New Manzamine Alkaloids with Potent Activity against Infectious Diseases

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Abstract: The isolation of the new enantiomers of 8-hydroxymanzamine A (**1**), manzamine F (**2**), along with the unprecedented manzamine dimer, *neo*-kauluamine from an undescribed genus of Indo-Pacific sponge (family *Petrosiidae*, order *Haplosclerida*) is reported. The relative stereochemistry of *neo*-kauluamine was established through detailed analysis of NOE-correlations combined with molecular modeling. The significance of the manzamines as in vivo antimalarial agents with superior activity to the clinically used drugs artemisinin and chloroquine is discussed along with the activity in vitro against the AIDS-opportunistic infectious diseases tuberculosis and toxoplasmosis. Reexamination of the sponges identified as *Prianos*, and *Pachypellina*, in earlier publications has confirmed that these are members of the same genus as the sponge described here, but differ at the species level.

Introduction

The manzamines are complex polycyclic marine-derived alkaloids first reported in 1986 from the Okinawan sponge genus *Haliclona*.[1] These compounds possess a fused and bridged tetra- or pentacyclic ring system, which is attached to a β-carboline moiety. Since the first report of manzamine A, an additional 30 manzamine-type alkaloids have been reported from nine different sponge genera (four orders).[2,3] The occurrence of manzamine alkaloids in a diversity of unrelated sponges points to a microbial origin of the biosynthesis for these compounds. Most of the currently available medications for many infectious diseases, for example, malaria, tuberculosis (TB), and toxoplasmosis, are either unable to eradicate these diseases, have developed resistance, or yield severe side effects. Hence, there is an urgent need to discover new lead compounds with improved activity and less toxicity. Malaria accounts for over a million deaths each year worldwide with 273 million cases reported in 1998.[4] The most dangerous malaria parasite, *Plasmodium falciparium* which causes cerebral malaria, is expected to spread in the central or northern regions of Europe and North America within a few decades.[4] The pathogenic synergy with HIV has increased the overall incidence of many opportunistic infectious diseases.[5] For example, the incidence of tuberculosis infection in HIV positive cases is 50-fold over HIV negative ones,[5] and the same holds true in the case of *T. gondii*.[6] One-third of the human population worldwide conceals latent tuberculosis and is expected to generate more than three million mortality cases this year.[5] The emergence of drug-resistant and multidrug-resistant TB and malaria strains as well as the lack of any current chemotherapy which targets the latent form of *T. gondii* augmented the necessity to search for new and better anti-TB, -malaria, and -toxoplasmosis drug leads.[4−6]

The manzamines have previously exhibited a diverse range of bioactivities including cytotoxicity,[1] insecticidal,[7] antibacterial,[8] and antimalarial.[9] We now report the new enantiomers of 8-hydroxymanzamine A (**1**),[10] manzamine F (**2**),[11] along with the unprecedented manzamine dimer, *neo*-kauluamine from an undescribed genus of Indo-Pacific sponge (family *Petrosiidae*, order *Haplosclerida*). The importance of manzamines as possible treatments for malaria, tuberculosis, and toxoplasmosis is also highlighted. Reexamination of the sponges identified as *Prianos*[12] and *Pachypellina*[10] in earlier publications has confirmed that these are members of the same genus as that of the sponge described here, but they differ at the species level and there are at least four species known to the authors.

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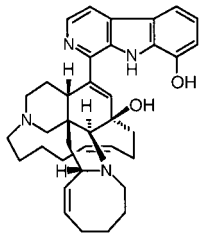
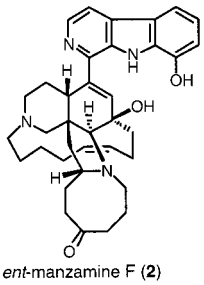
Results and Discussion

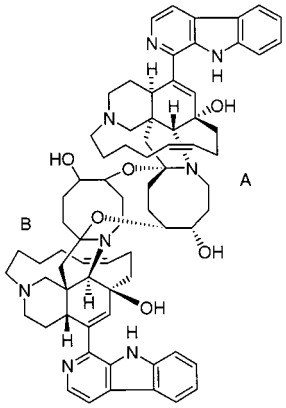
Isolation and Structure Elucidation. The lipophilic extract of the freeze-dried sponge (145 g) afforded, after repeated flash chromatography on Si gel 60, the known manzamines A (0.66%), E (0.003%), J (0.0017%), ircinal A (0.008%) and 6-deoxymanzamine X (0.0021%) along with the new *ent*-8-hydroxymanzamine A (**1**) (1.24%), *ent*-manzamine F (**2**) (0.055%), and *neo*-kauluamine (**3**) (0.0048%).[13] Compounds **1** and **2** show comparable physical and spectral data (±2.0 ppm for all but 2 reported ^{13}C NMR signals) to the known 8-hydroxymanzamine A[10] and manzamine F[11] with the exception of their optical rotations and biological activity. Compound **1**



ent-8-hydroxymanzamine A (**1**)



ent-manzamine F (**2**)



neo-kauluamine (**3**)

has an $[\alpha]_D^{25}$ −112.0°, while 8-hydroxymanzamine A rotates +118.5°, in the same solvent. Similarly, **2** provides an $[\alpha]_D^{25}$ −44.6°, while the value for manzamine F is +59.9°, both measured in $CHCl_3$. An authentic sample of (+)-8-hydroxymanzamine A, kindly provided by professor Scheuer, was subjected to preparative TLC, using the same NH_4OH-containing solvent system used to isolate compound **1**. Comparison of ^{1}H NMR, ^{13}C NMR, optical rotation, TLC, and melting point data for compound **1** and the authentic (+)-8-hydroxymanzamine A free base (both separately and mixed) revealed identical physical properties with the exception of opposite optical rotation values. These data support our conclusion that **1** and **2** are enantiomers, rather than diastereoisomers, of the known 8-hydroxymanzamine A and manzamine F. *ent*-8-Hydroxymanzamine A exhibits improved activity against P-388 with an IC_{50} 0.25 μg/mL when compared with the previously published value of >20 μg/mL for the (+) compound, further supporting the existence of an enantiomeric pair.[10]

Intriguing is the fact that manzamine A and the antipode of 8-hydroxymanzamine A are both isolated with comparable yields. Ircinols A and B represent the first report of antipodes in the manzamine-related alkaloids, based on their inverted optical rotation value and the chemical conversion of (+)-ircinals A and B to (+)-ircinols A and B.[14] (−)-Ircinols A and B were reported to co-occur with (+)-ircinals A and B, as well as (+)-manzamines A and B in the same *Amphimedon* sp.[14] The co-occurrence of both enantiomers of keramaphidin, a manzamine-related alkaloid, in the marine sponge *Amphimedon* sp. and their chiral resolution are also reported.[15] Consequently, the report of **1** and **2** is supported by previous examples of antipodes in the manzamine-related alkaloids.

The HRFABMS data of **3** provided a molecular ion peak $(M + H)^+$ at *m/z* 1161.6956, which combined with detailed analysis of ^{1}H-, ^{13}C-, and ^{15}N NMR data, suggested a molecular formula of $C_{72}H_{89}N_8O_6$ and 33 double bond equivalents. The IR spectrum ($CHCl_3$) of **3** shows absorption bands at 3592, 3475−3250 cm^{-1}, indicating the presence of OH and NH functionality. The ^{1}H- and ^{13}C NMR spectra of **3** (Table 1) provided a data set for nonidentical twins indicating the dimeric nature of **3**. The segments C-1−C-25, and C-1′−C-25′ were closely related to those of the known manzamine A.[1] The HMBC correlation of the proton singlets H-26 and H-26′ with C-11−13, -24, -25, -34−36, and C-11′−13′, -24′, -25′, -34′−36′, respectively, (Figure 1) further supported this relationship. It was then clear that dimerization and additional oxygenation were present in the eight-membered ring segments N-27−C-34 and N-27′−C-34′. The proton doublets resonating at δ 4.41 and 4.14 (Table 1) are assigned as H-31 and H-30′, respectively, on the basis of the ^2J-HMBC correlation of their carbons with the broad doublets resonating at δ 3.76 and 3.69 (H-30 and H-31′), respectively. The proton signals resonating at δ 3.15 (2H, m) and 3.57, 3.18 (each 1H, m) are assigned as H_2-28 and H_2-28′, respectively, on the basis of their ^3J-HMBC correlation to the downfield quaternary carbons resonating at δ 89.7 and 104.5 (C-34 and C-34′), respectively. H_2-28 and -28′ also show ^2J-^{15}N-GHMQC correlations to the nitrogens resonating at δ 57.4 and 37.4, which are assigned as N27 and 27′, respectively. H_2-28 and -28′ also show COSY couplings to the upfield proton signals at δ 2.05, 1.56 and 2.27, 2.12 assigned as H_2-29 and -29′, respectively. H_2-29 and -29′ show strong COSY couplings with H-30 and H-30′. The four upfield multiplets resonating at δ 2.07, 1.55 and 1.75, 1.45 are assigned as H_2-32 and H_2-33, respectively, on the basis of their ^2J- and ^3J-HMBC correlations to the downfield quaternary carbon C-34. Similarly, both H_2-32′ and -33′ show ^2J- and ^3J-HMBC correlations to C-34′. In addition, H_2-32 shows strong COSY coupling to the downfield H-31 doublet. ^{1}H−^{1}H-TOCSY data further supported the assignments of N-27−C-34 and N-27′−C-34′ segments by displaying correlations between spin systems H_2-28−33 and H_2-28′-33′. The ^3J-HMBC correlation between H-31 and C-34′, as well as between H-30′ and C-34, provides the important connections between monomers A and B. The relative stereochemistry for the segments C-1−C-25 and C-1′−C-25′ was shown to be the same as that for manzamine A on the basis of NOESY data and comparison of NMR chemical shift values and coupling constants with those of manzamine A.[1] Protons H-30, and H-30′ show strong NOESY correlations with H-31 and H-31′, respectively, suggesting the same relative stereochemistry. In monomer A H-31 (4.41) shows a key NOESY correlation with H-26 (3.85), requiring that the ether linkage at C-34 is alpha and H-31 are beta in configuration. The conformational analysis of monomer A and its C-34 epimer provided structures with the respective distances of 2.1 and 6.1 Å between

(13) The name *neo*-kauluamine is derived from the manzamine dimer in an earlier report.[12]

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Table 1. ^{13}C-, ^{15}N-, and ^{1}H NMR Data of *Neo*-Kauluamine (**3**)[a]

	monomer A			monomer B	
	^{13}C or ^{15}N	^{1}H		^{13}C or ^{15}N	^{1}H
1	142.8, s	–	1′	143.0, s	–
N2	273.7, S	–	N2′	273.7, S	–
3	138.7, d	8.45, d (5.1)	3′	138.8, d	8.46, d (5.1)
4	113.4, d	7.83, d (5.3)	4′	113.5, d	7.83, d (5.3)
4a	129.3, s	–	4′a	129.4, s	–
4b	121.8, s	–	4′b	121.8, s	–
5	121.6, d	8.10, d (7.9)	5′	121.6, d	8.10, d (7.9)
6	120.1, d	7.29, dd (8.0, 7.9)	6′	120.1, d	7.29, dd (8.0, 7.9)
7	128.4, d	7.55, dd (8.3, 8.0)	7′	128.4, d	7.55, dd (8.3, 8.0)
8	111.5, d	7.51, d (8.4)	8′	111.5, d	7.51, d (8.4)
8a	139.8, s	–	8′a	139.9, s	–
N9	83.6, P	8.6, s	N9′	83.6, P	8.70, s
9a	133.5, s	–	9′a	133.6, s	–
10	139.8, s	–	10′	140.8, s	–
11	137.2, d	6.37, s	11′	137.2, d	6.41, s
12	70.8, s	–	12′	69.3, s	–
13	40.4, t	2.11, 2H, m	13′	41.6, t	2.13, m 1.70, m
14	21.7, t	2.37, m 2.09, m	14′	21.8, t	2.37, m 2.11, m
15	128.0, d	5.64, m	15′	128.3, d	5.64, m
16	132.5, d	5.58, m	16′	132.8, d	5.58, m
17	25.9, t	1.78, m 1.59, m	17′	25.8, t	1.77, m 1.55, m
18	26.7, t	1.44, 2H, m	18′	26.7, t	1.44, 2H, m
19	25.6, t	1.76, m 1.39, m	19′	26.1, t	1.77, m, 1.41, m
20	44.6, t	2.64, m 2.49, m	20′	53.1, t	2.46, m 1.96, m
N21	13.5, S	–	N21′	13.0, S	–
22	49.6, t	2.75, m 1.98, m	22′	49.7, t	2.76, m 1.99, m
23	32.4, t	1.88, m 1.54, m	23′	32.2, t	1.89, m 1.53, m
24	39.8, d	3.06, m	24′	39.2, d	3.05, m
25	45.3, s	–	25′	45.3, s	–
26	75.5, d	3.85, s	26′	75.9, d	3.66, s
N27	57.4, S	–	N27′	37.4, S	–
28	47.2, t	3.15, 2H, m	28′	44.6, t	3.57, m 3.18, m
29	29.7, t	2.05, m 1.56, m	29′	30.0, t	2.27, m 2.12, m
30	72.2, d	3.76, brd (6.1)	30′	72.7, d	4.14, d (7.5)
31	84.4, d	4.41, d (8.7)	31′	67.2, d	3.69, brd (6.1)
32	39.5, t	2.07, m 1.55, m	32′	22.8, t	2.12, m 1.52, m
33	26.5, t	1.75, m 1.45, m	33′	26.6, t	1.78, m 1.40, m
34	89.7, s	–	34′	104.5, s	–
35	53.1, t	2.65, m 2.45, m	35′	51.2, t	2.36, d (13.1) 2.05, d (12.9)
36	68.7, t	3.42, dd (11.3, 2.0) 2.30, m	36′	67.0, t	3.10, dd (11.8, 2.1) 2.28, d (11.9)

[a] In $CDCl_3$, 400 MHz for ^{1}H-, 100 MHz for ^{13}C-, and 50 MHz for ^{15}N NMR. Carbon multiplicities were determined by DEPT135° experiments. s = quaternary, d = methine, t = methylene carbons. Coupling constants (*J*) are in Hz. Nitromethane was used as external standard for ^{15}N NMR. S = quaternary, P = protonated nitrogens.

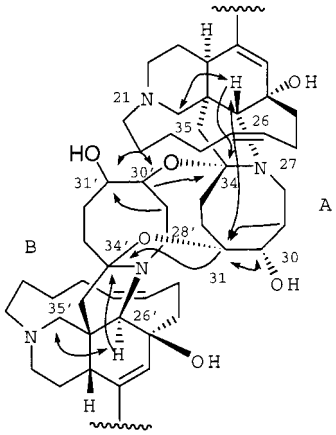

Figure 1. Key HMBC (one arrow) and NOESY (two arrow) correlations of **3**.

H-31 and H-26, respectively. This further supported the assignment of C-34 as alpha in configuration in monomer A. Molecular modeling and NOE measurements suggest that the distance between H-26′ and H-31′ in monomer B would be 4.9 Å (Figure 2) when the distance between H26 and H-31 in monomer A is assigned as 3.0 Å or less. As a result, the relative stereochemistry for both monomers A and B may be identical despite the absence of a comparable NOE correlation between H-26′ and H-31′. The difference between hemispheres A and B yielding unlike chemical shift values may be limited to the nature of the dimerization that has occurred asymmetrically at C-31 and C-30′. In the absence of supporting NOE data, the stereochemistries of C-30′, -31′, and -34′ have been left unassigned despite the fact that the minimized structure indicates that monomer A and B may be identical.

Bioactivity. In vitro analysis of **1**, **2**, and manzamine A against *T. gondii* indicated significant activity. Compound **2** showed a 37% inhibition of the parasite (10 μM concentration) without toxicity to the host. Manzamine A displayed 70% inhibition of the parasite at 0.1 μM concentrations without host cell toxicity. The activity was significantly increased at concentrations of 1 and 10 μM even though it was accompanied by an increase in the toxicity for the host cells. Compound **1** inhibited 71% of the parasite at 1 μM with 38% inhibition of the host cell. Hence, manzamine A was selected for in vivo analysis since it was the most active and least toxic in vitro. A daily i.p. dose of 8 mg/kg of manzamine A, for 8 consecutive days, beginning on day 1 following the infection prolonged the survival of SW mice to 20 days, as compared with 16 days for

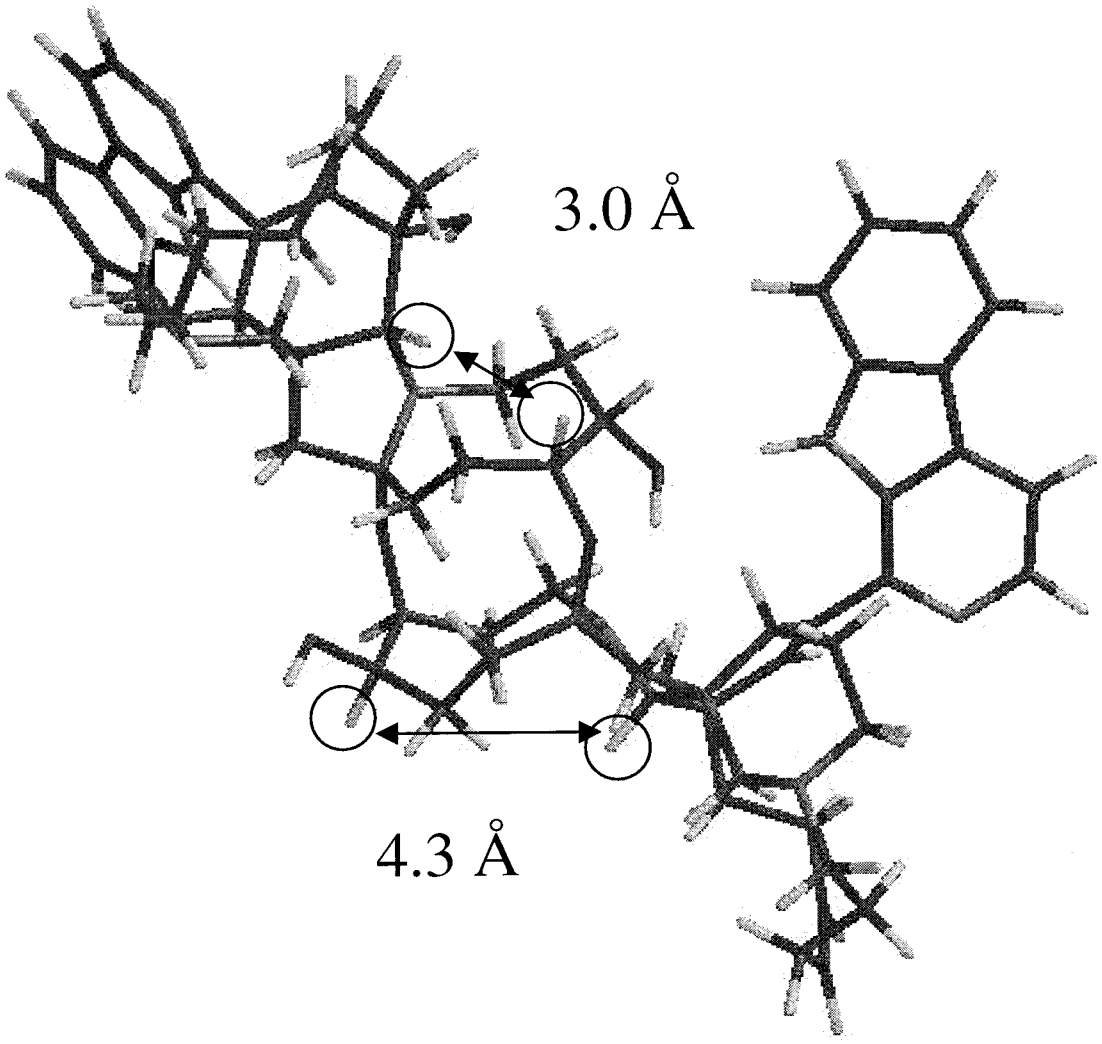

Figure 2. 3D structure of the most stable conformer of **3** with the distance between H-26 and H-31 established by NOE.

the untreated control. Additional manzamine isolations, SAR, and optimized dosing studies will be of significant value to improve the in vivo efficacy of the manzamines against *T. gondii*. All new and known manzamines with the exception *neo*-kauluamine, induced 98−99% inhibition of *Mycobacterium tuberculosis* (H37Rv) with MIC <12.5 μg/mL. Manzamine A, E, and *ent*-8-hydroxymanzamine A exhibit MIC endpoints of 1.56, 3.13, and 3.13 μg/mL, respectively.

Manzamines **1**, **2**, and **3** were assayed in vivo against *Plasmodium berghei* with a single intraperitoneal (i.p.) dose of 100 μmoles/kg and exhibited no apparent toxicity.[9] *ent*-8-Hydroxymanzamine A (**1**) and *neo*-kauluamine (**3**) efficiently reduced parasitemia with an increase in the average survival days of *P. berghei*-infected mice (9−12 days), as compared with: untreated controls (2−3 days), mice treated with artemisinin (2 days) and chloroquine (6 days). The increase in survival days in mice treated with manzamines appears to be attributed in part to an observed immunestimulatory effect.[9] Three 50 μmol/kg i.p. doses of **1** were found to be curative and totally cleared the parasite, and two oral doses (100 μmol/kg) provided a notable reduction of parasitemia. The pharmacokinetic properties of manzamine A, and possibly the rest of manzamines, with its rapid onset of action (2 h) followed by continuous sustained release ensure better bioavailability and effective parasite eradication. These data indicate that manzamines are more active and less toxic than the currently available antimalarial drugs artemisinin and chloroquine. *neo*-Kauluamine (**3**) exhibits cytotoxicity with an IC_{50} 1.0 μg/mL, against human lung and colon carcinoma cells, unlike the first manzamine dimer kauluamine, which was inactive in anticancer assays.[12]

Conclusions

In the first round of murine studies the manzamines have been shown to be more effective antimalarial agents than the currently used drugs artemisinin and chloroquine. With improved pharmacokinetic properties, significant immunostimulatory activity, and the lack of significant in vivo toxicity,[9] the manzamines are clearly valuable candidates for further investigations and development as promising leads against malaria and several other serious infectious diseases. Despite its structural complexity, *neo*-kauluamine with its potent in vivo activity is still an extremely promising antimalarial lead. The potential of dimerizing the readily available manzamine monomers after additional oxygenation provides promise for large-scale production through a combination of mariculture and semisynthesis.

Experimental Section

General Experimental Procedure. Melting points are uncorrected. The ^{1}H- and ^{13}C NMR spectra were recorded in $CDCl_3$, on NMR spectrometers operating at 400 or 500 MHz for ^{1}H-, and 100 or 125 MHz for ^{13}C NMR. The HRMS spectra were measured using a Bioapex

FTMS with electrospray ionization or VG Autospec Q with FAB. TLC analyses were carried out on precoated silica gel G_{254} or aluminum oxide ALOX-100 UV_{254} 500 μm, with the following developing system: cyclohexanes−EtOAc−NH_4OH (100:50:0.1) or C18-reversed phase plates, 200 μm using MeOH−H_2O−NH_4OH (80:20:0.1). For column chromatography, Si gel 60, 40 μm was used.

^{15}N NMR. Inverse detected ^{15}N NMR spectra were recorded using a 500 MHz NMR spectrometer equipped with a 3 mm inverse-detection gradient probe. A gradient HMBC pulse sequence with 1 ms Gaussian *Z*-axis gradient pulses (70:30:50) was used. Referencing of the indirectly detected ^{15}N dimension was accomplished using nitromethane as an external standard. A GHMBC experiment was performed with nitromethane and the ^{15}N correlation was calibrated to 380.2 ppm. This same calibration value was then used for the manzamines. The acquisition time was 12−24 h for each compound.

Sample Material. The sponge was collected on 13 November, 1994, in Manado Bay, Sulawesi, Indonesia, from a depth of −20 m. It is irregularly massive with a rough surface, the texture is tough and crumbly, and the colors in life are maroon externally and yellow internally. The skeleton is very irregular and composed of small, round meshes set in irregular, curving fascicles. The spicules are irregularly curved strongyles, 80−140 μm. The sample represents an undescribed genus and species of the family *Petrosiidae* (order *Haplosclerida*). This group of sponges is characterized by a skeletal architecture that is reminiscent of that of *Xestospongia* and *Petrosia* spp but is more irregular and fasciculate, by the presence of irregularly curved strongyles that range from 80 to 160 μm, by a delicate fibrous texture, and by a deep mustard yellow internal coloration. A voucher specimen has been deposited at the Natural History Museum, London (BMNH 1997.11.11.9). Careful reexamination of specimens, whose manzamine chemistry has been published, such as the *Haliclona* spp, *Xestospongia* spp, and *Pellina* spp, may reveal taxonomic congruence of these specimens and thus a chemical marker for the genus group.

Molecular Modeling. All computational molecular models were performed with SPARTAN version 5.0 program (Wavefunction Inc., CA) mounted on the Indigo2 Workstation (Silicon Graphics, Inc. CA). Conformational analysis was carried out using Osawa method in conjunction with MMFF94 molecular mechanics.

In Vivo Antimalarial and in Vitro Antituberculosis Assays. The detailed materials and methods used for in vivo antimalarial and in vitro antituberculosis assays were reported elsewhere.[9,16]

In Vitro Anti-*Toxoplasma gondii* Assay. In vitro anti-*T. gondii* assays were carried out using [^{3}H]uracil incorporation in cultures of human foreskin fibroblast (HFF) or L929 cells infected with 4×10^4 tachyzoites/well in 96-well flat bottom tissue culture microtiter plates.[6] The toxicity of the tested compound for HFF and L929 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation assay using Cell Titer 96 Kit (Promega Corporation, Madison, Wisconsin).[6]

In Vivo Anti-*Toxoplasma gondii* Assay. Outbred, female Swiss Webster mice (Simonsen Laboratories, Gilroy, California), weighing approximately 20 g at the beginning of each experiment, were used. Food and water were available to the mice at all times. Mice were infected with 2500 tachyzoites of the RH strain i.p. or with 10 cysts of the C56 strain orally.[17] Treatment of mice was initiated 24 h after infection and lasted for 10 days. For oral or intraperitoneal (i.p.) administration, the tested manzamines were dissolved in 0.25% carboxymethylcellulose (CMC) or sterile saline, respectively. Mice were observed for 30 days from the day of infection for mortality and time to death.

***ent*-8-Hydroxymanzamine A (1):** yellowish powder from EtOH, mp 196−198 °C dec, $[\alpha]_D^{25}$ −112.0° (*c* 0.12, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 266 (2.95), 282 (2.94), 390 (2.85) nM; IR υ_{max} ($CHCl_3$) 3499−3267, (NH and OH), 3017−2807, 1680, 1563, 1446 1220 cm^{-1}; ESIFTMS *m*/*z* calculated for $C_{36}H_{45}N_4O_2$ $(M + H)^+$ 565.3543, found 565.3433.

***ent*-Manzamine F (2):** yellowish powder from EtOH, mp 194 °C dec, $[\alpha]_D^{25}$ −44.6° (*c* 0.11, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 266 (3.04), 300 (3.02), 380 (2.92) nM; IR υ_{max} ($CHCl_3$) 3498−3260, (NH and OH), 3026−2802, 1699 (C═O), 1670, 1564, 1446 1221 cm^{-1}; ESIFTMS *m*/*z* calculated for $C_{36}H_{45}N_4O_3$ $(M + H)^+$ 581.3492, found 581.3434.

***neo*-Kauluamine (3):** colorless needles from EtOH, mp 184 °C, $[\alpha]_D^{25}$ +94.6° (*c* 0.1, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 252 (4.20), 357 (3.85) nM; IR υ_{max} ($CHCl_3$) 3592, 3475−3250 (NH and OH), 3007−2802, 1626, 1560, 1454, 1215 cm^{-1}; ^{1}H- and ^{13}C NMR, see Table 1; HRFABMS *m*/*z* calculated for $C_{72}H_{89}N_8O_6$ $(M + H)^+$ 1161.6905, found 1161.6956.

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Supporting Information Available: ^{1}H-, ^{13}C NMR, HMQC, ^{15}N-GHMQC, HMBC spectra of **1**−**3**, in addition to DEPT 135, ^{1}H−^{1}H-COSY, TOCSY, and NOESY spectra of **3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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