*-methylation of the iminium moiety of the makaluvamine series increased antimalarial activity from 9.9 to 30.5-fold compared to the non-methylated skeleton present in **5**. A similar trend in activity was also observed for the makaluvamine series **2-5** in the Dd2 *P. falciparum* line.

Comparing the makaluvamine analogs **2-5** to the simplified 1,2-dicarbonyl tricyclic compounds, damirones A (**6**) and B (**7**), showed an almost complete loss of malaria growth inhibition, indicating that the iminoquinone of **2-5** rather than the 1,2-dicarbonyl of **6-7** is essential for antimalarial activity.

Furthermore, when comparing the biological data of **2-5** that all have more than two rotatable bonds in their *N*-alkyl side chain to tsitsikammamine C (**1**) the data indicates that restricting the configuration of the pendant phenol motif improved activity slightly. This suggests that an out-of-plane conformation of the phenol aromatic ring due to steric interactions with the methyl on the iminium nitrogen is preferred and establishes the likely preferred conformation.

Compounds **1-3** were also examined for stage-specific activity against ring versus trophozoite stage *P. falciparum* 3D7 parasites following exposure times of 4, 6, and 10 h. Compounds **1** and **2** displayed similar activity profiles, with greatest growth inhibition of both rings and trophozoites observed after 10 h exposure (Figure 3 A-D). In contrast, compound **3**

was more effective in inhibiting the growth of trophozoite stage parasites than rings, with no significant inhibition observed when ring stage parasites were exposed to up to ~10 times the IC₅₀ concentrations of this compound for up to 10 h (Figure 3 E and F). Most clinically used antimalarial drugs that target blood-stage *P. falciparum* parasites do not act on multiple life cycle stages. An exception to this is the rapidly acting artemisinins, which act on both ring and trophozoite stage parasites. Thus our finding that compounds **1** and **2** have, at least *in vitro*, similar activity against rings and trophozoites is very promising for future development of this class of compounds for malaria.

Sufficient quantities of compounds **2** and **3** were available for additional biological studies to be performed. First, *in vitro* studies in hepatic microsomes were conducted to assess the extent of metabolic degradation and then *in vivo* studies were conducted in mice to determine the systemic exposure following subcutaneous administration. In both human and mouse liver microsomes, compound **3** was more rapid degraded compared than **2**, suggesting that the *in vivo* hepatic clearance would be higher for **3** than for **2** (Table 3). There was also evidence for a higher rate of degradation of **2** in mouse microsomes relative to human. *In vivo* studies were conducted using an abbreviated sampling protocol with only three time points and two mice per time point due to the limited availability of each of the compounds. Each compound was administered at a dose level of 8 mg/kg in 0.1 mL of a formulation composed of 5-7 % (v/v) DMSO in saline. At this formulation concentration (~2.2 mg/mL for **2** and ~2.6 mg/mL for **3**) compound **2** was a solution while **3** was a fine suspension. Plasma concentrations of **2** reached approximately 1.7 μ M at 1 h post-dose and declined over the 4 h post-dose sampling period. In comparison, plasma concentrations of **3** were consistently low (less than 0.04 μ M) over the same duration. Even with this limited data, it was evident that the systemic exposure of **3** was markedly lower than that for **2** (Figure 4). Assuming that hepatic metabolism is a major determinant of the *in vivo* clearance of these two compounds, this result is consistent with the

in vitro microsomal stability data which suggested **2** was more metabolically stable than **3**. Furthermore, the observed differences in plasma exposure may also result from differences in the rate and/or extent of absorption, given that **2** was dosed as a solution, whereas **3** was dosed as a suspension.

Based on the DMPK data, *in vivo* tolerability and efficacy studies were subsequently undertaken on makaluvamines J (**2**) and G (**3**). Due to the limited supply of each compound, the maximum dosage assessed for tolerability was 8 mg/kg/day for 4 days. No physical distress was seen in healthy mice administered subcutaneously with makaluvamine J up to 4 mg/kg/day, however at 8 mg/kg/day the mice showed reduced activity, ruffled coat and weight loss >10%. In contrast, makaluvamine G was well tolerated in mice at 8 mg/kg/day. When compound **2** was tested for efficacy at 4 mg/kg/day, no parasite suppression was observed in mice infected with *P. berghei* using the Peter's 4-day test.⁵² In contrast, when administered at 8 mg/kg/day, compound **3** suppressed *P. berghei* infection by 10% on day 1 post infection (p.i.), and by 35%, 47%, and 48% at day 2, 3, and 4 p.i., respectively, relative to the control group. The comparator, chloroquine, suppressed *P. berghei* infection by 35% at day 4 p.i., which is in accord with previous findings, with ED₅₀ (effective dose at 50% suppression) of 1.9 mg/kg/day.⁵² The apparent *in vivo* activity of **3** is interesting in light of the low exposure in mice after oral dosing, raising the possibility that there could be an active metabolite for this compound. Further work to characterize the metabolic profile of **3** would be required to explore this hypothesis.

The *in vivo* data for makaluvamine G (**3**) indicates that this structure class has promising antimalarial activity. The new metabolite tsitsikammamine C (**1**) displayed 2-3 fold greater *in vitro* potency, had selectivity indices in excess of 200 and inhibited both ring and trophozoite stages of the parasite life cycle compared to makaluvamine G (**3**) that had selectivity indices

around 50 and mainly inhibited the trophozoite stage. Synthesis focused on the tsitsikammamine motif is warranted in order to enable further antimalarial evaluation.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were recorded on a Jasco V-650 UV/Vis spectrophotometer. NMR spectra were recorded at 30 °C on Varian INOVA 500 and 600 MHz NMR spectrometers. The latter spectrometer was equipped with a triple resonance cold probe. The ^1H and ^{13}C NMR chemical shifts were referenced to the solvent peak for DMSO- d_6 at δ_{H} 2.49 and δ_{C} 39.5. Standard parameters were used for the 2D experiments, which included gCOSY, gHSQC ($^1J_{\text{CH}} = 140$ Hz) and gHMBC ($^nJ_{\text{CH}} = 8.3$ Hz). LRESIMS were recorded on a Waters ZQ mass spectrometer. HRESIMS were measured on a Bruker Daltonics Apex III 4.7e Fourier Transform Mass Spectrometer, fitted with an Apollo API source. A Thermo Scientific C₁₈ Betasil 5 μm 143 Å column (21.2 mm $\times$ 150 mm) was used for semi-preparative HPLC. A Waters 600 pump fitted with a 996 Photodiode Array Detector and 717 plus Autosampler was used for the semi-preparative HPLC separations. End-capped Septra C₁₈ bonded silica (Phenomenex) was used for preadsorption work. A BIOLINE orbital shaker was used for the large-scale extraction of sponge material. All compounds were analyzed for purity by analytical HPLC using a Phenomenex C₁₈ ONYX column (4.6 mm $\times$ 100 mm) and shown to be >95%. Water was Millipore Milli-Q PF filtered, while all other solvents used were Lab-Scan HPLC grade. Parasite strains 3D7 and Dd2 were from the Queensland Institute of Medical Research. O+ Erythrocytes were obtained from the Australian Red Cross Blood Service. Cell Carrier polylysine coated imaging plates were from PerkinElmer. 4',6-Diamidino-2-phenylindole (DAPI) stain and Alamar Blue were from Invitrogen. Triton-X, saponin, puromycin, chlorquine and artemisinin were all from Sigma

Aldrich. HEK293 cells were purchased from the American Tissue Culture Collection. The 384-well Falcon sterile tissue culture treated plates were from BD.

Sponge Material. The sponge *Zyzya* sp. (Acanthidae) was collected by SCUBA diving (-43 m) at Rodda Reef, Queensland, Australia on 30 June 2003, and kept frozen prior to freeze-drying and extraction. A voucher sample (G320528) has been lodged at the Queensland Museum, Brisbane, Australia. A description of the sponge and a photographic image can be found in the Supporting Information.

Extraction and Isolation. The freeze-dried and ground sponge (10 g) was sequentially extracted with *n*-hexane (250 mL), CH₂Cl₂/CH₃OH (4:1, 250 mL) and CH₃OH (250 mL × 2). All CH₂Cl₂/CH₃OH and CH₃OH extractions were combined and dried down under reduced pressure to yield a brown solid (0.32 g). This extract was subsequently preadsorbed to C₁₈-bonded silica (1.0 g), then packed into a stainless steel guard cartridge (10 × 30 mm) and attached to a C₁₈ semi-preparative HPLC column. Isocratic HPLC conditions of 90% H₂O (0.1% TFA)/10% CH₃OH (0.1% TFA) were initially employed for the first 10 min, then a linear gradient to CH₃OH (0.1% TFA) was run over 40 min, followed by isocratic conditions of CH₃OH (0.1% TFA) for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min then analyzed using the antimalarial bioassay. Activity was identified in fractions 31-39. ¹H NMR and LRESIMS analysis of these bioactive fractions showed that fraction 32 contained pure makaluvamine J (**2**, 2.3 mg, 0.023% dry wt), while fraction 31 (4.6 mg) and fractions 36-39 (21.6 mg) contained semi-pure mixtures of related natural products. Both of these fractions were further purified by semi-preparative C₁₈ HPLC using identical conditions to those described above to afford tsitsikammamine C (**1**, 0.2 mg, 0.002% dry wt), and makaluvamine G (**3**, 0.4 mg, 0.004% dry wt). Further large-scale

extraction and isolation work was subsequently undertaken on the remaining 145 g of the freeze-dried and ground sponge material. Identical extraction protocols to those used for the 10 g sample were employed, albeit larger volumes of solvent were used. The resulting CH₂Cl₂/CH₃OH and CH₃OH extractions were combined then subjected to extensive C₁₈ bonded silica HPLC (CH₃OH/H₂O/0.1% TFA), using MS and UV data to guide purification. This yielded more of tsitsikammamine C (**1**, 9.0 mg, 0.006% dry wt), makaluvamines J (**2**, 30 mg, 0.021% dry wt) and G (**3**, 24 mg, 0.017% dry wt), along with the additional, and previously reported compounds, makaluvamines K (**4**, 3.0 mg, 0.002% dry wt) and L (**5**, 6.9 mg, 0.004% dry wt), and damirones A (**6**, 3.3 mg, 0.002% dry wt) and B (**7**, 0.7 mg, 0.0005% dry wt).

TFA Salt of Tsitsikammamine C (1): isolated as a pink gum; UV (CH₃OH) λ_{max} (log ϵ) 243 (3.91), 197 sh (3.45), 343 (2.93) nm; ¹H and ¹³C NMR data see Table 1; (+)-LRESIMS m/z (rel. int.) 332 (100) [M-TFA]⁺; (+)-HRESIMS m/z 332.1390 [M-TFA]⁺ (calcd for C₂₀H₁₈N₃O₂, 332.1394 Δ 1.1 ppm).

***In vitro* Antimalarial Assay.** Compounds were incubated in the presence of 2 or 3% parasitemia (3D7 or Dd2) and 0.3% hematocrit in a total assay volume of 50 μ L, for 72 h at 37°C and 5% CO₂, in poly-*D*-lysine coated CellCarrier Imaging plates. After incubation plates were stained with DAPI in the presence of saponin and Triton X-100 and incubated for a further 5 h at room temperature in the dark before imaging on the OPERA HTS confocal imaging system (PerkinElmer). The digital images obtained were analyzed using the PerkinElmer Acapella spot detection software where fluorescent spots which fulfill the criteria established for a stained parasite are counted. The percent inhibition of parasite replication was calculated using DMSO and 2 μ M artemisinin control data. Artemisinin [IC₅₀

= 0.021 μ M (Dd2); IC₅₀ = 0.021 μ M (3D7)] and chloroquine [IC₅₀ = 0.130 μ M (Dd2); IC₅₀ = 0.025 μ M (3D7)] were used as positive controls.

***In vitro* Stage Specific Assays.** Synchronous *P. falciparum* 3D7 ring (~10-16 h post invasion) or trophozoite (~24-30 h post invasion) stage parasites (0.5% parasitemia; 5% hematocrit) were incubated with $\sim 5 \times \text{IC}_{50}$ or $\sim 10 \times \text{IC}_{50}$ concentrations of each compound, or vehicle control (0.04% DMSO), for 4, 6, and 10 h. After gently washing with pre-warmed parasite media, cultures were seeded into triplicate wells of a 96 well tissue culture plate and 0.5 μ Ci ³H-hypoxanthine added per well. ³H-hypoxanthine incorporation was determined after ~48-72 h by harvesting onto 1450 MicroBeta filter mats (Wallac) and counting using a 1450 MicroBeta liquid scintillation counter. Percentage growth compared to the matched untreated vehicle controls was determined. At least two independent experiments were performed for each treatment. Concentrations of compounds used were as follows: compound **1** at 204 and 408 nM; compound **2** at 125 and 250 nM, and compound **3** at 180 and 360 nM. Artemisinin was used as a positive control at 75 and 150 nM.

***In Vitro* Cytotoxicity Assay.** Compounds were added to Falcon 384 well black/clear tissue treated assay plates containing 3000 adherent cells/well (HEK293) in an assay volume of 45 μ L. The plates were incubated for 72 h at 37 °C and 5% CO₂. After incubation the supernatant was aspirated out of the wells and 40 μ L of 10% Alamar Blue added per well. Plates were incubated for a further 5-6 h and measured for fluorescence at 535 nm excitation and 590 nm emission using a VICTOR II (PerkinElmer). The percent inhibition of cell growth was calculated using DMSO and 10 μ M puromycin control data. IC₅₀ values were obtained by plotting % inhibition against log dose using Prism4 graphing package and non-linear regression with variable slope plot.

DMPK Assays.

***In vivo* Pharmacokinetic Studies.** Pharmacokinetic studies in mice were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the study protocols were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. The systemic exposures of compounds **2** and **3** were studied following subcutaneous administration at a nominal dose of 8 mg/kg to non-fasted male Swiss outbred mice weighing 26 – 34 g. Mice had access to food and water continuously throughout the pre- and post-dose phases of the study. Compounds were administered subcutaneously into the dorsal skinfold of each mouse via a 29G × ½" syringe, as either a solution (**2**) or suspension (**3**) in a saline based vehicle containing 5 – 7% DMSO (0.1 mL dose volume per mouse). At 0.25, 1 or 4 h post-dose, blood samples were collected from mice (n = 2 mice per time point for each compound) via cardiac puncture (following anaesthetization with gaseous isoflurane). Blood was transferred to heparinised tubes containing a stabilization cocktail (Complete® inhibitor cocktail, potassium fluoride and EDTA) to minimize the potential for *ex vivo* degradation of compound. Samples were immediately centrifuged to collect plasma for analysis. Quantitative analysis of **2** and **3** in plasma was conducted by UPLC-MS (Waters Corporation Acquity UPLC coupled to a Micromass Quattro Premier triple quadrupole MS) against calibration standards prepared in blank mouse plasma. All samples and standards were processed by precipitation with acetonitrile, followed by centrifugation and analysis of the supernatant. The analytical lower limits of quantitation in plasma were 0.016 µM for compound **2** and 0.006 µM for compound **3**.

***In vitro* Microsomal Stability Assay.** Compounds were incubated at 37 °C and 1 µM concentration in human or mouse liver microsomes (BD Gentest) suspended in 0.1 M

phosphate buffer (pH 7.4) at a final protein concentration of 0.4 mg/mL. Metabolic reactions were initiated by the addition of an NADPH-regenerating system (1 mg/mL NADP, 1 mg/mL glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase) and MgCl₂ (0.67 mg/mL) and were quenched at various time points up to 60 min by the addition of ice-cold acetonitrile. Quenched samples were centrifuged and the relative loss of parent compound over the course of the incubation (and formation of potential metabolites) was monitored by LC-MS using a Micromass QTOF mass spectrometer (Waters Corporation). Concentration versus time data for each compound were fitted to an exponential decay function to determine the first order rate constant for substrate depletion which was then used to calculate the degradation half-life, an *in vitro* intrinsic clearance value (*in vitro* CL_{int}) and subsequently a predicted *in vivo* hepatic CL_{int} value according to the methods of Obach,⁵³ *In vivo* hepatic CL_{int} values were converted to predicted *in vivo* hepatic extraction ratio (E_H) using the following equation: $E_H = CL_{int} / (Q + CL_{int})$ where Q is liver blood flow, which was assumed to be 20.7 mL/min/kg and 90 mL/min/kg for humans and mice, respectively.⁵⁴

***In Vivo* Efficacy Testing.** The animal studies were approved by the Army Malaria Institute Animal Ethics Committee (AEC No. 02/08) in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Male and female Swiss albino mice (7-9 weeks old from Animal Resource Centre, Murdoch, Western Australia) weighing 25-35 g were used for both tolerability and efficacy assessment. Compounds were initially dissolved in Tween 80/ethanol (7/3 v/v) and further diluted (10-fold) in distilled water prior to daily administration by the subcutaneous route (~0.2 mL) for 4 consecutive days. Chloroquine as a comparator for the efficacy studies was prepared in deionized distilled water. For tolerability healthy mice were evaluated before and after daily administration of the compounds and monitored for physical distress (i.e. weight loss >10%, ruffled coat, shaking, severe pallor, reduced activity, and behavior changes). For efficacy assessment, female mice were inoculated intraperitoneally with 20×10^6 parasitized erythrocytes with the chloroquine-

sensitive *P. berghei* ANKA strain. Mice (treatment groups of 6) were dosed daily beginning on the day of infection. The control group were administered the vehicle of Tween 80/ethanol/water (7/3/90 v/v/v). Parasitemia was determined from thin blood smears collected from the tail and stained with Giemsa. Parasitemia was determined 24 h after inoculation (D+1) and then daily for 3 consecutive days (D+2 to D+4).

ASSOCIATED CONTENT

Supporting Information. A photograph of the sponge and NMR spectra for tsitsikammamine C (**1**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

PFT, protein fold topology; ACT, Artemisinin-based combination therapy; HCT-116, human colon cells; xrs-6, Chinese hamster ovary cells; 3D7, chloroquine-sensitive *P. falciparum* cell line; Dd2 chloroquine-resistant *P. falciparum* cell line.

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Table 1. NMR Data for Tsitsikammamine C (**1**).^a

position	¹³ C, mult.	¹ H (mult., <i>J</i> in Hz)	HMBC	ROESY
1	130.0, CH	7.26 (d, 8.4)	3, 5, 7	2, 18-CH ₃
2	115.5, CH	6.85 (d, 8.4)	3, 4, 6	1
3	157.3, C			
3-OH		^b		
4	115.5, CH	6.85 (d, 8.4)	2, 3, 6	5
5	130.0, CH	7.26 (d, 8.4)	1, 3, 7	4, 18- CH ₃
6	125.0, C			
7	127.5, C			
8	125.8, CH	7.31 (d, 2.9)	7, 10, 20	9
9		13.46 (br s)		8
10	135.0, C			
11	166.5, C			
12	125.2, C			
13-CH ₃	35.8, CH ₃	3.95 (s)	12, 14	14
14	127.6, CH	7.16 (s)	12, 13-CH ₃ , 15, 21	13-CH ₃ , 16
15	117.5, C			
16	18.2, CH ₂	2.99 (t, 7.8)	14, 15, 17, 21	14, 17
17	55.1, CH ₂	3.98 (t, 7.8)	15, 16, 19	16, 18- CH ₃
18-CH ₃	46.1, CH ₃	2.98 (s)	17, 19	1, 5, 17
19	158.2, C			
20	113.9, C			
21	123.3, C			

^a Recorded in DMSO-*d*₆ at 30°C. ^b Not observed

Table 2. Physicochemical Parameters and Biological Profiles of Compounds **1-7**.

Compound	Physicochemical Parameters ^a				IC ₅₀ (nM) ^b			SI ^c	
	MW	cLogP	HBA	HBD	3D7	Dd2	HEK293	3D7	Dd2
1	332	-0.75	2	2	13	18	3,600	276	200
2	322	-1.76	3	3	25	22	1,200	48	54
3	334	-1.44	5	2	36	39	2,000	55	51
4	320	-1.67	8	3	40	21	1,400	35	66
5	321	1.98	4	2	396	300	1,100	3	4
6	216	0.89	3	0	1880	360	>120,000	>63	>333
7	202	0.67	3	1	12250	3800	>120,000	>10	>32
Chloroquine	319	3.93	4	1	25	130	-	-	-

^aIn silico calculations performed using Instant JChem software.⁴⁹ MW = molecular weight (Da) of free base or quaternary ammonium cation, HBA = H-bond acceptors, HBD = H-bond donors; ^b50% Inhibitory concentration *in vitro* against *P. falciparum* chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) lines; cSI = mammalian cell IC₅₀/*P. falciparum* IC₅₀.

Table 3. *In vitro* metabolism of compounds **2** and **3** in human and mouse liver microsomes

Compound	Species	Half-life min	<i>In vitro</i> CL _{int}	
			$\mu\text{L}/\text{min}/\text{mg}$ protein	E _H
2	<i>human</i>	>350	<5	<0.2
	<i>mouse</i>	42	41	0.64
3	<i>human</i>	8.8	197	0.92
	<i>mouse</i>	7.4	235	0.91

E_H – Predicted *in vivo* hepatic extraction ratio, calculated according to published methods.
53, 54

Figure 1. Chemical Structures of Natural Products **1-9**.

Figure 2. Key HMBC and ROESY correlations for compound **1**

Figure 3. Effect of compounds on *P. falciparum* ring versus trophozoite stage-parasites. Synchronous ring (A, C, E, and G) or trophozoite (B, D, F, and H) stage *P. falciparum* 3D7 parasites were exposed to $\sim 5 \times$ (black bars) or $\sim 10 \times$ (white bars) IC₅₀ concentrations of compounds for 4, 6, and 10 h. After washing and culturing for a further 48-72 h in the presence of ³H-hypoxanthine, percentage growth compared to untreated control cultures (100% growth) was determined. Additional controls received each compound continuously. Effects of artemisinin are shown as a positive control (G & H). Mean % growth ($\pm$ SD) is shown for at least 2 independent experiments for each treatment.

Figure 4. Plasma concentrations of compound **2** (squares) and compound **3** (circles) following subcutaneous administration to mice at a dose level of 8 mg/kg. Symbols represent concentrations from two individual animals at each time point.

TOC Graphic

Tsitskikammamine C

$IC_{50} = 18 \text{ nM}$, *Plasmodium falciparum* (Dd2)

$IC_{50} = 13 \text{ nM}$, *Plasmodium falciparum* (3D7)