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ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · AUGUST 2006

Impact Factor: 3.8 · DOI: 10.1021/np0601399 · Source: PubMed

Manzamine B and E and Ircinal A Related Alkaloids from an Indonesian *Acanthostrongylophora* Sponge and Their Activity against Infectious, Tropical Parasitic, and Alzheimer's Diseases

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Received March 27, 2006

Four new manzamine-type alkaloids, 12,28-oxamanzamine E (**2**), 12,34-oxa-6-hydroxymanzamine E (**3**), 8-hydroxymanzamine B (**5**), and 12,28-oxaircinal A (**11**), were isolated from three collections of an Indonesian sponge of the genus *Acanthostrongylophora* together with 13 known manzamine alkaloids, ircinal A, ircinol A, xestomanzamine A, manzamines A, E, F, J, and Y, manadomanzamines A and B, *neo*-kauluamine, 8-hydroxymanzamine A, and manzamine A *N*-oxide. The structures of the new compounds were elucidated by means of 1D and 2D NMR spectroscopic methods. Three of these compounds (**2**, **3**, and **11**) possess a unique manzamine-type aminal ring system generated through an ether linkage between carbons 12−28 or between carbons 12−34. In the case of manzamine B and related metabolites, carbons 11 and 12 of the typical manzamine structure have an epoxide group and add to our growing understanding of manzamine structure−activity relationships (SAR) and metabolism. The bioactivity and SAR for a number of previously reported manzamine-related metabolites against malaria, leishmania, tuberculosis, and HIV-1 are also presented. Manzamine Y (**9**) showed significant inhibitory activity of GSK3, an enzyme implicated in Alzheimer's disease pathology. The toxicity of manzamine A and *neo*-kauluamine was evaluated against both medaka fry and eggs.

A common Indo-Pacific sponge, *Acanthostrongylophora* sp., has been shown to be a highly rich source of bioactive manzamine-related alkaloids.[1−3] This class of alkaloids has been reported previously to show a number of significant biological activities including cytotoxic,[4] insecticidal,[5] antibacterial,[6] anti-inflammatory,[7] anti-infective,[8] and antiparasitic[9] activities, with the greatest potential for possible clinical applications existing for the control of *Plasmodium falciparum* and *Mycobacterium tuberculosis*.[10]

As part of our ongoing investigations to identify new manzamines and to define the SAR as well as utilize the natural products as synthetic starting materials,[1−3,11−13] extracts of the Indonesian sponge *Acanthostrongylophora* sp. were investigated. In an earlier investigation, this sample yielded two new manzamine alkaloids, named 12,28-oxamanzamine A and 12,28-oxa-8-hydroxymanzamine A, and a unique ircinol A analogue together with manzamines A and F and *neo*-kauluamine.[2] Manzamine A (**1**) exhibits potent in vitro bioactivity against chloroquine-sensitive (D6, Sierra Leone) and -resistant (W2, Indo-China) strains of *P. falciparum.* Reisolation of manzamine A for pharmacokinetic and toxicology studies as well as for preparation of analogues necessitated a major collection of *Acanthostrongylophora* sp., yielding four new manzamine alkaloids (**2**, **3**, **5**, and **11**) together with the 13 known manzamine alkaloids, which are the subject of this report.

In addition, most of the manzamines obtained have been evaluated for activity against glycogen synthase kinase 3 (GSK3), a therapeutic target for the development of drugs for the control of diabetes and Alzheimer's disease (AD).[14] AD remains the most common of the neurodegenerative disorders without any highly effective therapeutic interventions. As the population ages, the social and economic relevance of AD becomes more apparent, which drives the need for effective treatments. Symptoms of AD include memory loss, language deterioration, impaired ability to mentally manipulate visual information, poor judgment, confusion, restlessness, and mood swings. Eventually AD destroys cognition, personality, and the ability to function.[15]

Abnormal increases in GSK3 levels and activity have been associated with neuronal death, neurite retraction and a decline in cognitive performance.[14] Abnormal activity in GSK3 is also implicated in strokes. In fact, lithium, a widely used drug for bipolar disorders, inhibits GSK3 at therapeutically relevant concentrations. Thus, a selective inhibitor of GSK3 could be a potential lead for Alzheimer's disease and other CNS disorders. Only few pharmacological inhibitors of GSK3 are available. In an effort to identify new selective kinase inhibitors with increased potency, the manzamine-type alkaloids have emerged as potential GSK3 inhibitors.

Results and Discussion

The sample of the sponge *Acanthostrongylophora* sp. was collected in May 2002 from Manado, Indonesia, and exhaustively extracted with acetone, after which the chloroform-soluble part of the acetone extract was subjected to silica gel vacuum-liquid chromatography followed by column chromatography and reversed-phase HPLC to yield compounds **2** and **3**.

Compound **2** was obtained as a pale yellow amorphous solid and showed a molecular $[M + H]^+$ ion peak at *m*/*z* 563.3404 in the HRESIMS, and the resulting molecular formula was determined to be $C_{36}H_{42}N_4O_2$ with 18 degrees of unsaturation. The IR spectrum showed a strong absorption at 1718 cm^{-1}, indicating the presence of a carbonyl group. The ^1H NMR data of **2** (Table 1) showed signals of a 1-substituted β-carboline moiety[16] at δ_H 8.44 (1H, d, J = 5.1 Hz, H-3), 8.08 (1H, d, J = 7.8 Hz, H-5), 7.84 (1H, d, J = 5.1 Hz, H-4), 7.53 (1H, d, J = 8.0 Hz, H-8), 7.29 (1H, t, J = 7.4 Hz, H-7), and 7.26 (1H, t, J = 8.0 Hz, H-6), as determined on the basis of correlations of $^1H-^1H$ COSY, HMQC, and HMBC spectra.

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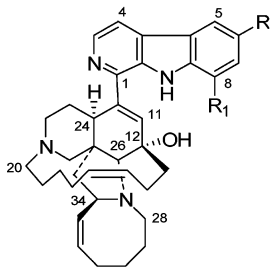
10.1021/np0601399 CCC: $33.50
Published on Web 06/02/2006



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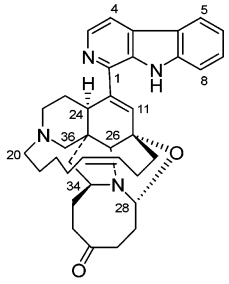

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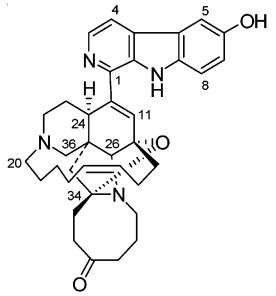

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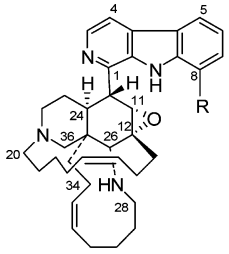

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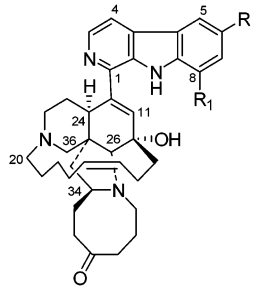

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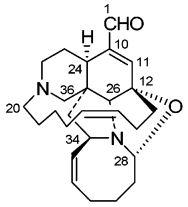

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The olefinic signals at δ 6.56 (s), 5.66 (dt, J = 4.7, 10.3 Hz), and 5.53 (dt, J = 4.3, 11.0 Hz) revealed the presence of one tri- and one disubstituted double bond, and the locations of the two double bonds at C-10/C-11 and C-15/C-16 were clarified by analysis of the HMBC spectrum. These spectroscopic features suggested that compound **2** has a skeleton similar to that of the common manzamine alkaloids, and comparison with the literature data indicated that **2** has the same framework as 12,34-oxamanzamine E.[12] The ^{13}C NMR signals in both compounds matched closely, with the exception of C-28 and C-31 to C-34, which differed significantly, supporting that these compounds have the same skeleton but have differences in functionalities and oxygen substitution. The HMBC spectra of both of the compounds showed similar correlations except for C-28 and C-34, confirming the same skeleton. The proton singlet resonating at δ 4.65 showed a correlation to the nitrogenated methine carbon at δ 76.5 (C-26) in the HMQC spectrum and was assigned to H-26. This proton showed correlations to a quaternary carbon (C-12, δ 77.9) and a methine carbon (C-28, δ 94.5). The downfield shift of C-28 and its appearance as a CH signal in the DEPT spectrum suggested the presence of a new ether bridge between C-12 (C) and C-28 (CH). Data from the ^{1}H−^{1}H-COSY, HMQC, and HMBC spectra provided additional support to justify the gross structure shown for **2**, which was assigned as 12,28-oxamanzamine E. The proposed mechanism of formation of the 12,28-oxaether bridge in **2** is illustrated in an earlier publication.[2]

The positive-ion high-resolution ESIMS of **3** showed an accurate $[M + H]^+$ ion peak at m/z 579.3314, in accordance with a molecular formula of $C_{36}H_{42}N_4O_3$, implying 18 degrees of unsaturation, which was supported by the ^{13}C NMR spectrum and DEPT data. The UV absorption maxima suggested the presence of a β-carboline chromophore.[17] The IR spectrum exhibited an absorption band at 1722 cm^{-1}, supporting the presence of a carbonyl functionality in **3**. The ^{1}H NMR spectrum of **3** showed five characteristic signals in the aromatic region [δ_H 8.21 and 7.82 (1H each, d, J = 5.2 Hz)] assigned to H-3 and H-4 [δ_H 7.50, d (J = 2.5 Hz), 7.10, dd (J = 2.5 and 8.6 Hz), and 7.53 d (J = 8.6 Hz), 1H each] and to the H-5, H-7, and H-8 protons, indicating the presence of a 1,6-disubstituted β-carboline moiety.[16] The olefinic signals at δ 6.26 (s), 5.65 (dt, J = 4.5, 11.4 Hz), and 5.52 (dd, J = 4.8, 8.3 Hz) revealed the presence of one tri- and one di-substituted double bond. The ^{1}H and ^{13}C NMR spectra of **3** (Table 1) suggested close structural homology with that of 6-hydroxymanzamine E (**7**)[1] with one additional double-bond equivalent. The chemical shift of the carbonyl carbon (δ_C 206.2, C-31) is inconsistent with a ketone functionality as observed in **6**−**8** but would fit that of an oxa-analogue as observed in **2**. The downfield shift of C-12 and C-34 (δ 101.8) in **3** and the appearance of the latter carbon as a quaternary signal based on the DEPT spectrum compared with that of **7** suggested the presence of a new ether bridge between C-12 and C-34. Analysis of ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR data, along with a comparison of chemical shift values with those of **6** and **7**, allowed us to establish the structure of **3** as 12,34-oxa-6-hydroxymanzamine E. The 12,34-oxaether bridge was assigned with an α-orientation based on retention of configuration of the C-12 oxygen in the parent compound **7** during the formation of the ether bridge, since in an enzyme-catalyzed reaction H would be oxidatively cleaved as a hydride ion with the formation of a carbocation stabilized by the tertiary nitrogen (enamine). Subsequent attack of OH in an S_N1 fashion and loss of the proton would result in the formation of the 12,34-oxaether bridge. The relative configuration for the segments C-1−C-26 was shown to be the same as that for **8** on the basis of NOESY data and comparison of NMR chemical shift values and coupling constants.

The lipophilic extract of a sponge sample of *Acanthostrongylophora* sp. (04IND35) collected in March 2004 afforded the known ircinal A,[18] manzamine A (**1**),[19] 8-hydroxymanzamine A (**10**),[20] manzamine E (**6**),[21] manzamine F (**8**),[21] ircinol A,[22] and the new manzamine analogue, 8-hydroxymanzamine B (**5**).

Compound **5** was obtained as a brown amorphous powder. Its molecular formula, $C_{36}H_{46}N_4O_2$, was established via HRESIMS, ^{13}C NMR, and DEPT spectroscopic data. The ^{1}H NMR spectrum of **5** showed characteristic signals assignable to a disubstituted β-carboline moiety[16] at δ_H 8.64 (1H, d, J = 5.6 Hz, H-3), 7.24 (1H, d, J = 5.6 Hz, H-4), 7.08 (1H, d, J = 7.7 Hz, H-5), 6.56 (1H, t, J = 8.0 Hz, H-6), and 6.89 (1H, d, J = 7.8 Hz, H-7), which was confirmed unambiguously by the ^{1}H−^{1}H COSY spectrum, which displayed vicinal coupling correlations, while the HMBC spectrum demonstrated correlations of H-3 with C-1 and C-4a, H-4 with C-4b and C-9b, H-5 with C-4a and C-9a, H-6 with C-4b and C-8, and H-7 with C-5 and C-9a. The olefinic signals at δ 5.35 (dt, J = 4.3, 10.8 Hz) and 5.33 (br t, J = 7.5 Hz) revealed the presence of only two isolated, disubstituted double bonds instead of the three found in manzamine A (**1**). Consequently, the trisubstituted double bond in **1** must be saturated in **5** since the diagnostic olefinic singlet (ca. δ 6.50, H-11) of **1** was missing from the ^{1}H NMR spectrum of **5**. The signals at δ 61.5 (s) and 64.9 (d) and the doublet at δ 3.56 (J = 5.5 Hz) are consistent with an epoxy group. The $^1J_{CH}$ value (180 Hz of C-11) suggested the presence of an epoxide, and the epoxy ring was placed at the C-11 and C-12 positions as a result of the observed correlations between H-11/C-1, C-24, and C-26 in the

Table 1. ^{1}H and ^{13}C NMR Data of Compounds **2**, **3**, **5**, and **11** (δ in ppm, J in Hz, $CDCl_3$)[a]

	2		**3**		**5**		**11**	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	143.4 s		143.9 s		140.8 s		193.3 d	9.48 s
3	139.3 d	8.44 d (5.1)	138.8 d	8.21 d (5.2)	137.3 d	8.64 d (5.6)		
4	114.6 d	7.84 d (5.1)	114.2 d	7.82 d (5.2)	122.6 d	7.24 d (5.6)		
4a	130.2 s		129.9 s		115.2 s			
4b	122.3 s		122.0 s		126.3 s			
5	122.2 d	8.08 d (7.8)	121.8 d	7.50 d (2.5)	120.2 d	7.08 d (7.7)		
6	120.6 d	7.26 t (8.0)	150.4 s		123.6 d	6.56 t (8.0)		
7	128.3 d	7.29 t (7.4)	128.8 d	7.10 dd (2.5, 8.6)	108.3 d	6.89 d (7.8)		
8	117.5 d	7.53 d (8.0)	112.3 d	7.53 d (8.6)	139.9 s			
8a	141.2 s		140.8 s		141.2 s			
9a	133.5 s		133.8 s		133.5 s			
10	143.1 s		142.8 s		44.1 d	3.39 m	143.8 s	
11	134.9 d	6.56 s	132.7 d	6.26 s	64.9 d	3.56 d (5.5)	155.4 d	6.75 s
12	77.9 s		80.5 s		61.5 s		77.0 s	
13	40.4 t	2.34 m, 1.65 m	40.3 t	1.80 m, 1.89 m	30.4 t	1.38m, 1.85 m	39.1 t	1.50 m, 2.0 m
14	22.9 t	2.91 m, 2.31 m	23.1 t	2.11 m, 2.21 m	22.4 t	1.91 m, 2.29 m	21.7 t	2.10 m, 2.60 m
15	129.2 d	5.66 dt (4.7, 10.3)	129.9 d	5.65 dt, 4.5, 11.4	128.9 d	5.35 dt (4.3, 10.8)	127.9 d	5.60 m
16	133.4 d	5.53 dt (4.3, 11.0)	129.8 d	5.52 dd, 4.8, 8.3	129.4 d	5.33 br t (7.5)	132.9 d	5.55 m
17	25.9 t	1.86 m, 1.72 m	25.4 t	1.67 m, 2.48 m	29.9 t	2.86 m, 2.92 m	25.7 t	1.8 m, 2.50 m
18	26.2 t	1.56 m, 1.31 m	30.0 t	1.36 m, 1.39 m	29.2 t	1.54 m, 1.89 m	26.1 t	1.20 m, 1.40 m
19	24.4 t	1.44 m, 1.42 m	30.1 t	1.34 m, 1.72 m	29.6 t	1.24 m, 1.73 m	29.9 t	1.80 m
20	52.5 t	2.87 m, 2.33 m	59.3 t	2.34 m, 2.63 m	52.9 t	2.83 m, 2.93 m	52.6 t	2.10 m, 2.60 m
22	49.3 t	3.23 m, 2.12 m	50.1 t	1.81 m, 2.72 m	49.8 t	1.93 m, 2.89 m	49.0 t	1.9, 2.4 dd (4.0, 10.4)
23	32.9 t	2.35 m, 2.64 m	32.1 t	1.45 m, 1.84 m	32.3 t	1.15 m, 1.84 m	32.7 t	1.1, 1.8 ddd (4.8, 12.4)
24	43.3 d	2.45 dd (5.3, 11.4)	46.3 d	3.12 dd (7.3, 11.0)	46.3 d	2.45 m	37.3 d	2.6 dd (6.0, 12.0)
25	38.8 s		38.6 s		43.8 s		42.0 s	
26	76.5 d	4.65 s	67.2 d	3.65 s	57.5 d	3.71 s	76.8 d	4.2 s
28	94.5 d	4.67 t, 8.1	54.1 t	2.64 m, 3.54 m	59.5 t	2.37 m, 2.62 m	94.8 d	4.65 t (7.9)
29	26.6 t	1.73 m, 1.80 m	23.3 t	1.80 m, 1.91 m	29.6 t	1.69 m, 1.81 m	24.3 t	1.40 m, 1.90 m
30	37.1 t	2.45 m, 1.89 m	33.1 t	1.65 m, 1.98 m	29.3 t	1.29 m, 1.89 m	26.7 t	1.50 m, 1.70 m
31	204.5 s		206.2 s		25.8 s	1.51 m, 1.70 m	23.3 t	1.8 m, 3.2 m
32	30.9 t	2.65 m, 3.20 m	30.9 t	1.80 m, 2.21 m	132.6 d	5.65 m	132.4 d	5.40 m
33	30.5 t	2.17 m, 2.26 m	30.5 t	1.79 m, 2.60 m	129.2 d	5.37 m	124.0 d	5.32 bt
34	61.1 d	5.32 m	101.8 s		38.1 t	2.19 m, 2.71 m	60.7 d	3.40 m
35	47.6 t	2.25 m, 2.31m	47.4 t	2.14 m, 2.38 m	37.6 t	1.39 m, 2.22m	48.9 t	1.50 m, 2.0 m
36	66.5 t	3.20 d, 2.15 m	66.3 t	2.61 m, 3.31 m	65.5 t	2.07 d, 2.29 m	68.3 t	2.2 d, 2.7 d (1.5, 11.5)

[a] 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. Carbon multiplicities were determined by DEPT experiments. s = C, d = CH, t = CH_2. Coupling constants (J) are in Hz.

HMBC spectrum and confirmed by the observed vicinal coupling correlations between H-10 and H-11 in the ^{1}H−^{1}H COSY spectrum. A comparison of the data for compound **5** with the literature indicated the same structural features of manzamine B (**4**).[23] The HMBC spectra of both compounds showed similar correlations, confirming the same skeleton. This evidence suggested that the two compounds possess the same configuration with regard to the epoxide group. The ^{13}C NMR and DEPT data of **5** were similar to those of **4** except for the C-8 signal at δ_C 139.9 (s), which suggested that these compounds have the same skeleton but have some differences in oxygen functionalities and revealed that the 8-position of the β-carboline moiety of **5** is substituted by a hydroxyl group.[16] The DQFCOSY and HMQC spectra connected all protons and the protonated carbons in the molecule. HMBC correlations, such as the correlations of H-11 to C-1, C-24, and C-26; H-15 to C-13 and C-17; H-26 to C-11, C-24, and C-35; and H_2-34 to C-32 and C-25, led to the assignment of the gross structure shown for **5**, and thus this compound was characterized as 8-hydroxymanzamine B (**5**). Relative configurations at C-10, C-11, C-12, C-24, C-25, and C-26 of **5** were elucidated to be the same as those of manzamine B (**4**) by NOESY data of **5** as well as by the similarity of the ^{13}C NMR chemical shifts of **5** to those of **4**, whose structure was established by X-ray crystallography.[23]

The sample of *Acanthostrongylophora* sp. MD01 was collected in March 2003 at Manado Bay, Indonesia, by scuba, and the wet sponge (11 kg) was exhaustively extracted with acetone followed by partitioning between chloroform and water to provide 95 g of chloroform extract. Further workup on this extract yielded the known manzamines ircinal A,[18] ircinol A,[22] xestomanzamine A,[22] manzamines A (**1**),[19] E (**6**),[21] F (**8**),[21] J,[4] and Y,[9] manadomanzamines A and B,[3] *neo*-kauluamine,[11] 8-hydroxymanzamine A (**10**),[20] and manzamine A *N*-oxide,[4] along with the new alkaloid 12,28-oxaircinal A (**11**).

12,28-Oxaircinal A (**11**) was obtained as a yellowish powder, and the molecular formula was determined as $C_{26}H_{36}N_2O_2$ by HRESIMS (*m*/*z* 409.2855 $[M + H]^+$), which is consistent with 10 degrees of unsaturation. The IR spectrum provided an absorption band at 1687 cm^{-1}, which was indicative of a carbonyl stretching frequency for an α,β-unsaturated aldehyde functionality. Furthermore, the UV spectrum showed an absorption at λ_{max} 208 nm, consistent with the presence of an α,β-unsaturated aldehyde moiety. ^{1}H NMR and ^{13}C NMR signals resonating at δ 9.48 and 193.3 ppm, respectively, confirmed the presence of the aldehyde. The ^{13}C NMR spectrum revealed the presence of 26 carbons, of which six are olefinic (Table 1). The ^{1}H NMR spectrum indicated the presence of one trisubstituted double bond (δ 6.75, s) and two disubstituted double bonds (δ 5.60, 5.55 and δ 5.32, 5.40) in the molecule. A comparison of the data of **11** with the literature data for ircinal A indicated that they have similar structures.[18] The ^{13}C NMR signals in both compounds matched closely except for C-12 and C-28 to C-34. The downfield shift of C-12 (δ 77.0) and C-28 (δ 94.8) compared with ircinal A suggested the presence of an ether bridge between C-12 and C-28. In addition, a strong COSY correlation between the proton resonating at δ 4.65 ppm (H-28) and the protons resonating at δ 1.90 and 1.40 ppm that were assigned to H_2-29 confirmed the position of the ether linkage. The structure of **11** was finally confirmed by comparing its ^{1}H and ^{13}C NMR data with those of the 12,28-oxamanzamines[2] and was thus characterized as 12,28-oxaircinal A.

Table 2. In Vitro Activity of Manzamine Alkaloids against *Mycobacterium tuberculosis*, *Plasmodium falciparum*, and *Leishmania donovani*

	activity in vitro[a]					
				Leishmania donovani		cytotoxicity (Vero cells)
compound	*M. tuberculosis* (H37Rv) MIC μg/mL	*P. falciparum* (chloroquine sensitive D6 clone) IC_{50} ng/mL	*P. falciparum* (chloroquine resistant W2 clone) IC_{50} ng/mL	IC_{50} μg/mL	IC_{90} μg/mL	IC_{50} μg/mL
manzamine A (**1**)	1.5	4.5	8.0	0.9	1.8	1.2
(+)-8-hydroxymanzamine A (**10**)	0.9	6.0	8.0	6.2	11	1.1
manzamine Y (**9**)	5.2	420	850	1.6	8.0	3.9
manzamine E (**6**)	3.8	3400	4760	3.8	6.8	NC
6-hydroxymanzamine E (**7**)	0.4	780	870	2.5	4.3	4.3
manzamine F (**8**)	2.6	780	1700	4.2	7.0	NC
12,34-oxamanzamine A	NA	4760	NA	14	40	NC
12,28-oxamanzamine A	NT	NA	NA	7.8	50	NC
12,28-oxa-8-hydroxy-manzamine A	NT	NA	NA	24	4.0	NC
12,34-oxamanzamine E	NT	NA	NA	NA	NA	NC
ent-12,34-oxamanzamine F	12.5	840	1100	NA	NA	NC
12,28-oxamanzamine E (**2**)	NT	NA	NA	18	40	NC
12,34-oxa-6-hydroxymanzamine E (**3**)	NT	NA	NA	NA	NA	NC
rifampicin	0.5	NT	NT	NT	NT	NT
chloroquine	NT	15.5	170	NT	NT	NT
artemisinin	NT	10	6.3	NT	NT	NT
pentamidine	NT	NT	NT	2.1	10	NT
amphotericin B	NT	NT	NT	0.06	0.15	NT

[a] NA = not active (concentration: *P. falciparum*, 4.76 mg/mL; *M. tuberculosis*, 64 μg/mL; *L. donovani*, 40 μg/mL; NT = not tested; NC = no cytotoxicity (concentration: 4.76 μg/mL).

Table 3. Activity of Manzamines against AIDS Opportunistic Pathogens and HIV-1

	activity/IC_{50} (μg/mL)[a]				
compound	*S. aureus*	MRS	*C. neoformans*	*M. intracellulare*	HIV-1 EC_{50} (μM)
manzamine A (**1**)	0.5	0.7	3.0	0.35	4.2
8-hydroxymanzamine A (**10**)	0.9	4.0	3.0	1.0	0.59
manzamine Y (**9**)	15	15	NA	3.5	NT
manzamine E (**6**)	NA	NA	NA	12.5	13.1
6-hydroxymanzamine E (**7**)	NT	NT	5.5	3.5	NT
manzamine F (**8**)	NA	NA	6.5	6.25	7.3
12,34-oxamanzamine A	NA	NA	NA	NA	NT
12,28-oxamanzamine A	NA	NA	NA	NA	22.2
12,28-oxa-8-hydroxymanzamine A	NA	NA	NA	NA	NT
12,34-oxamanzamine E	NA	NA	NA	NA	17.5
ent-12,34-oxamanzamine F	NA	NA	NA	NA	14.9
12,28-oxamanzamine E (**2**)	NA	NA	NA	NA	NT
12,34-oxa-6-hydroxymanzamine E (**3**)	NA	NA	NA	NA	NT
amphotericin B	NT	NT	0.15	NT	NT
ciprofloxacin	0.10	0.10	NT	0.25	NT
AZT[29]	NT	NT	NT	NT	0.004

[a] NA = not active; NT = not tested.

The relative configurations of **2** and **11** were deduced from NOESY experiments, which showed NOE correlations between H-26 (s) and H-28 [t, J = 8.1 (**2**), 7.9 (**11**) Hz] as well as between H-24 [dd, J = 5.3 and 11.4 (**2**), 6.0 and 12.0 (**11**) Hz] and H-35. The 12,28-oxaether bridge was assigned with an α-orientation on the basis of retention of stereochemistry of the C-12 oxygen in the parent compounds manzamine E (**6**)[21] and ircinal A[18] during the formation of the ether bridge through a proposed enzymatic oxidation followed by intramolecular quenching of the cation by the OH-12 group through an S_N1 mechanism.[1,2] The relative configuration at the remaining chiral centers of **2** and **11** would then be analogous to those of manzamine E (**6**) and ircinal A on the basis of ^{13}C NMR shifts and NOE data.

Finally, the relative configuration at C-25 and C-26 in **2**, **3**, **5**, and **11** was deduced from *W*-type correlations in their NMR spectra [H-36a/C-12 in HMBC and H-11/H-26 in COSY].

The promising antituberculosis, antimalarial, and antimicrobial activities of the manzamines have been reported earlier, with the exception of manzamine Y (**9**).[1−3,11−13] In Table 2, the in vitro activity of manzamine Y (**9**) is reported along with those of related manzamine analogues and corresponding oxamanzamine derivatives, against *M. tuberculosis* (H37Rv), the malaria parasite, *P. falciparum*, and *Leishmania donovani*, the causative agent for visceral leishmaniasis. All the reported oxamanzamines were inactive against *M. tuberculosis*, *P. falciparum*, and *L. donovani*. The significant differences in biological activities of manzamines A, E, and F and 8-hydroxymanzamine A and the corresponding oxa-derivatives indicate that the C-12 hydroxy, the C-34 methine, or the conformation of the lower aliphatic rings plays a key role in the antimalarial and leishmanicidal activity and provides valuable insight into the structural moieties required for activity with this compound class against the malaria and leishmania parasites. The activity of manzamine Y (**9**) against both the D6 clone and W2 clone of the malarial parasite *P. falciparum* (IC_{50} 420 and 850 ng/mL, respectively) is significantly lower than that of 8-hydroxymanzamine A (**10**) (IC_{50} 6.0 and 8.0 ng/mL, respectively). This difference indicates that the change of the hydroxyl substitution from the C-8 position of the β-carboline moiety to the C-6 position decreases the antimalarial activity dramatically; however, it improves the antileishmanial activity.

The antimicrobial and HIV-1 activities of the manzamines and the corresponding oxa-derivatives are given in Table 3. From this table, it is evident that manzamine A (**1**) and 8-hydroxymanzamine A (**10**) are more potent than manzamines E (**6**), F (**8**), and Y (**9**), and these results provide valuable data to show that the nature of the eight-membered ring and hydroxyl functionality position on the β-carboline moiety are essential for antimicrobial and HIV-1 activity. This observation also suggests that reduction of the C-32−C-33 olefin and oxidation of C-31 to the ketone reduced the antimicrobial activity for the manzamine alkaloids. Significant

Table 4. Biological Activity of Some Manzamine Analogues

compound	$Ca2^+$ channel blocking[a]	GSK3 inhibition in vitro[b]	GSK3 inhibition cell-based assay
manzamine A (**1**)	1 μM	73.2%	+++
8-hydroxymanzamine A (**10**)	1 μM	86.7%	+++
manzamine Y (**9**)		74.29%	n.d.
manzamine E (**6**)		53.6%	−
manzamine F (**8**)		29.9%	++
neo-kauluamine		82.0%	+
ircinal A		0.0%	−

[a] For the calcium channel blocking activity, the minimum concentration inhibiting >20% of the cytosolic calcium increase is given. [b] The % of GSK-3 inhibition at a fixed compound concentration of 25 μM is given.

Table 5. ED_{50} and LD_{50} Values for Medaka Exposed to Manzamine A (**1**), *neo*-Kauluamine, and Chloroquine[a]

	manzamine A (**1**)	*neo*-kauluamine	chloroquine
fry LD_{50}	0.074 ± 0.007	0.82 ± 0.06	121 ± 12
egg ED_{50}	0.17 ± 0.045	2.45 ± 0.47	

[a] Values (μM) are mean ± standard errors ($n = 3$). [b] ED_{50} values determined for eggs failing to hatch following an 11 day exposure. [c] LD_{50} values determined for fry following a 96 h exposure.

differences in biological activities of manzamine A and 8-hydroxymanzamine A and the corresponding oxa-derivatives further indicate that the C-12 hydroxy, the C-34 methine, or the conformation of the lower aliphatic rings plays a key role in the antimicrobial and HIV-1 activity and provides valuable insight into the structural moieties required for activity.

To further explore the bioactivity profile of the manzamines and manzamine-derived compounds, the isolated compounds were evaluated in a series of assays relevant to nervous system function and pathology. The manzamines did not show any effect on acetylcholinesterase (AChE) or β-amyloid cleaving enzyme (β-secretase, BACE1) using in vitro enzymatic assays. Likewise, the compounds did not exhibit any significant ability to protect human neuroblastoma SH-SY5Y cells against oxidative stress-induced cell death.

However, as shown in Table 4, a moderate effect was observed with some compounds on the ability to block calcium channels. More specifically, manzamine A (**1**) was able to block the cytosolic calcium increase induced by KCl depolarization on human neuroblastoma SH-SY5Y cells. The maximum effect was observed at 1.0 μM, inhibiting by 31.5%. 8-Hydroxymanzamine A (**10**) also showed a similar effect, whereas none of the other compounds showed any significant effect whatsoever.

Interestingly, when tested in an in vitro enzymatic assay, most of the compounds did show a moderate, but significant effect in inhibiting human GSK3β activity,[24] as shown in Table 4. Furthermore, when tested in a cell-based assay that measures GSK3β-dependent tau phosphorylation, most of the compounds, but especially manzamine A and 8-hydroxymanzamine A, showed a strong ability to inhibit tau phosphorylation within cells at concentrations as low as 5 μM in the absence of any cytotoxicity. Together these data suggest that the manzamines may be interesting prototypes to potentially develop novel drugs for the treatment of AD and other tauopathies.

Manzamine A (**1**), *neo*-kauluamine, and chloroquine were evaluated against both medaka fry and eggs (see Table 5) and were found to be more toxic than ethanol alone in both medaka fry and eggs (in control groups, percent fry survival and eggs hatching were over 94% and 90%, respectively). Medaka fry were 2.3 and 3.0 times more sensitive than eggs when exposed to manzamine A and *neo*-kauluamine, respectively. Manzamine A was about 11 times more toxic than *neo*-kauluamine to medaka fry ($p = 0.0017$, *t*-test) and about 14.4 times more toxic (unsuccessful hatch) to eggs ($p = 0.012$, *t*-test) than *neo*-kauluamine. Chloroquine was approximately 150 and 1600 times less toxic than *neo*-kauluamine and manzamine A, respectively. Up to a 10.6 μM concentration of chloroquine did not affect medaka hatch relative to a control.

To date, 15 different species of sponges from five families have been shown to produce manzamine-related alkaloids. The fact that the same types of compounds are isolated from such an array of sponges suggests the likelihood that they are produced by sponge-associated microorganisms. Furthermore, in 2003, the manadomanzamines with the novel skeleton were isolated from a morphologically different species of *Acanthostrongylophora* collected from the same area, and there was no manzamine A skeleton in the sample.[3] However, the sample studied in this paper contains both manzamine A and manadomanzamine skeletons, suggesting that the two skeletons may be produced through biotransformations by unique microbial communities associated with individuals of the same species.

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-310 digital polarimeter. UV and IR spectra were respectively obtained using a Perkin-Elmer Lambda 3B UV/vis spectrophotometer and an AATI Mattson Genesis Series FTIR spectrometer. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ and methanol-d_4 using NMR spectrometers operating at 400 or 500 MHz for ^1H and 100 or 125 MHz for ^{13}C NMR. The HRMS were measured using a Bioapex FTESI-MS with electrospray ionization. TLC analysis was carried out on precoated silica gel G_{254} or aluminum oxide ALOX-100 UV_{254} 500 μm.

Biological Material. The samples of *Acanthostrongylophora* sp. were collected in May 2002 (02IND35), March 2003 (MD01), and March 2004 (04IND35) from vertical slopes between 33 and 40 m from Knife Cape Manado, Indonesia. The sponge is massive and encrusting as well as extremely fragile.[12] The sponge was identified as a species of the genus *Acanthostrongylophora* (order Haplosclerida, family Petrosiidae) by Michelle Kelly of the National Institute of Water and Atmospheric Research Ltd., Auckland, New Zealand. Voucher specimens designated as 02IND35 (May 2002), MD01 (March 2003), and 04IND35 (March 2004) are deposited at National Institute of Water and Atmospheric Research Ltd., Auckland, New Zealand, and the Department of Pharmacognosy, The University of Mississippi.

Extraction and Isolation. A fraction obtained from the lipophilic extract of the freeze-dried sponge *Acanthostrongylophora* sp.[2] (02IND35) after purification on silica gel, alumina, and RP-HPLC gave two compounds that were characterized as 12,28-oxamanzamine E (**2**, 6.0 mg, 1.3×10^{-4}% dry wt) and 12,34-oxa-6-hydroxymanzamine E (**3**, 4.0 mg, 8.9×10^{-5} dry wt).

The sample of the sponge *Acanthostrongylophora* sp. designated as 04IND35 (23 kg, wet wt) was crushed, homogenized, and then extracted with acetone at room temperature. The extract was concentrated under reduced pressure, and the resultant aqueous acetone extract was treated with chloroform. TLC analysis of the chloroform extract indicated that it contained manzamine A (**1**), together with various related minor alkaloids as detected by Dragendorff's reagent. The chloroform extract (500 g) was subjected to silica gel vacuum-liquid chromatography and eluted beginning with hexane (100%), hexane−acetone (9:1, 3:1, 1:1), acetone (100%), chloroform−methanol (1:1), and finally methanol (100%). A total of 15 major fractions were collected, and the elution of the metabolites was monitored by TLC.

Fraction 3 (14 g) was rechromatographed on silica gel and eluted with hexane−acetone to obtain ircinal A (1.2 g, 5.22×10^{-5}% wet wt). Fraction 4 (35 g) was rechromatographed over silica and eluted with a gradient system of hexane−acetone and then with methanol to obtain manzamine A (**1**, 12.4 g, 5.4×10^{-4}% wet wt), 8-hydroxymanzamine A (6.6 g, 2.87×10^{-4}% wet wt), 3 g of manzamine E (**6**, 1.3×10^{-4}% wet wt), and 8-hydroxymanzamine B (**5**, 6 mg, 2.6×10^{-7}% wet wt).

Purification of fraction 5 (18 g) over a silica gel column (gradient elution with chloroform−methanol) gave manzamine F (**8**, 8.0 g, 3.5×10^{-4}% wet wt). Flash column chromatography of fraction 6 (26 g) over silica gel, by eluting with a hexane−acetone gradient, acetone,

and ethyl acetate, gave 20 fractions. Ircinol A (4.5 g, 1.96×10^{-4}% wet wt) was obtained from fraction 6.

The sponge sample MD01 (11 kg, wet weight) was extracted with acetone three times. The crude extract was then partitioned between chloroform and water to yield 95 g of chloroform extract. The latter was passed over a VLC column and eluted with hexane−acetone followed by acetone−methanol using a gradient system with 10% increment increases.

The fraction eluted with 10% acetone−hexane (3 g) was chromatographed on a gravity silica column with hexane−2-propanol (9:1) to yield a mixture of manzamine J and 12,28-oxaircinal A. The two compounds were separated by HPLC (Phenomenex, Luna C_8 5 μM, 250 × 22.2 mm, λ 254 nm) eluting with a gradient of acetonitrile−water from 20 to 100% over 40 min (flow rate 5 mL/min) to obtain 7 mg of manzamine J (6.3×10^{-5}% wet wt) and 1 mg of 12,28-oxaircinal A (**11**, 9×10^{-6}% wet wt). Another fraction of the gravity column was subjected to alumina column chromatography using hexane−acetone (9.5:0.5), yielding ircinal A (200 mg, 1.8×10^{-3}% wet wt) and manzamine A (**1**, 1 g, 9×10^{-3}% wet wt).

Elution of the extract with 20% acetone−hexane resulted in six fractions. Fraction 1 (9 g) was subjected to a silica VLC column using a hexane−ethyl acetate gradient system. 8-Hydroxymanzamine A (**10**, 600 mg, 5.4×10^{-3} % wet wt) was obtained and crystallized from methanol. Fraction 5 (1.4 g) was chromatographed using an alumina column and eluted with a hexane−acetone gradient system to obtain a mixture of manadomanzamines A and B and pure manzamine E (**6**, 129 mg, 11.7×10^{-4}% wet wt). The mixture of the manadomanzamines was separated by preparative TLC on alumina using a hexane−acetone system (8.5:1.5) to yield manadomanzamine A (5 mg, 4.5×10^{-5}% wet wt) and manadomanzamine B (3 mg, 2.7×10^{-5}% wet wt).

The fraction eluted with 40% acetone−hexane (970 mg) was passed over a Sephadex LH-20 column eluting with methanol and then chromatographed using HPLC (Phenomenex, Luna C_8 5 μM, 250 × 22.2 mm, λ 254 nm) eluting with a gradient of acetonitrile and water to yield three compounds: *neo*-kauluamine, manzamine F (**8**, 400 mg, 3.6×10^{-3}% wet wt), and manzamine Y (**9**, 10 mg, 9×10^{-5}% wet wt).

The fraction eluted with 50% acetone−hexane (500 mg) was subjected to HPLC chromatography (Phenomenex Prodigy ODS 10 μM 250 × 50.0 mm column, flow rate 15 mL/min, λ 254, 360 nm) using a gradient solvent system of acetonitrile and water to obtain xestomanzamine A (6.5 mg, 6×10^{-5}% wet wt) with 60% water in acetonitrile.

The fraction eluted with 100% acetone (1.8 g) was subjected to separation over a normal-phase silica column using dichloromethane and methanol as a gradient solvent system. Fractions 46−57 were chromatographed on an alumina column using gradient elution with hexane−acetone mixtures. Ircinol A (86 mg, 7.8×10^{-4}% wet wt) eluted with 20% acetone in hexane.

The fraction eluted with 70% methanol in acetone (300 mg) was chromatographed on a normal-phase silica column using a gradient eluting system from 1% to 10% methanol−dichloromethane. Fractions 71−77 were purified by a small silica column using 20% acetone−hexane−2% triethylamine (TEA) as an isocratic system to yield 20 mg (1.8×10^{-4}% wet wt) of manzamine A *N*-oxide.

12,28-Oxamanzamine E (2): pale yellow amorphous solid; $[\alpha]^{25}_D$ +29.4 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 250 (3.80), 281 (3.63), 352 (3.31), 361 (3.42) nm; IR ($CHCl_3$) ν_{max} 3645, 2931, 1718, 1561, 1464, 1196, 678 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS (positive mode) *m*/*z* 563.3404 ($[M + H]^+$, calcd for $C_{36}H_{43}N_4O_2$, 563.3386).

12,34-Oxa-6-hydroxymanzamine E (3): yellow powder; mp 163 °C (dec); $[\alpha]_D^{25}$ +44.3 (*c* 0.4, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 252 (3.82), 275 (3.67), 356 (3.41) nm; IR ($CHCl_3$) ν_{max} 3655, 3001−2821, 1722, 1622, 1593, 1535, 1454, 1264, 1146, 1054 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS (positive mode) *m*/*z* 579.3314 ($[M + H]^+$, calcd for $C_{36}H_{43}N_4O_3$, 579.3335).

8-Hydroxymanzamine B (5): brown amorphous powder; $[\alpha]^{25}_D$ +39.6 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 241 (3.78), 252 (3.65), 278 (3.61), 291 (3.19), 334 (3.26), 352 (3.41) nm; IR ($CHCl_3$) ν_{max} 3635, 3212, 3143, 2992, 2852, 1625, 1511, 1469, 1438, 1324, 1196, 628 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS (positive mode) *m*/*z* 567.3652 ($[M + H]^+$, calcd for $C_{36}H_{47}N_4O_2$, 567.3644).

12,28-Oxaircinal A (11): yellow powder; $[\alpha]^{25}_D$ +56.6 (*c* 0.06, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 208 (3.63), 268 (3.78) nm; IR (film) ν_{max} 2918, 2849, 2365, 2321,1687, 1460 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS (positive mode) *m*/*z* 409.2855 ($[M + H]^+$, calcd for $C_{26}H_{37}N_2O_2$, 409.2850).

In Vitro Evaluation of Antiparasitic, Antimicrobial, and Cytotoxic Activities. Antimalarial activity of the compounds was determined in vitro on chloroquine-sensitive (D6, Sierra Leone) and -resistant (W2, Indo-China) strains of *P. falciparum*. The 96-well microplate assay is based on evaluation of the effect of the compounds on growth of asynchronous cultures of *P. falciparum*, determined by the assay of parasite lactate dehydrogenase (pLDH) activity.[25] The appropriate dilutions of the compounds were prepared in RPMI 1640 medium and added to the cultures of *P. falciparum* (2% hematocrit, 2% parasitemia) set up in clear flat-bottomed 96-well plates. The plates were placed in a modular incubation chamber, flushed with a gas mixture of 90% N_2, 5% CO_2, and 5% O_2, and incubated at 37 °C for 72 h. Growth of the parasite in each well was determined by pLDH assay using Malstat reagent.[25] The medium and RBC controls were included on each plate. The standard antimalarial agents, chloroquine and artemisinin, were used as the positive controls, while DMSO was tested as the negative control.

Antileishmanial activity of the compounds was tested in vitro on a culture of *L. donovani* promastigotes. In a 96-well microplate assay compounds with appropriate dilution were added to the leishmania promastigotes culture (2×10^6 cell/mL). The plates were incubated at 26 °C for 72 h, and growth of leishmania promastigotes was determined by the Alamar blue assay.[26] Pentamidine and amphotericin B were used as the standard antileishmanial agents.

All the analogues were simultaneously tested for cytotoxicity to mammalian cells (Vero cells: monkey kidney fibroblasts) by a Neutral Red assay.[27] The IC_{50} value for each compound was computed from the growth inhibition curve.

Antimicrobial activity against opportunistic pathogens, antituberculosis activity against *M. tuberculosis*, and anti-HIV activity were evaluated by previously published procedures.[28,29]

In Vitro Enzymatic Assays. AChE activity was evaluated in vitro essentially as described by Ellman et al.[30] using AChE from bovine erythrocytes (Sigma) and acetylthiocholine iodide as substrate. BACE1 activity was determined in a FRET-based assay using human recombinant BACE (Sigma). GSK3β activity was measured in vitro via incorporation of ^{33}P-γ-ATP into the GS2 peptide substrate using human recombinant GSK3β (Upstate).

Cell-Based Assays. Human neuroblastoma SH-SY5Y were exposed for 24 h to 100 μM H_2O_2, and the ability of compounds to protect against the oxidative stress was evaluated measuring cellular viability using the LDH assay (Roche). To measure the effect on changes in cytosolic Ca^{2+} in response to depolarization with 60 mM KCl, compounds were assayed in SH-SY5Y cells loaded with Fluo-4. To measure the effect of compounds on GSK3β activity within cells, SH-SY5Y cells were treated for 24 h and tau phosphorylation at the GSK3β-dependent residue Ser396 epitope was determined by an ELISA sandwich method using a phospho-specific antibody.

Fish Source, Care, and Handling. The Japanese medaka (*Oryzias latipes*) culture has been maintained at The University of Mississippi for many years under the University Institutional Animal Care and Use Committee (IACUC) approved conditions. Sexually mature fish are bred and kept in Nanopure BSS (Balanced Salt Solution, Yamamoto, 1965), 82.5 mg/L $CaCO_3$ hardness, 34.3 mg/L $CaCO_3$ alkalinity, 6.6 pH, 23.5 °C temperature, 8.83 mg/L dissolved oxygen, and 1.64 g/L NaCl salinity. The fish are maintained at a 16:8 h light/dark cycle. Adult fish are fed twice daily with tropical flake fish food (Tetramin, Tetra Werke, Germany) and live brine shrimp. Fry and hatchlings are fed only brine shrimp once or twice daily depending on their size.

Eggs Collecting, Handling, and Hatching. Eggs were collected by stripping the egg clutch from the ventral side of the female medaka. The eggs were then transferred to 500 mL jars containing Yamamoto's hatchling solution.[31] The jars were incubated at 26.5 °C on a 16:8 h light/dark cycle and aerated. Hatchling solution was exchanged as needed. When the eggs hatched after10−13 days, the fry were transferred into a Carolina dish containing regular BSS, maintained in the incubator for use (26.5 °C, 16:8 h light/dark cycle).

Fry Exposure. Six-well plates (Falcon, Franklin Lakes, NJ) were used to expose eggs and fry. For fry, seven exposure wells (one ethanol control and six doses) were filled with 5.0 mL of BSS. At 24 h following hatching, 6 fry were transferred to each exposure well for 24 h acclimatization. Alkaloid exposure began 2 days post hatch and

continued for a 96 h exposure. Every 24 h, the fish were checked, dead fish were removed, water was changed (100% static water renewal), and fish were re-dosed. At 120 h after first exposure, final results were recorded and LD_{50}'s calculated. Manzamine A (**1**, 1 mM) and *neo*-kauluamine stock solutions (1 mM) were made in 95% ethanol and diluted to obtain the various concentrations. Nominal concentrations (30 μL of ethanol containing chemicals added to each 5 mL of BSS) were 0, 0.03, 0.06, 0.12, 0.3, 0.48, and 0.6 μM for manzamine A and 0, 0.47, 0.58, 1.17, 2.34, 3.51, and 4.68 μM for *neo*-kauluamine. Chloroquine stock solution (1.006 mM) was made in BSS water and diluted to obtain the appropriate concentrations. Nominal concentrations were 0, 62.5, 125, 250, 375, 435, and 500 μM for chloroquine (30 μL of chemical + 30 μL of EtOH added to 4.97 mL of BSS).

Egg Exposure. Eggs were collected as described above. Seven exposure wells (one control and six dosed) were filled with 3.0 mL of Yamamoto's hatchling solution and 10 eggs per well (26.5 °C on a 16:8 h light/dark cycle). Manzamine A (**1**) and *neo*-kauluamine stock solutions (1 mM) were made in 95% ethanol and diluted to obtain the various concentrations. The test chemical in 30 μL of ethanol was added to 3.0 mL exposure wells to achieve nominal concentrations (control eggs received ethanol alone). Medaka eggs were exposed to nominal concentrations: manzamine A at 0, 0.05, 0.1, 0.2, 0.5, 0.8, and 1.0 μM; *neo*-kauluamine at 0, 0.78, 0.97, 2.92, 5.85, 7.79, and 9.74 μM; and chloroquine at 0, 0.106, 0.424, 0.848, 1.06, 5.3, and 10.6 μM. Exposures were started 24 h after the eggs were collected. Thereafter, the wells were checked, water was changed (100% static water renewal), and eggs were re-dosed every 24 h. Dead eggs and hatched fish were removed from the wells. Eggs were dosed for 11 days, then at day 13, final results were collected, ED_{50}'s were calculated, and the exposure was ended.

Statistical Analyses. Exposures were done in triplicate per exposure. Survival and hatch data were converted to probit units, and E/LD_{50}'s were calculated from a linear regression of probit values versus log concentration. The mean and standard error ($n = 3$) of E/LD_{50} were calculated, and data were compared between treatment groups using Student's *t*-test with $p < 0.05$ being statistically significant.

Acknowledgment. We thank M. Jacob for testing the antimicrobial activities. We also thank F. T. Wiggers and C. D. Dunbar for spectroscopic analysis; M. K. Reddy and M. Yousaf for extraction and purification; J. M. Trott and M. Orugnati for antimalarial and antileishmanial assays; Haitao Yang for technical support with the Medaka exposures; and M. Kelly for taxonomic identification. Financial support was provided by NIH (R01 A136596 and K02 A101502), Center for Disease Control (U50/CCU423310-03 and 1 U01 CI000211-02), USDA-ARS, and Medicines for Malaria Venture, Geneva. The work was supported in part by the Egyptian Government (predoctoral fellowship for M.S.D.). This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program (C06 RR-14503-01) from the National Center for Research Resources, NIH.

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NP0601399