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Thiaplakortones A–D: Antimalarial Thiazine Alkaloids from the Australian Marine Sponge *Plakortis lita*

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ABSTRACT: A high-throughput screening campaign using a prefractionated natural product library and an in vitro antimalarial assay identified active fractions derived from the Australian marine sponge *Plakortis lita*. Bioassay-guided fractionation of the CH₂Cl₂/CH₃OH extract from *P. lita* resulted in the purification of four novel thiazine-derived alkaloids, thiaplakortones A–D (**1–4**). The chemical structures of **1–4** were determined following analysis of 1D/2D NMR and MS data. Comparison of the chiro-optical data for **3** and **4** with literature values of related *N*-methyltryptophan natural products was used to determine the absolute configuration for both thiaplakortones C and D as 11*S*. Compounds **1–4** displayed significant growth inhibition against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *Plasmodium falciparum*, (IC₅₀ values <651 nM) and only moderate cytotoxicity against HEK293 cells (IC₅₀ values >3.9 μM). Thiaplakortone A (**1**) was the most active natural product with IC₅₀ values of 51 and 6.6 nM against 3D7 and Dd2 lines, respectively.

INTRODUCTION

Malaria is a significant infectious disease caused by *Plasmodium* parasites. This disease results in the deaths of 0.8–1.2 million people annually, and it is estimated that over 3.2 billion people living in Central and South America, Sub-Saharan Africa, South East Asia, the Middle East, and India and are at risk from this disease.¹ Despite recent preliminary data on a partially effective pre-erythrocytic stage vaccine (RTS, S),² malaria prevention and treatment currently relies on small molecule drugs and vector control. It is predicted that even if the RTS, S vaccine is approved for clinical use, it will be implemented as part of a multi-pronged approach that will also include antimalarial drugs in order to eradicate the parasite. Whilst a number of drugs are used therapeutically to treat malaria, all have now succumbed to parasite drug resistance and subsequently display reduced efficacy.³ Consequently, new antimalarial drugs with novel targets are urgently needed in order to combat the global problem of parasite drug resistance.

Historically natural products and their synthetic derivatives have played a significant role in the discovery and development of antimalarial drugs.⁴⁻⁶ Notable examples include artemisinin from the Chinese medicinal plant *Artemisia annua*, and quinine, which was isolated from the bark of the South American tree *Cinchona succirubra*.⁶ These compounds have subsequently led to the development of numerous other antimalarial drugs that are based on these natural product pharmacophores.

Natural products have an inherent understanding of biology space. Our lead discovery program is based on the drug-like natural product metabolome.^{7, 8} Natural products and their analogues have had high impact as drugs because of the embedded biosynthetic molecular recognition that transfers to therapeutic targets as described by protein fold topology (PFT).⁹ Molecular recognition during biosynthesis (the biosynthetic imprint) has been shown to carry through to a similar binding motif with therapeutic targets. PFT

describes cavity recognition points unrelated to fold and sequence similarity, and defines a natural product's ability to recognize biology space.⁹⁻¹² The discovery library was generated by HPLC fractionation of 18,453 optimized extracts obtained from plant and marine invertebrates sourced from tropical and temperate Australia, China and Papua New Guinea. Fractionation of each biota extract was accomplished using analytical reversed-phase C₁₈ HPLC (11 fractions collected per extract) to generate the 202,983 fraction library.⁷ In this current study we undertook HTS¹³ of this optimized screening library in order to identify new antimalarial lead compounds from nature. HTS data analysis identified three fractions from the sponge *Plakortis lita* (Plakinidae) that displayed significant growth inhibition against *P. falciparum* 3D7 parasites and showed no toxicity towards a human embryonic kidney cell line (HEK293). Bioassay-guided fractionation on the crude organic extract of *P. lita* resulted in the isolation of four novel thiazine-derived alkaloids, which we have named thiaplakortones A–D (1–4) (Figure 1). Herein we report the isolation, and structure elucidation of 1–4 along with their in vitro antimalarial activity and mammalian toxicity.

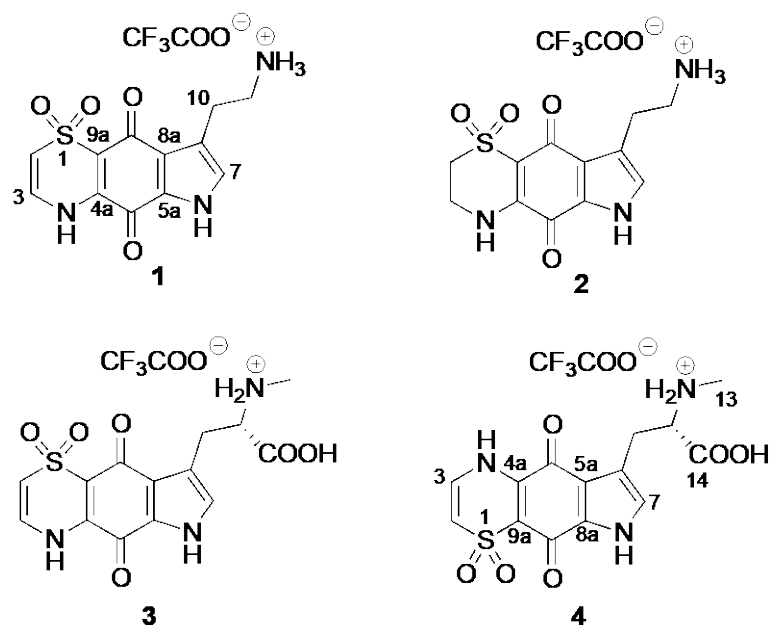


Figure 1. Structures of Thiaplakortones A–D (1–4).

RESULTS and DISCUSSION

The freeze-dried and ground sponge *P. lita* (de Laubenfels, 1954) was extracted with *n*-hexane, CH₂Cl₂, and CH₃OH. Bioassay-guided fractionation was performed on the combined CH₂Cl₂ and CH₃OH extracts using several iterations of reversed-phase C₁₈ HPLC (CH₃OH/H₂O/CF₃COOH) and biological screening. This process yielded the antimalarial thiaplakortones A–D (**1–4**) as their trifluoroacetate salts.

Thiaplakortone A was isolated as a stable amorphous solid. The molecular formula of the free base of **1** was determined to be C₁₂H₁₁N₃O₄S by (+)-HRESIMS of the [M - CF₃COO⁻]⁺ ion at *m/z* 294.0543. The ¹H NMR data (Table 1) of **1** showed only eight unique signals, three of which (δ_{H} 13.09, 11.07 and 7.84) were determined to be exchangeable, following a deuterium (D₂O) exchange NMR experiment. The remaining proton signals at δ_{H} 7.36, 7.06, 6.43, 3.05 and 2.97 were from protons attached to carbons resonating at δ_{C} 129.2, 130.2, 111.7, 38.4 and 23.4, respectively, following HSQC data analysis. The ¹³C NMR data of **1** displayed 12 signals (Table 2), which included seven sp² quaternary signals that resonated between δ_{C} 114.1 and 178.5. Analysis of the COSY spectrum of **1** readily established three unique spin systems, which included -CH₂CH₂NH₃⁺, -NHCH=CH- and -NHCH=

substructures (Figure 2a).

Table 1. ¹H NMR (600 MHz) Data for Thiaplakortones A–D (**1–4**) in DMSO-*d*₆.

Position	1 ^a	2 ^a	3 ^a	4 ^a
2	6.43, d (9.0)	3.28, m	6.46, d (9.6)	6.52, d (8.4)
3	7.06, dd (9.0, 5.4)	3.81, m	7.07, dd (9.6, 6.0)	7.10, dd (8.4, 5.4)
4	11.07, d (5.4)	8.92, s	11.10, d (6.0)	11.09, d (5.4)
6	13.09, s	12.89, s	13.14, s	
7	7.36, d (2.4)	7.32, d (2.4)	7.35, d (2.4)	7.13, d (2.4)
8				13.00, s
10	2.97, t (7.8)	2.94, t (7.2)	3.24, dd (14.4, 7.8)	3.26, dd (15.0, 6.6)
			3.29, dd (14.4, 6.6)	3.32, dd (15.0, 6.0)
11	3.05, m	3.04, m	4.18, br s	4.18, br s
12	7.84, br s	7.76, br s	8.98, br s	8.80, br s
				8.93, br s
13			2.64, s	2.60, s
14			<i>b</i>	<i>b</i>

^a δ_H, mult. (*J* in Hz). ^b Exchangeable proton was not observed.

Table 2. ¹³C NMR (125 MHz) Data for Thiaplakortones A–D (**1–4**) in DMSO-*d*₆.

Position	1 ^a	2 ^a	3 ^a	4 ^a
2	111.7, CH	48.4, CH ₂	111.8, CH	111.8, CH
3	130.2, CH	39.0, CH ₂	130.2, CH	130.2, CH
4a	139.9, C	147.3, C	140.0, C	140.5, C
5	167.8, C	167.9, C	167.8, C	174.9, C
5a	128.0, C	127.6, C	128.0, C	118.1, C
6				119.8, C
7	129.2, CH	129.6, CH	129.9, CH	126.4, CH
8	121.7, C	121.4, C	119.2, C	
8a	123.3, C	124.2, C	123.5, C	131.5, C
9	178.5, C	176.1, C	178.6, C	172.0, C
9a	114.1, C	108.6, C	114.1, C	113.2, C
10	23.4, CH ₂	23.4, CH ₂	24.9, CH ₂	24.8, CH ₂
11	38.4, CH ₂	38.6, CH ₂	59.6, CH	60.0, CH
13			31.3, CH ₃	31.5, CH ₃
14			169.6, C	169.6, C

^a δ_C, mult.

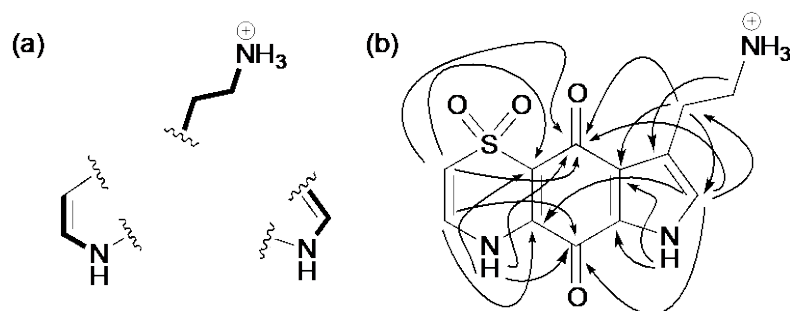


Figure 2. (a) COSY (—) identified fragments for **1**; (b) Key HMBC (→) correlations for **1**.

The ethylamine moiety was attached to a trisubstituted double bond on the basis of HMBC correlations (Figure 2b) from the ethyl protons at δ_{H} 2.97 (H-10) to carbon resonances at δ_{C} 123.3, 121.7 and 129.2; the signal at δ_{C} 129.2 was known to be associated with the --NHCH= fragment. Furthermore, the NH proton (δ_{H} 13.09, H-6) of this fragment showed HMBC correlations to δ_{C} 123.3, 121.7, 129.2 and 128.0 that allowed construction of a 1,2-disubstituted-3-ethylaminopyrrolo moiety. HMBC correlations from the pyrrolo proton at δ_{H} 7.36 (H-7) to carbonyl signals at δ_{C} 167.8 (C-5) and 178.5 (C-9), suggested a 1,4-quinone pyrrolo substructure.¹⁴ The remaining COSY-derived fragment of **1** (--NHCH=CH--) was expanded to a unsaturated 1,1-dioxo-1,4-thiazine moiety based on HMBC data and the ¹³C chemical shifts of the associated methine carbons [δ_{C} 130.2 (C-3) and 111.7 (C-2)], which were identical to those previously reported.¹⁵ Fusion of this moiety to the remaining unassigned quaternary carbons [δ_{C} 139.9 (C-4a) and 114.1 (C-9a)] was deduced by HMBC correlations from δ_{H} 11.07 (H-4) and 6.43 (H-2) to δ_{C} 114.1, and δ_{H} 11.07 and 7.06 (H-3) to δ_{C} 139.9. Furthermore, HMBC correlations from δ_{H} 11.07 (H-4) to the quinone carbonyl carbons established a tricyclic thiazine-fused pyrroloquinone substructure. At this stage all atoms for **1** had been accounted for, but the regiochemistry of the thiazine moiety had not been determined. Fortunately, the HMBC data secured this assignment via long-range

correlations. While several four-bond correlations to the quinone carbonyls were identified (Figure 2b), it was the correlation from both δ_{H} 6.43 (H-2) and 2.97 (H-10) into the carbonyl at δ_{C} 178.5 (C-9) that unequivocally established the regiochemistry. Thus thiaplakortone A was assigned to structure **1**.

The free base of the minor compound, thiaplakortone B (**2**), was assigned the molecular formula $\text{C}_{12}\text{H}_{13}\text{N}_3\text{O}_4\text{S}$ on the basis of (+)-HRESIMS and NMR data (Tables 1 and 2). Comparison of the ^1H and ^{13}C NMR data of **2** with thiaplakortone A readily identified that these two secondary metabolites belonged to the same structure class. The only major differences observed were that **2** lacked the thiazine *Z* double bond signals [δ_{H} 7.06/ δ_{C} 130.2 and δ_{H} 6.43/ δ_{C} 111.7], but contained additional upfield signals [δ_{H} 3.81/ δ_{C} 39.0 and δ_{H} 3.28/ δ_{C} 48.4]. These data in conjunction with the MS information, and literature values for related 1,1-dioxo-1,4-thiazine moieties¹⁵⁻²⁰ indicated that **2** was the 2,3-dihydro derivative of **1**. HMBC data analysis confirmed this assignment with multiple correlations identified between the thiazine-associated protons and the quinone moiety. The regiochemistry of the thiazine for **2** was determined to be identical to **1** based on NMR data comparison, thus thiaplakortone B was assigned as structure **2**.

Thiaplakortone C (**3**) was isolated as an optically active amorphous solid. The molecular formula of the free base of **3** was determined to be $\text{C}_{14}\text{H}_{13}\text{N}_3\text{O}_6\text{S}$ based on (+)-HRESIMS of a pseudomolecular ion $[\text{M} - \text{CF}_3\text{COO}]^+$ ion at m/z 352.0608. Analysis of both the 1D and 2D NMR data for **3** confirmed that the thiazine-pyrroloquinone moiety of **1** was also present in **3**; only minor differences were noted when comparing the ^{13}C ($\Delta < 0.8$ ppm) and ^1H ($\Delta < 0.06$ ppm) chemical shifts for the tricyclic systems of **1** and **3**. ^1H NMR and COSY data analysis for the unassigned ^1H signals for **3** established a 2-methylaminopropanoic acid side chain. HMBC data confirmed this moiety, and showed that it was attached to C-8 of the pyrrolo ring. Hence thiaplakortone C was assigned to structure

3. In order to determine the absolute configuration of **3** we compared the specific rotation data for thiaplakortone C with literature values of related *N*-methyltryptophan natural products, whose absolute configuration had been unequivocally determined.²¹⁻²³ The specific rotations for the hydrochloride salts of the plant metabolites (*S*)-*N*-methyltryptophan²¹ and (*S*)-*N*-methyltryptophan methyl ester²² were reported as $[\alpha]_{\text{D}}^{25} +46$ (*c* 1.554, 0.5 M HCl)²¹ and $[\alpha]_{\text{D}} +51$ (*c* 0.82, CH₃OH),²² respectively. We recorded $[\alpha]_{\text{D}}^{27} +56$ (*c* 0.025, CH₃OH) and $[\alpha]_{\text{D}}^{25} +129$ (*c* 0.007, 0.5 M HCl) for **3**; these data indicated that thiaplakortone C had the same absolute configuration as the plant natural products mentioned above and hence **3** was assigned as 11*S*.

Thiaplakortone D (**4**) was deemed to be an isomer of **3** based on NMR and MS data analysis. While the ¹³C NMR resonances for C-7 to C-14 of **4** were essentially identical to those of **3** ($\Delta < 0.8$ ppm), several major differences were noted in the quinone-thiazine bicyclic system. Most notably the quinoid carbonyl signals of **3** (δ_{C} 167.8 and 178.6) resonated at δ_{C} 172.0 and 174.9 in **4**, while the pyrrolo-quinone bridging carbon signals of **3** (δ_{C} 128.0 and 123.5) had shifted to δ_{C} 131.5 and 118.1 in **4**. These data indicated that **3** and **4** were regioisomers, which was supported by literature values,^{15,16} and HMBC data analysis. Thus thiaplakortone D was assigned to structure **4**. The absolute configuration of thiaplakortone D at C-11 was also assigned as *S* based on comparison of specific rotation data of **4** with literature values.²¹⁻²³

Thiaplakortones A–D were all tested against the 3D7 (chloroquine-sensitive) and Dd2 (chloroquine-resistant) *P. falciparum* lines, and preliminary cytotoxicity data was also acquired using the human cell line HEK293. Physicochemical properties (log P, HBA, HBD, MW) for alkaloids **1–4** were calculated in silico using Instant JChem software,²⁴ in order to assess these molecules for lead- and drug-like properties.^{25,26} Comparison of the data with Lipinski's drug-like "rule of five",²⁵ and Hann and Oprea's lead-like criteria,²⁶ identified that

thiaplakortones A–D were all compliant. Details of the calculated physicochemical properties, antimalarial activity, cytotoxicity and selectivity indices, compounds **1–4** are shown in Table 3.

Table 3. Physicochemical and Biological Profiles of Thiaplakortones A–D (**1–4**).

Compound	Physicochemical Parameters ^a				IC ₅₀ (nM) ^b			SI ^c	
	MW	cLogP	HBA	HBD	3D7	Dd2	HEK293	3D7	Dd2
1	293	-2.06	6	3	51	6.6	3,900	76	591
2	295	-2.21	6	3	650	92	> 40,000	> 62	> 435
3	351	-4.55	8	4	309	171	> 40,000	> 129	> 233
4	351	-4.55	8	4	279	159	> 80,000	> 285	> 500

^a In silico calculations performed using Instant JChem software,²⁴ MW = molecular weight of the free base, cLogP = calculated logarithm of the partition coefficient (n-octanol/H₂O), HBA = H-bond acceptors, HBD = H-bond donors; ^b50% Inhibitory concentration in vitro against *P. falciparum* drug-sensitive (3D7) and drug-resistant (Dd2) lines and a human embryonic kidney cell line (HEK293); ^cSI = HEK293 IC₅₀/*P. falciparum* IC₅₀

Thiaplakortone A (**1**) showed the most promising biological profile with IC₅₀ values of 51 and 6.6 nM against the 3D7 and Dd2 lines, respectively. Furthermore, **1** displayed only moderate toxicity against HEK293 cells, which translated to selectivity indices of 76 and 591 against the 3D7 and Dd2 strains, respectively. The replacement of the unsaturated thiazine moiety of **1** with the thiazine motif in **2** resulted in 12.7- and 13.9-fold reductions in 3D7 and Dd2 antimalarial activity, respectively.

Thiaplakortones C and D both displayed similar antiparasitic activity against the 3D7 and Dd2 lines, suggesting that the regiochemistry of the thiazine is not critical for antimalarial activity. Furthermore, while the 2-methylaminopropanoic acid side chain of **3**

and **4** reduced antimalarial activity, compared to the ethylamine chain of **1**, there seems to be some tolerance to variation in this side chain.

Natural products containing a thiazine-fused quinone fragment are rare.²⁷ A substructure search of the Dictionary of Natural Products database²⁷ using this bicyclic fragment identified only fourteen compounds which included: thiaplidiaquinones A and B,¹⁶ conicaquinones A and B,¹⁹ aplidinones A–C,¹⁷ and conithiaquinones A and B,¹⁸ which were all isolated from the ascidian *Aplidium conicum*, adociaquinone A and 3-oxoadociaquinone A from the sponge *Adocia* sp.,²⁰ xestoquinolide B from the sponge *Xestospongia* cf. *carbonaria*,²⁸ and ascidiathiazones A and B from the ascidian *Aplidium* sp.¹⁵ A variety of biological activities have been reported for these secondary metabolites such as topoisomerase II inhibition,²⁹ anticancer,^{16,18,20,29} and anti-inflammatory properties.¹⁵

Although thiaplakortones A–D share some structural homology with the above listed compounds, their tricyclic alkaloid skeleton is without precedent among known natural products. We proposed that these metabolites are most likely biosynthesized from L-tryptophan, which could undergo oxygenation to form a pyrroloquinone moiety (**i**) that could react with L-cysteine to afford two potential thiazine-pyrroloquinone regioisomers,¹⁵ only one of which (intermediate **ii**) is shown in Figure 3.

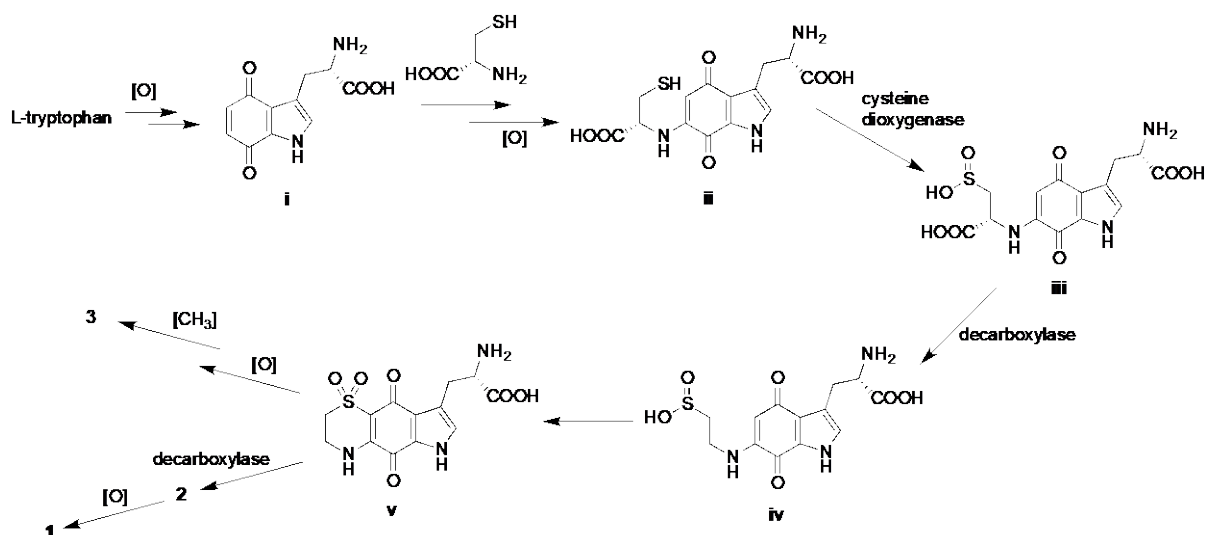


Figure 3. Proposed Biogenesis of Thiaplakortones A–C (1–3).

Oxidation by a dioxygenase similar to cysteine dioxygenase³⁰ to the sulfinic acid derivative (**iii**), followed by decarboxylation using an enzyme similar to cysteine sulfinic decarboxylase³¹ would yield the intermediate **iv**. Cyclization of **iv** to form the thiazine **v** and subsequent methylation and oxidation would yield thiaplakortone C (**3**), while decarboxylation of intermediate **v** would afford thiaplakortone B (**2**) that upon oxidation of the thiazine ring could produce **1**. The biogenesis of the thiazine-containing natural products pheomelanin and pheofungin from the coupling of cysteine with activated tyrosine and orsellinic acid, respectively, has been previously reported.^{32,33}

CONCLUSIONS

The significant *in vitro* antimalarial activity of this novel alkaloid class combined with the low cytotoxicity is encouraging, and we believe medicinal chemistry to expand the structure-activity relationships and generate potential candidates for *in vivo* evaluation is warranted.

EXPERIMENTAL SECTION

General Experimental Methods. NMR spectra were recorded at 30 °C on 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. LRESIMS were recorded on a ZQ mass spectrometer. HRESIMS were measured on a 4.7 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer fitted with an API source. An HPLC pump fitted with a Photodiode Array Detector and either a C₁₈-bonded silica 5 μm 143 Å column (21.2 mm \times 150 mm) or a C₁₈-bonded silica 5 μm 100 Å column (10 mm \times 250 mm) were used for the semi-preparative HPLC separations. End-capped C₁₈-bonded silica was used for pre-adsorption work, and the resulting material was packed into stainless steel guard cartridges (10 \times 30 mm). An orbital shaker was used for the large-scale extraction of sponge material. All compounds were analyzed for purity by analytical HPLC using a C₁₈ monolithic column (4.6 mm \times 100 mm) and shown to be >95%. H₂O was filtered, while all other solvents used were 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. Polylysine coated plates were used for *P. falciparum* assays and 4',6-diamidino-2-phenylindole (DAPI), Alamar Blue and Triton-X, saponin, puromycin, artemisinin, chloroquine and HEK293 cells were all purchased from commercial suppliers.

Sponge Material. The sponge *Plakortis lita* (family: Plakinidae) was collected by scuba diving (- 42 m) at Melville Passage, Tydeman Reef, Queensland, Australia on the 29th of June 2003 and kept frozen prior to freeze-drying and extraction. A voucher sample (QMG320465) has been lodged at the Queensland Museum, Brisbane, Australia.

Extraction and Isolation. The freeze-dried and ground sponge (10 g) was sequentially extracted with *n*-hexane (250 mL), CH₂Cl₂ (250 mL × 4) and CH₃OH (250 mL × 4). All the CH₂Cl₂ and CH₃OH extracts were combined and dried down under reduced pressure to yield a dark orange solid (2.4 g). This material was divided into four equal portions (~0.6 g), then individually pre-adsorbed on C₁₈-bonded silica (1 g), then packed into four guard cartridges. Each cartridge was attached to a C₁₈-bonded silica HPLC column (21.2 mm × 150 mm) and isocratic HPLC conditions of 90% H₂O (0.1% CF₃COOH)/10% CH₃OH (0.1% CF₃COOH) were employed for the first 10 min, then a linear gradient to CH₃OH (0.1% CF₃COOH) was run over 40 min, followed by isocratic conditions of CH₃OH (0.1% CF₃COOH) for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected by time from the start of the HPLC run. This HPLC process was repeated for all four cartridges, and the corresponding fractions from each separation were combined then assayed for antimalarial activity. Fractions 16–18 (20 mg, sample 1) and 19–26 (95 mg, sample 2) were combined on the basis on ¹H NMR, MS and bioassay data analysis. Both these samples were separately pre-adsorbed on C₁₈-bonded silica (1 g), and the resulting dry material packed into guard cartridges that were subsequently attached to a C₁₈-bonded silica HPLC column (21.2 mm × 150 mm). Each sample was separately fractionated using isocratic HPLC conditions of 95% H₂O (1.0% CF₃COOH)/5% CH₃OH (1.0% CF₃COOH) for the first 10 min, followed by a 40 min linear gradient to 60% H₂O (1.0% CF₃COOH)/40% CH₃OH (1.0% CF₃COOH), then isocratic conditions of 60% H₂O (1.0% CF₃COOH)/40% CH₃OH for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected by time from the start of the HPLC run. HPLC of sample 2 [i.e. fractions 19–26 (95 mg)] yielded the trifluoroacetate salts of thiaplakortones C (**3**, 5.4 mg, 0.054% dry wt, ^tR = 29.0–29.9 min) and D (**4**, 9.3 mg, 0.093% dry wt, ^tR = 30.1–31.0 min). HPLC of sample 1 [i.e. fractions 16–18 (20 mg)] yielded semi-pure thiaplakortones A (8.4 mg) and B (2.9 mg) in

fractions 27 and 26, respectively. These semi-pure fractions were individually pre-adsorbed on C₁₈-bonded silica (1 g), and the resulting dry material packed into individual guard cartridges that were subsequently attached to a C₁₈-bonded silica HPLC column (10 mm × 250 mm). Each sample was separately fractionated using isocratic HPLC conditions of 95% H₂O (0.1% CF₃COOH)/5% CH₃OH (0.1% CF₃COOH) for the first 10 min, followed by a 40 min linear gradient to 65% H₂O (0.1% CF₃COOH)/35% CH₃OH (0.1% CF₃COOH), then isocratic conditions of CH₃OH (0.1% CF₃COOH) for a further 10 min, all at a flow rate of 4 mL/min. This afforded the trifluoroacetate salts of thiaplakortones A (**1**, 2.8 mg, 0.028% dry wt, ¹R = 27.0–29.5 min), and B (**2**, 1.0 mg, 0.010% dry wt, ¹R = 24.8–26.0 min).

Trifluoroacetate Salt of Thiaplakortone A (1): stable orange-brown amorphous solid; UV (CH₃OH) λ_{max} (log ε) 228 (3.49), 248 (3.47), 276 sh (3.17), 375 (3.26) nm; IR ν_{max} (KBr) 3600–3300, 1683, 1632, 1515, 1261, 1206, 1127, 1109 cm⁻¹; ¹H and ¹³C NMR data (DMSO-*d*₆) see Tables 1 and 2; (+)-LRESIMS (rel. int.) *m/z* 294 (100) [M - CF₃COO]⁺; (+)-HRESIMS *m/z* 294.0536 (C₁₂H₁₂N₃O₄S [M - CF₃COO]⁺ requires 294.0543).

Trifluoroacetate Salt of Thiaplakortone B (2): stable orange-brown amorphous solid; UV (CH₃OH) λ_{max} (log ε) 225 (3.83), 299 (3.61), 357 (3.33) nm; IR ν_{max} (KBr) 3600–3300, 1671, 1637, 1585, 1266, 1204, 1120, 1110 cm⁻¹; ¹H and ¹³C NMR data (DMSO-*d*₆) see Tables 1 and 2; (+)-LRESIMS (rel. int.) *m/z* 296 (100) [M - CF₃COO]⁺; (+)-HRESIMS *m/z* 296.0698 (C₁₂H₁₄N₃O₄S [M - CF₃COO]⁺ requires 296.0699).

Trifluoroacetate Salt of Thiaplakortone C (3): stable orange-brown amorphous solid; [α]_D²⁷ +56 (*c* 0.025, CH₃OH); [α]_D²⁵ +129 (*c* 0.007, 0.5 M HCl); UV (CH₃OH) λ_{max} (log ε) 271 (3.57), 304 sh (3.29), 373 (3.00), 474 (3.29) nm; IR ν_{max} (KBr) 3600–3300, 1670, 1657, 1630, 1411, 1266 cm⁻¹; ¹H and ¹³C NMR data (DMSO-*d*₆) see Tables 1 and 2; (+)-

LRESIMS (rel. int.) m/z 352 (100) $[M - CF_3COO^-]^+$; (+)-HRESIMS m/z 352.0608 ($C_{14}H_{14}N_3O_6S [M - CF_3COO^-]^+$ requires 352.0598).

Trifluoroacetate Salt of Thiaplakortone D (4): stable orange-brown amorphous solid; $[\alpha]_D^{28} +80$ (c 0.025, CH_3OH); $[\alpha]_D^{25} +143$ (c 0.007, 0.5 M HCl); UV (CH_3OH) λ_{max} ($\log \epsilon$) 237 (3.67), 267 sh (3.37), 330 (3.22), 464 (2.87) nm; IR ν_{max} (KBr) 3600–3300, 1670, 1635, 1220 cm^{-1} ; 1H and ^{13}C NMR data (DMSO- d_6) see Tables 1 and 2; (+)-LRESIMS (rel. int.) m/z 352 (100) $[M - CF_3COO^-]^+$; (+)-HRESIMS m/z 352.0597 ($C_{14}H_{14}N_3O_6S [M - CF_3COO^-]^+$ requires 352.0598).

In vitro Antimalarial Image-based 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 μL , for 72 h at 37 °C and 5% CO_2 , in poly-*D*-lysine coated images plates. After incubation the 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 a HTS confocal imaging system. The digital images obtained were analyzed using spot detection software where fluorescent spots, which fulfilled the criteria established for a stained parasite were counted. The percent inhibition of parasite replication was calculated using 0.4% DMSO (0% inhibition) and 2 μM artemisinin (100% inhibition) control data. Artemisinin [$IC_{50} = 0.0043 \mu M$ (Dd2); $IC_{50} = 0.0058 \mu M$ (3D7)] and chloroquine [$IC_{50} = 0.413 \mu M$ (Dd2); $IC_{50} = 0.051 \mu M$ (3D7)] were used as positive controls.

In Vitro Cytotoxicity Assay. Compounds were added to 384 well black/clear tissue treated assay plates containing 3000 adherent cells/well (HEK293) in an assay volume of 45 μL . 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 μL 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. The percent inhibition of cell growth was calculated using 0.4%

DMSO (no inhibition) and 10 μM puromycin (100% inhibition) control data. IC_{50} values were obtained by plotting % inhibition against log dose using a graphing package and non-linear regression with variable slope plot. The positive control, puromycin, was shown to display an IC_{50} of 0.382 μM towards the HEK293 cell line.

ASSOCIATED CONTENT

Supporting Information. $^1\text{H}/^{13}\text{C}$ NMR spectra, and NMR data tables for thiaplakortones A–D (1–4); antimalarial and cytotoxicity dose response curves for thiaplakortones A–D (1–4) and the positive controls (artemisinin, chloroquine and puromycin) used during these studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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