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

Rohan A. Davis,[†] Malcolm S. Buchanan,[†] Sandra Duffy,[†] Vicky M. Avery,[†] Susan A. Charman,[‡] William N. Charman,[‡] Karen L. White,[‡] David M. Shackleford,[‡] Michael D. Edstein,[§] Katherine T. Andrews,[†] David Camp,[†] and Ronald J. Quinn*[,†]

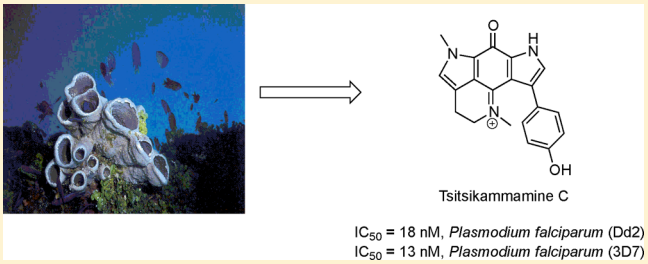
[†]Eskitis Institute, Griffith University, Brisbane, QLD 4111, Australia

[‡]Centre for Drug Candidate Optimisation, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia

[§]Australian Army Malaria Institute, Brisbane, QLD 4051, Australia

Supporting Information

**ABSTRACT:** A new bispyrroloiminoquinone alkaloid, tsitsikammamine C (**1**), displayed potent in vitro antimalarial activity with $IC_{50}$ values of 13 and 18 nM against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *Plasmodium falciparum*, respectively. Tsitsikammamine C (**1**) displayed selectivity indices of >200 against HEK293 cells and inhibited both ring and trophozoite stages of the malaria parasite life cycle. 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 (*Zyzzya* sp.). Compounds **2**−**4** displayed potent growth inhibitory activity ($IC_{50}$ < 100 nM) against both *P. falciparum* lines and only moderate cytotoxicity against HEK293 cells ($IC_{50}$ = 1−4 μM). Makaluvamine G (**3**) was not toxic to mice and suppressed parasite growth in *P. berghei* infected mice following subcutaneous administration at 8 mg $kg^{-1}$ $day^{-1}$.




## 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 Southeast 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 defense against malaria is drug prophylaxis or treatment; however, this is under threat because of parasite drug resistance[2] 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 are 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 druglike natural products following high-throughput screening (HTS) campaigns, we have shown that it is possible to develop a library of druglike natural products.[16] In order to increase the diversity, we recently reported a procedure to capture natural products with log *P* < 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 HTS[18] of the prefractionated 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*

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sp. (Acarnidae) 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 $CH_2Cl_2/CH_3OH$ extract of *Zyzzya* sp. (10 g dry wt) resulted in the purification of a new bispyrroloiminoquinone alkaloid, tsitsikammamine C (**1**, Figure 1), along with the previously

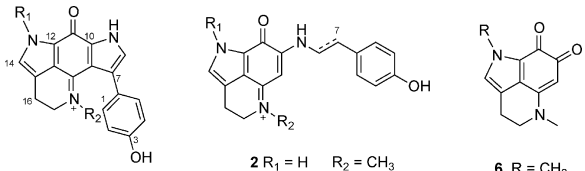


**Figure 1.** Chemical structures of natural products **1**–**9**.

isolated compounds makaluvamines J (**2**) and G (**3**). Subsequent chemical investigations on larger quantities of the $CH_2Cl_2/CH_3OH$ 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** and 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*-hexane, 4:1 $CH_2Cl_2/CH_3OH$, and $CH_3OH$. The $CH_2Cl_2/CH_3OH$ and $CH_3OH$ extracts were combined and chromatography was performed using $C_{18}$ bonded silica HPLC ($CH_3OH/H_2O$/0.1% TFA) to yield makaluvamine J (**2**, 2.3 mg, 0.023% dry wt) and two other semipure bioactive fractions. Both of these fractions were further purified by $C_{18}$ bonded silica HPLC ($CH_3OH/H_2O$/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). Because of 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 $C_{18}$ bonded silica HPLC ($CH_3OH/H_2O$/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 $^1$H NMR (Table 1) and COSY spectra of **1** allowed three spin systems to be assigned. These included a para-substituted phenyl substructure [$\delta_H$ 7.26 (2H, d, $J$ = 8.4 Hz) and 6.85 (2H, d, $J$ = 8.4 Hz)], two contiguous methylenes [$\delta_H$ 2.99 (2H, t, $J$ = 7.8 Hz) and 3.98

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

| position | $^{13}$C $\delta$, mult | $^1$H $\delta$ (mult, $J$ 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.


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(2H, t, $J$ = 7.8 Hz)] and a −NH−CH═ moiety [$\delta_H$ 13.46 (1H, s) and 7.31 (1H, s)]. The remaining unassigned proton signals included an isolated aromatic singlet at $\delta_H$ 7.16 (1H, s) and two methyl singlets at $\delta_H$ 2.98 (s, 3H) and 3.95 (s, 3H). The $^{13}$C NMR spectrum of **1** showed 18 unique signals that resonated between $\delta_C$ 18 and 167. HSQC data analysis allowed all 16 nonexchangeable protons to be assigned to their directly attached carbons. This identified that **1** contained 10 quaternary carbons. HMBC correlations (Figure 2) from the

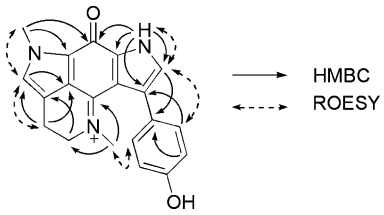


**Figure 2.** Key HMBC and ROESY correlations for compound **1**.

methine singlet proton ($\delta_H$ 7.31), associated with −NH−CH═ moiety, to carbons at $\delta_C$ 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 $\delta_H$ 3.95 to carbons at $\delta_C$ 125.2 and 127.6 and from the methine singlet at $\delta_H$ 7.16 to carbons resonating at $\delta_C$ 35.8, 125.2, 123.3, and 117.5. Furthermore, the *N*-methylpyrrolic system was shown to be substituted by the previously identified 1,2-disubstituted ethane unit [$\delta_H$ 2.99/3.98] via HMBC correlations from the protons at $\delta_H$ 2.99 to carbons at $\delta_C$ 117.5 and 127.6. A strong ROESY correlation between $\delta_H$ 2.99 and 7.16 further supported this substructure assignment. An *N*-methyliminium moiety was elucidated and attached to the ethane unit following HMBC data analysis for the proton resonance at $\delta_H$ 2.98. Although no phenolic signal was observed in the $^1$H 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 $\delta_C$ 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] $^1$H 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 analogue of tsitsikammamine B.[22,23] Confirmation of the structure of **1** was further supported by a $^3J_{CH}$ correlation from the proton at $\delta_H$ 7.26 to the $\beta$-pyrrole carbon at $\delta_C$ 127.5, as well as a strong ROESY correlation between these protons and the *N*-methyliminium protons at $\delta_H$ 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] By use of a decatenation inhibition assay, only makaluvamines A and F were shown to inhibit mammalian topoisomerase II, with $IC_{90}$ values of 41 and 25 $\mu$M, 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 $\mu$M.[27] In a similar manner, cytotoxicity data for makaluvamines A−F against xrs-6 cells had $IC_{50}$ values in the range 0.08−13.49 $\mu$M.[28] The pyrroloiminoquinone substructure not only has been isolated from *Zyzzya* spp.[19,27,29−31] but also has 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 analogues.[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 reinvestigation of other South African latrunculid sponges yielded N-18 oxime analogues 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 analogues reported.[47,48]

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

| | physicochemical parameter[a] | | | | $IC_{50}$ (nM)[b] | | | SI[c] | |
|---|---|---|---|---|---|---|---|---|---|
| compd | MW | cLogP | HBA | HBD | 3D7 | Dd2 | HEK293 | 3D7 | Dd2 |
| **1** | 332 | −0.75 | 2 | 2 | 13 | 18 | 3600 | 276 | 200 |
| **2** | 322 | −1.76 | 3 | 3 | 25 | 22 | 1200 | 48 | 54 |
| **3** | 334 | −1.44 | 5 | 2 | 36 | 39 | 2000 | 55 | 51 |
| **4** | 320 | −1.67 | 8 | 3 | 40 | 21 | 1400 | 35 | 66 |
| **5** | 321 | 1.98 | 4 | 2 | 396 | 300 | 1100 | 3 | 4 |
| **6** | 216 | 0.89 | 3 | 0 | 1880 | 360 | >120000 | >63 | >333 |
| **7** | 202 | 0.67 | 3 | 1 | 12250 | 3800 | >120000 | >10 | >32 |
| chloroquine | 319 | 3.93 | 4 | 1 | 25 | 130 | | | |

[a]In 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. [b]50% Inhibitory concentration in vitro against *P. falciparum* chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) lines. [c]SI = (mammalian cell $IC_{50}$)/(*P. falciparum* $IC_{50}$).


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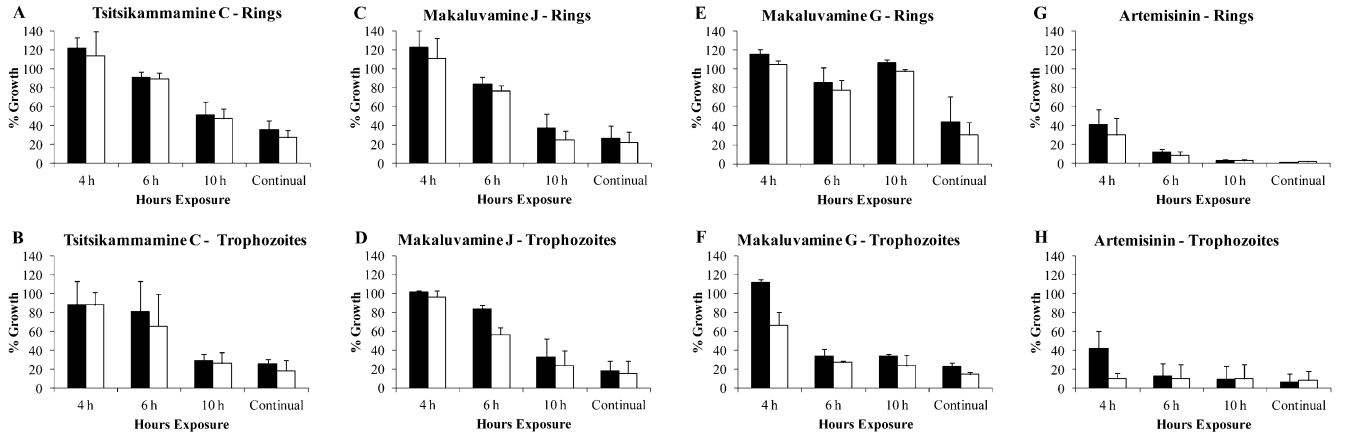


**Figure 3.** Effect of compounds on *P. falciparum* ring versus trophozoite stage parasites. Synchronous ring (A, C, E, G) or trophozoite (B, D, F, H) stage *P. falciparum* 3D7 parasites were exposed to ~5 × $IC_{50}$ (black bars) or ~10 × $IC_{50}$ (white bars) concentrations of compounds for 4, 6, and 10 h. After washing and culturing for a further 48−72 h in the presence of $^{3}$H-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 (±SD) is shown for at least two independent experiments for each treatment.

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 were also obtained for **1**−**7** using a human embryonic kidney cell line (HEK293). In order to assess the druglike and leadlike properties of alkaloids **1**−**7**, in silico physicochemical properties were calculated using Instant JChem software[49] and the data compared to Lipinski's druglike "rule of five"[50] and Hann and Oprea's leadlike 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 $IC_{50}$ < 100 nM against both the 3D7 and Dd2 lines and showed moderate cytotoxicity against the HEK293 cell line, with $IC_{50}$ values in the range 1.1−3.6 μM. The new compound tsitsikammamine C (**1**) showed the most promising in vitro biological profile with $IC_{50}$ values of 13 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 $IC_{50}$ of 3.6 μM.

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*-methyliminium analogues **2**−**4** all display $IC_{50}$ < 41 nM (3D7) compared to the protonated imimium makaluvamine K (**5**), with an $IC_{50}$ of 396 nM (3D7). N-Methylation of the iminium moiety of the makaluavmine series increased antimalarial activity from 9.9- to 30.5-fold compared to the nonmethylated 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 analogues **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** and **7** is essential for antimalarial activity.

Furthermore, from comparison of 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 indicate that restricting the configuration of the pendent 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 of exposure (Figure 3A−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 $IC_{50}$ concentrations of this compound for up to 10 h (Figure 3E,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 rapidly degraded compared to **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 because of the limited availability of each of the compounds. Each compound was


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**Table 3. In Vitro Metabolism of Compounds 2 and 3 in Human and Mouse Liver Microsomes**

| compd | species | half-life, min | in vitro $CL_{int}$, ($\mu$L/min)/(mg of protein) | $E_H$ [a] |
|---|---|---|---|---|
| **2** | human | >350 | <5 | <0.2 |
| | mouse | 42 | 41 | 0.64 |
| **3** | human | 8.8 | 197 | 0.92 |
| | mouse | 7.4 | 235 | 0.91 |

[a] $E_H$: predicted in vivo hepatic extraction ratio, calculated according to published methods.[53,54]

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 $\mu$M at 1 h postdose and declined over the 4 h postdose sampling period. In comparison, plasma concentrations of **3** were consistently low (less than 0.04 $\mu$M) over the same duration. Even with these 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

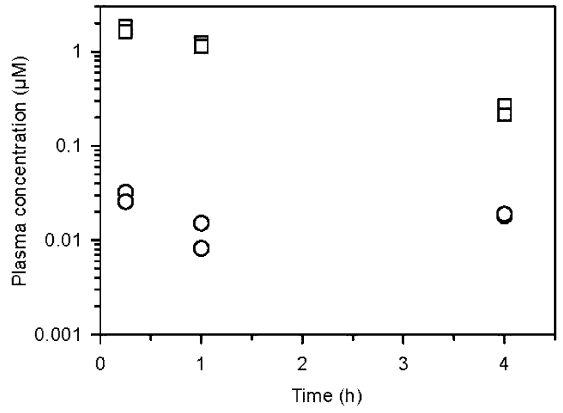


**Figure 4.** Plasma concentrations of compound **2** (squares) and compound **3** (circles) following subcutaneous administration to mice at a dose level of 8 mg/kg. Symbols represent concentrations from two individual animals at each time point.

determinant of the in vivo clearance of these two compounds, this result is consistent with the in vitro microsomal stability data that 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.

On the basis of the DMPK data, in vivo tolerability and efficacy studies were subsequently undertaken on makaluvamines J (**2**) and G (**3**). Because of the limited supply of each compound, the maximum dosage assessed for tolerability was 8 mg $kg^{-1}$ $day^{-1}$ for 4 days. No physical distress was seen in healthy mice administered subcutaneously with makaluvamine J up to 4 mg $kg^{-1}$ $day^{-1}$; however, at 8 mg $kg^{-1}$ $day^{-1}$ the mice showed reduced activity, ruffled coat, and weight loss of >10%. In contrast, makaluvamine G was well tolerated in mice at 8 mg $kg^{-1}$ $day^{-1}$. When compound **2** was tested for efficacy at 4 mg $kg^{-1}$ $day^{-1}$, 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 $kg^{-1}$ $day^{-1}$, compound **3** suppressed *P. berghei* infection by 10% on day 1 postinfection (pi) and by 35%, 47%, and 48% at days 2, 3, and 4 pi, respectively, relative to the control group. The comparator chloroquine suppressed *P. berghei* infection by 35% at day 4 pi, which is in accord with previous findings, with $ED_{50}$ (effective dose at 50% suppression) of 1.9 mg $kg^{-1}$ $day^{-1}$.[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**) indicate that this structure class has promising antimalarial activity. The new metabolite tsitsikammamine C (**1**) displayed 2- to 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 of ~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 $\delta_H$ 2.49 and $\delta_C$ 39.5. Standard parameters were used for the 2D experiments, which included gCOSY, gHSQC ($^1J_{CH}$ = 140 Hz), and gHMBC ($^nJ_{CH}$ = 8.3 Hz). LRESIMS data were recorded on a Waters ZQ mass spectrometer. HRESIMS data were measured on a Bruker Daltonics Apex III 4.7e Fourier Transform mass spectrometer fitted with an Apollo API source. A Thermo Scientific $C_{18}$ Betasil 5 $\mu$m, 143 Å column (21.2 mm × 150 mm) was used for semipreparative HPLC. A Waters 600 pump fitted with a 996 photodiode array detector and 717 Plus autosampler was used for the semipreparative HPLC separations. End-capped Sepra $C_{18}$ 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 $C_{18}$ ONYX column (4.6 mm × 100 mm) and shown to be >95% pure. 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. CellCarrier 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 *Zyzzya* sp. (Acarnidae) was collected by scuba diving (−43 m) at Rodda Reef, Queensland, Australia, on June 30, 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), $CH_2Cl_2$/$CH_3OH$ (4:1, 250 mL), and $CH_3OH$ (250 mL × 2). All $CH_2Cl_2$/$CH_3OH$ and $CH_3OH$ extractions were combined and dried under reduced pressure to yield a brown solid (0.32 g). This extract was subsequently preadsorbed to $C_{18}$-bonded silica (1.0 g), then packed into a stainless steel guard cartridge (10 mm × 30 mm) and attached to a $C_{18}$ semipreparative HPLC column. Isocratic HPLC conditions of 90% $H_2O$ (0.1% TFA)/10% $CH_3OH$ (0.1% TFA) were initially


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employed for the first 10 min. Then a linear gradient to $CH_3OH$ (0.1% TFA) was run over 40 min, followed by isocratic conditions of $CH_3OH$ (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 and then analyzed using the antimalarial bioassay. Activity was identified in fractions 31−39. $^1$H 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 semipure mixtures of related natural products. Both of these fractions were further purified by semipreparative $C_{18}$ HPLC using conditions identical 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. Extraction protocols identical to those used for the 10 g sample were employed, albeit larger volumes of solvent were used. The resulting $CH_2Cl_2/CH_3OH$ and $CH_3OH$ extractions were combined and then subjected to extensive $C_{18}$ bonded silica HPLC ($CH_3OH/H_2O$/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) and 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).** **1** was isolated as a pink gum. UV ($CH_3OH$) $\lambda_{max}$ (log $\varepsilon$) 243 (3.91), 197 sh (3.45), 343 (2.93) nm. For $^1$H and $^{13}$C NMR data, see Table 1. (+)-LRESIMS $m/z$ (rel intens) 332 (100) $[M - TFA]^+$; (+)-HRESIMS $m/z$ 332.1390 $[M - TFA]^+$ (calcd for $C_{20}H_{18}N_3O_2$, 332.1394, $\Delta$ = 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 $\mu$L for 72 h at 37 °C and 5% $CO_2$ 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 that fulfill the criteria established for a stained parasite are counted. The percent inhibition of parasite replication was calculated using DMSO and 2 $\mu$M artemisinin control data. Artemisinin [$IC_{50}$ = 0.021 $\mu$M (Dd2); $IC_{50}$ = 0.021 $\mu$M (3D7)] and chloroquine [$IC_{50}$ = 0.130 $\mu$M (Dd2); $IC_{50}$ = 0.025 $\mu$M (3D7)] were used as positive controls.

**In Vitro Stage Specific Assays.** Synchronous *P. falciparum* 3D7 ring (∼10−16 h after invasion) or trophozoite (∼24−30 h after invasion) stage parasites (0.5% parasitemia, 5% hematocrit) were incubated with ∼5 × $IC_{50}$ or ∼10 × $IC_{50}$ concentrations of each compound or vehicle control (0.04% DMSO) for 4, 6, and 10 h. After the cultures were gently washed with prewarmed parasite medium, the cultures were seeded into triplicate wells of a 96-well tissue culture plate and 0.5 $\mu$Ci $^3$H-hypoxanthine was added per well. $^3$H-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 $\mu$L. The plates were incubated for 72 h at 37 °C and 5% $CO_2$. After incubation the supernatant was aspirated out of the wells and 40 $\mu$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 $\mu$M puromycin control data. $IC_{50}$ values were obtained by plotting % inhibition against log of the dose using the Prism4 graphing package and nonlinear 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 nonfasted male Swiss outbred mice weighing 26−34 g. Mice had access to food and water continuously throughout the pre- and postdose phases of the study. Compounds were administered subcutaneously into the dorsal skinfold of each mouse via a 29G × 1/2 in. 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 postdose, blood samples were collected from mice ($n$ = 2 mice per time point for each compound) via cardiac puncture (following anesthetization with gaseous isofluorane). Blood was transferred to heparinized 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 instrument coupled to a Micromass Quattro Premier triple quadrupole MS instrument) 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 $\mu$M for compound **2** and 0.006 $\mu$M for compound **3**.

**In Vitro Microsomal Stability Assay.** Compounds were incubated at 37 °C and 1 $\mu$M 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 $MgCl_2$ (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 $CL_{int}$ was 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 and 90 mL min$^{-1}$ kg$^{-1}$ 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 of >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 was 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

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.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Phone: +61-7-3735-6000. Fax: +61-7-3735-6001. E-mail: r.quinn@griffith.edu.au.

### Notes

The authors declare no competing financial interest.

The opinions expressed herein are those of the authors' and do not necessarily reflect those of the Australian Defence Force, Joint Health Command or any extant policy.

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## ■ ABBREVIATIONS USED

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

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