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12,34-Oxamanzamines, novel biocatalytic and natural products from manzamine producing Indo-Pacific sponges

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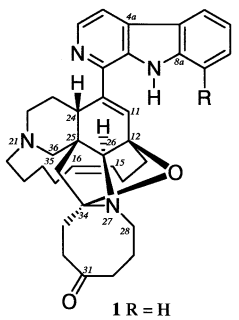
Abstract—*ent*-12,34-Oxamanzamines E (**1**) and F (**2**), as well as 12,34-oxamanzamine A (**4**) were isolated from three Indo-Pacific sponges and their structures were assigned on the basis of spectroscopic data. The biocatalytic transformation of *ent*-8-hydroxymanzamine A (**3**) to **2**, using *Nocardia* sp. ATCC 21145 and *Fusarium oxysporium* ATCC 7601, has also been achieved. These compounds possess a novel ring system generated through a new ether bridge formed between carbons 12 and 34 of the typical manzamine structure. Ten heterotrophic bacterial isolates, including actinomycetes and α-proteobacteria, were isolated from one of these sponges in a preliminary effort to identify a possible microbial origin for these compounds. The potent activity of the manzamines against malaria and the AIDS OI pathogen, *Mycobacterium tuberculosis*, is also presented.

1. Introduction

The manzamines are a group of sponge-derived alkaloids characterized by a complex heterocyclic ring system attached to a β-carboline moiety. Since the first report of manzamine A,[1] more than 40 additional manzamine-type alkaloids have been isolated from nine different genera (four orders).[2,3] Manzamines have been reported to exhibit antitumor,[1] antibacterial,[4] cytotoxic,[5] and immunestimulatory[6] activities and activity against AIDS OI-pathogens, (e.g. *Cryptosporidium parvum* and *Toxoplasma gondii*)[4] as well as the exciting curative activity against malaria in animal models.[6] In search for manzamine-related alkaloids from Indo-Pacific sponges, three manzamine alkaloids with a novel ring system, *ent*-12,34-oxamanzamine E (**1**), *ent*-12,34-oxamanzamine F (**2**) and 12,34-oxamanzamine A (**4**) have been isolated. Here we describe the isolation and structure elucidation of **1**, **2** and **4**, and in addition the biocatalytic transformation of *ent*-8-hydroxymanzamine A[4] (**3**) to **2**. A microbial community analysis for one of the common manzamine producing sponges was initiated in order to identify a sponge associated microbe that could be responsible for the production of these new metabolites.



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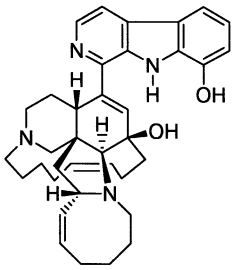
2 R = OH



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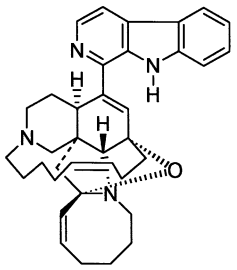

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Keywords: 12,34-oxamanzamines; manzamine; Indo-Pacific sponges.

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Table 1. ^{13}C and ^{1}H NMR data of **1**, **2** and **4**

Position	*ent*-12,34-Oxamanzamine E (**1**)		*ent*-12,34-Oxamanzamine F (**2**)		12,34-Oxamanzamine A (**4**)	
	^{13}C	^{1}H	^{13}C or ^{15}N	^{1}H	^{13}C	^{1}H
1	143.9, s	–	142.6, s	–	143.8, s	–
N2	298.0, S		299.0, S	–	ND	
3	138.8, d	8.41, d (5.2)	138.3, d	8.39, d (5.6)	138.2, d	8.46, d (5.0)
4	114.2, d	7.84, d (5.2)	114.3, d	7.82, d (5.6)	113.9, d	7.84, d (5.0)
4a	129.9, s	–	130.1, s	–	130.1, s	–
4b	122.0, s	–	123.4, s	–	112.0, s	–
5	121.8, d	8.08, d (7.8)	111.9, d	7.63, d (7.7)	122.1, d	8.12, d (7.7)
6	120.4, d	7.26, t (8.0)	120.9, d	7.13, dd (7.8, 7.7)	120.6, d	7.29, t (7.5)
7	128.8, d	7.51, t (7.4)	113.6, d	7.02, d (7.8)	128.4,d	7.53, t (7.6)
8	112.3, d	7.55, d (8.0)	143.6, s	–	111.9, d	7.49, d (7.6)
8a	140.8, s	–	130.6, s	–	140.3, s	–
N9	109.0, P	8.85, s	105.4, P	9.12, s	ND	
9a	133.8, s	–	133.2, s	–	133.2, s	–
10	142.8, s	–	140.1, s	–	142.9, s	–
11	132.7, d	6.24, s	132.2, d	6.33, s	135.5, d	6.58, s
12	80.5, s	–	80.3, s	–	80.4, s	–
13	40.3, t	2.35, m; 1.66, m	39.8, t	2.27, m; 2.09, m	41.4, t	2.25, m; 2.12, m
14	23.1, t	2.85, m; 2.45, m	22.5, t	2.24, m; 1.83, m	23.6, t	2.31, m; 2.01, m
15	129.9, d	5.34, br s	129.3, d	5.33, br s	127.8, d	5.65, m
16	129.8, d	5.29, br s	129.4, d	5.30, br s	133.2, d	5.57, m
17	25.4, t	1.86, m; 1.73, m	25.0, t	1.61, m; 1.49, m	24.6, t	1.65, m; 1.53, m
18	30.0, t	1.52, m; 1.24, m	29.7, t	1.81, m; 1.63, m	29.7, t	1.64, m; 1.73, m
19	30.1, t	1.46, m; 1.38, m	29.6, t	1.79, m; 160, m	30.1, t	1.81, m; 1.67, m
20	59.3, t	2.71, m; 2.28, m	58.9, t	2.67, m; 2.36, m	58.8, t	2.65, m; 2.34, m
N21	36.1, S		NO	–	ND	
22	50.1, t	3.02, m; 2.04, m	49.7, t	3.03, br d (9.3); 2.07, m	49.3, t	3.05, m; 2.15, m
23	32.1, t	2.59, m; 2.67, m	32.8, t	2.59, m; 2.20, m	33.8, t	2.46, m; 2.31, m
24	46.3, d	2.52, dd (11.8, 5.5)	45.9, d	2.57, dd (12.0, 5.6)	43.2, d	2.47, dd (12.0, 5.4)
25	38.6, s	–	38.0, s	–	39.9, s	–
26	67.2, d	4.36, s	66.8, d	4.39, s	68.8, d	4.38, s
N27	73.5, S		73.2, S	–	ND	
28	54.1, t	3.38, dd (12.5, 11.3); 2.84, dd (12.5, 4.4)	53.7, t	3.37, dd (12.8, 11.9); 2.84, dd (12.8, 4.7)	54.1, t	3.35, dd (12.7, 11.6) 2.83, dd (12.8, 4.6)
29	23.3, t	1.72, m; 1.76, m	22.7	1.57, m; 1.48, m	22.4, t	1.63, m; 1.46, m
30	33.1, t	1.64, m; 1.78, m	31.7, t	1.83, 2H, m	33.9, t	1.58, m; 1.82, m
31	206.2, s	–	205.1, s	–	29.6, t	2.32, m; 1.86, m
32	30.9, t	3.20, m; 2.75, m	30.5, t	1.79, m; 1.51, m	133.4, d	5.37, br s
33	30.5, t	2.25, m ; 2.15, m	30.0, t	2.53, m; 1.57, m	124.1, d	5.38, br s
34	101.8, s	–	101.6, s	–	94.9, s	–
35	47.4, t	2.27, d (12.5); 2.34, d (12.5)	47.2, t	2.34, d (12.4); 2.24, d (12.4)	49.1, t	2.35, d (12.5); 2.21, d (12.3)
36	66.3, t	3.15, d (11.0); 2.24, d (11.0)	66.0, t	3.16, d (11.1); 2.30, d (11.1)	69.9, t	3.14, d (11.2); 2.26, d (11.1)

In $CDCl_3$, 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR and 50 MHz for ^{15}N NMR. Nitromethane was used as external standard for ^{15}N NMR. Carbon multiplicities were determined by DEPT experiments. s=quaternary, d=methine, t=methylene carbons. Coupling constants (J) are in Hz. NO=not observed, ND=not determined.

2. Results and discussion

The lipophilic extract of the freeze-dried sponge 01IND 35 (4.5 kg) afforded, after repeated chromatography on Si gel, alumina and RP-HPLC, the known (+)-manzamine A,[1] (+)-8-hydroxymanzamine A,[7] (+)-manzamine E,[8] (+)-manzamine F,[8] (+)-ircinal A,[9] (−)-ircinol A,[10] (+)-6-deoxymanzamine X[11] along with the new (−)-12,34-oxamanzamine E (**1**) and (−)-12,34-oxamanzamine F (**2**).

The lipophilic extract of the freeze-dried sponge 00IND 76 (0.8 kg) afforded the known (+)-manzamine A,[1] (+)-8-hydroxymanzamine A,[7] (+) manzamine F,[8] *neo*-kauluamine,[4] (+) ircinal A[9] and (−)-12,34-oxamanzamine E (**1**).

The HRFTMS spectrum of **1** displayed a molecular ion peak (M+1) at *m/z* 563.3414, which combined with ^{1}H, ^{13}C NMR data (Table 1) suggested a molecular formula of $C_{36}H_{42}N_4O_2$ and 18 degrees of unsaturation. The IR spectrum of **1** provided an absorption band at 1714 cm^{-1}, which was indicative of a ketone functionality. The ^{1}H, ^{13}C NMR spectra of **1** suggested a close structural homology with that of manzamine E[8] with one additional double bond equivalent. The proton singlet resonating at δ 4.36 correlated to the nitrogenated methine carbon at δ 67.2 (C-26) was assigned H-26. This proton showed HMBC correlations to the quaternary carbons resonating at δ 80.5 and 101.8, which were assigned as C-12 and C-34, respectively. The downfield shift of C-12 (δ 80.5) and C-34 (δ 101.8) in **1** as compared with that of manzamine E[8] suggested the presence of a new ether bridge between C-12 and C-34. The downfield quaternary carbon signal at δ 206.2 is assigned as the C-31 ketone group, based on its HMBC correlations with H_2-29 and H_2-33.

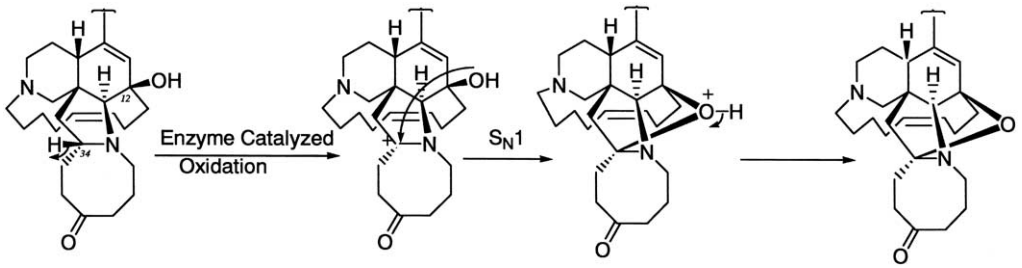

Figure 1. Plausible mechanism of formation of 12,34-oxaether bridge.

^{1}H and ^{13}C NMR data of compound **2** revealed that it differed from **1** only in the carbocyclic ring of the β-carboline moiety (C-5 to C-8a, Table 1). An additional oxygen atom in the molecular formula of **2** ($C_{36}H_{42}N_4O_3$) suggested a phenolic hydroxyl, which is also shown by the ^{1}H NMR spectrum exhibiting five aromatic proton signals instead of six, as well as a new downfield oxygenated aromatic quaternary carbon at δ 143.6 (C-8) in **2**.

The generation of new more active, biocatalytic products of marine-derived compounds has recently been reported for cembrane diterpenes and furanosesterterpenes.[12,13] In order to model the human metabolism of the manzamines and to generate additional analogs for biological evaluation, microbial transformation studies were performed for *ent*-8-hydroxymanzamine A[4] (**3**). Thirty-three growing microbial cultures were screened for their potential to bioconvert **3** to new metabolites. Only *Nocardia* sp. ATCC 21145 and *Fusarium oxysporium* ATCC 7601 were able to exhaustively metabolize **3** with the emergence of a new, less polar metabolite, when monitored by TLC. Both cultures were selected for preparative scale fermentation of **3** to afford the same metabolite which showed identical spectral and physical data, including the $[\alpha]_D^{25}$ value of −49.2 in $CHCl_3$, to that of *ent*-12,34-oxamanzamine F (**2**), isolated from the sponge 01IND 35. The proposed mechanism of formation of 12,34-oxaether bridge is illustrated in Fig. 1. 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 enantiomeric nature of **3** has previously been established based on comparison of its spectral and physical data with authentic (+)-8-hydroxymanzamine A, hence, the stereochemistry of **2** would be comparable to that of **3**.[4] The 12,34-oxaether bridge was assigned β-oriented based on retention of stereochemistry of the C-12 oxygen in the parent compound (**3**) during the formation of the ether bridge, presumably through the proposed S_N1 mechanism. The absolute and relative stereochemistry of **1** would then be analogous to that of **3** and is further supported by its optical rotation value ($[\alpha]_D^{25}=-54.6$ in $CHCl_3$).[4]

The culturable microbial community associated with sponge 01IND 35 were investigated to obtain isolates that could be screened for manzamine production and for their potential to bioconvert manzamines to the new metabolites presented here. Culturable isolates of heterotrophic bacteria were obtained and unequivocally identified by 16 S ribosomal RNA gene sequence analysis as described previously.[14] Ten isolates were obtained and the nearest relative of each isolate was found by BLAST analysis[15] (Table 2). Phylogenetic trees were then inferred for selected isolates by comparing homologous nucleotides using the neighbour-joining,[16] Fitch–Margoliash[17] and maximum parsimony[18] algorithms in the PHYLIP package.[19] Evolutionary distance matrices for the neighbor-joining and Fitch–Margoliash methods were generated as described by Jukes and Cantor.[20] Tree topologies were evaluated after 1000 bootstrap re-samplings of the neighbor-joining data. Isolates included α-proteobacteria (Table 2), a group previously found to be important in culturable sponge-associated bacteria[14] and actinomycetes. Actinomycetes have recently been found to be a significant component of sponge-associated microbiota[21] and are of particular interest considering the excellent track-record of these microbes in production of bioactive compounds. As a result this data supports the potential that sponges may provide a good source of novel actinomycetes for screening programs. The ten isolates from

Table 2. Nearest relatives of isolates from sponge 01IND 35[7] based on BLAST analysis

Isolate number	16 S rRNA sequence length (bp)	Nearest relative	GenBank accession number of nearest relative
M28	774	*Bacillus* sp. VAN35	AF286486
M29	597	*Staphylococcus arlettae*	AB009933
M30	642	*Brevibacillus borstelensis*	D78456
M31	768	α-Proteobacterium MBIC3368	AB012864
M34	635	Unidentified firmiculite strain HTE831	AB010863
M36	714	α-Proteobacterium MBIC3368	AB012864
M37	678	*Pseudomonas* sp. PB1	AF482708
M39	622	Unidentified eubacterium clone BSV04	AJ229178
M40	521	*Bacillus* sp. VAN35	AF286486
M41	653	*Microbacterium barkeri* DSM20145	X77446

Table 3. Bioactivity data for manzamines

Compounds	Assay		
	Mycobacterium tuberculosis (H37Rv) MIC (μg/mL)	*Plasmodium falciparum* (D6 clone) in vitro IC 50 (ng/mL)	*P. falciparum* (chlorine-resistant W2 clone) in vitro IC 50 (ng/mL)
ent-12,34-Oxamanamine E (**1**)	128	NA	NA
ent-12,34-Oxamanzamine F (**2**)	12.5	840	1100
ent-8-Hydroxymanzamine A (**3**)	3.13	NT	NT
12,34-Oxamanzamine A (**4**)	NT	4760	NA
Manzamine A	1.53	4.5	8.0
Ircinal A	30.2	NA	NA
(+)-8-Hydroxymanzamine A	0.91	6.0	8.0
Manzamine E	3.76	3400	4760
Manzamine F	2.56	780	1700
6-Deoxymanzamine X	1.77	1300	1400
Ircinol A	1.93	2400	3100
Rifampin	0.5	NT	NT
Chloroquine	NT	15.5	170
Artemisinin	NT	10	6.3

NA=not active, NT=not tested.

sponge 01IND 35 are currently under investigations for manzamine production and biotransformations.

The lipophilic extract (3.4 kg) of the sponge 01IND 51 afforded compounds **1** and **4**. The ^{1}H and ^{13}C NMR data of compound **4** showed close resemblance to that of manzamine A.[1] The HRFTMS of **4** suggested the molecular formula $C_{36}H_{42}N_4O$ and indicated the presence of one more double bond equivalent as compared with that of manzamine A. This suggested the presence of a new ether bridge between C-12 and C-34, similar to that of **1** and **2**, which was confirmed by ^{13}C NMR data. The relative stereochemistry was inferred to be analogous to that of manzamine A,[1] based on the 2D NOE data and an optical rotation value ($[\alpha]_D^{25}=+40.0$ in $CHCl_3$).

The enormous potential of the marine environment to provide new structural classes with activity against tuberculosis has been recently reported.[22] The in vitro activity of manzamines against *Mycobacterium tuberculosis* (H37Rv) using the microplate Alamar Blue assay[23] and malaria parasite *Plasmodium falciparum* is reported in Table 3. Most manzamines were active against *M. tuberculosis* with MICs <12.5 μg/mL. (+)-8-Hydroxymanzamine A had an MIC 0.91 μg/mL, indicating improved activity for the (+) over the (−) enantiomer. The significant activity of ircinol A (1.93 μg/mL) indicates that the β-carboline moiety is not essential for activity against Mtb in vitro. This result suggests the candidacy of ircinol A as a possible antituberculosis lead for further development since it showed minimal toxicity and reduced structural complexity. The decrease in activity of **1**, **2** and **4** against *M. tuberculosis* and *P. falciparum* is clearly associated with the changes in the molecule that result during the formation of the new C-12, C-34 oxygen bridge (Table 3).

3. Conclusions

The manzamine alkaloids are clearly viable antituberculosis and antimalarial leads based on the results reported herein. Despite the necessity of the β-carboline moiety for in vitro antimalarial activity, it has little effect on the antituberculosis activity suggesting several different possible mechanisms of action are likely to exist. Although further investigations are required to completely understand the SAR for this class of compounds, the absence of activity associated with the new C-12, C-34 oxygen bridge system provides valuable insight into the structural moieties required for activity against Mtb and malaria. The significant reduction in biological activity observed against *P. falciparum* for the new compounds **1**, **2** and **4** indicate that the C-12 hydroxy, C-34 methine or the conformation of the lower aliphatic rings play a key role in the antimalarial activity. Our previous report[6] indicates that reduction of the C32–C33 olefin and oxidation of C-31 also significantly reduces the antimalarial activity for the manzamine alkaloids in vivo. These data combined strongly suggest that the ability of the C-34 allylic carbon to form a stabilized carbocation after oxidation both in cell culture and in animals followed by the inherent nucleophilic attack may play a critical role in the biological activity of the manzamine alkaloids against the malarial parasite.

4. Experimental

4.1. General experimental procedures

The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, on a Bruker DRX NMR spectrometer operating at 400 MHz for ^{1}H, and 100 MHz for ^{13}C NMR. Chemical shift (δ) values are expressed in parts per million (ppm) and are referenced to the residual solvent signals of $CDCl_3$ at δ_H/δ_C 7.26/77.0. The HRMS spectra were measured on a Bioapex FTMS with electrospray ionization. The IR spectra were recorded on ATI Mattson Genesis Series FTIR spectrophotometer. UV spectra were scanned on a Perkin–Elmer Lambda 3B UV/Vis spectrometer. Silica gel (200–400 mesh) and alumina (63–200 μm) were obtained from Natland International Corporation (www.natland.com) and Scientific Adsorbents Incorporated (www.saisorb.com),

respectively. TLC was performed on aluminum sheets (silica gel 60 F_{254}, Merck KGaA, Germany).

4.2. Sponge collection, identification and taxonomy

01IND 35. The sponge was collected from reef slopes and vertical surfaces between 6 and 33 m from Black Reef Point, Manado Bay, Indonesia, on March 19, 2001, where it was extraordinarily abundant. The sponge is irregularly massive to thickly encrusting with a crumbly texture. The external color in life is brownish maroon, the interior mustard yellow, the sponge appears slightly greenish under water. The skeleton is made up of relatively regular round-meshed tracts of small curved strongyles. The sponge is a species of an undescribed *Petrosiidae* genus (Order Haplosclerida, Family Petrosiidae), very similar to the sponge 94IND 136 (BMNH 1997.11.11.9) described in Ref. 4. A voucher specimen of 01IND 35 has been deposited at the Natural History Museum, London, United Kingdom (BMNH 2002.5.13.1).

00IND 76. The sponge was collected from reef slopes at a depth of 10–20 m from Manado Bay, Indonesia, on March 17, 2000, and is irregularly massive and crumbly texture. The external color in life is maroon, the interior yellow. The skeleton is made up of ragged irregular ladder-like tracts of small curved strongyles. The sponge is a species of an undescribed *Petrosiidae* genus (Order Haplosclerida, Family Petrosiidae), very similar to the sponge 94IND 136,[4] and 01IND 35 above, but differing from the latter in terms of the smaller size of the skeletal mesh, the more delicate nature of the fibers and the slightly different arrangement of the primary tracts which are more ladder-like. A voucher specimen of 00IND 76 has been deposited at the Natural History Museum, London, United Kingdom (BMNH 2001.7.20.11).

01IND 51. The sponge was collected from vertical slopes between 33 and 40 m from Knife Cape, Manado Bay, Indonesia, on March 20, 2001, and is massively encrusting and extremely fragile. The external and internal color in life is brown. The skeleton is delicate, unlike that of 94IND 136,[4] 01IND 35, being more like that of 00IND 76 above, with narrow curving tracts of small strongyles that are interconnected by occasional irregular secondary tracts. The skeleton is however, a lot less dense than in 00IND 76, and the tracts are much finer. The sponge is also a species of an undescribed *Petrosiidae* genus (Order Haplosclerida, Family Petrosiidae), but it differs markedly from 01IND 35 and 00IND 76 in morphology, color, texture and skeletal density. This species is less common than 01IND 35 and 00IND 76. A voucher specimen of 01IND 51 has been deposited at the Natural History Museum, London, United Kingdom (BMNH 2002.5.13.2).

The three sponges examined here (01IND 35, 00IND 76, and 01IND 51) differ slightly in their morphology and in manzamine alkaloid chemistry. 01IND 35 and 00IND 76 have a maroon exterior and yellow interior, while 01IND 51 is brown internally and externally. The morphology of the latter also differs as the sponge is encrusting and much more fragile. The more delicate skeleton reflects this difference quite clearly. These differences are not however, reflected in the size of the strongyle spicules, which in all three specimens range in length from 100–150 μm.

4.3. Extraction and isolation

01IND 35. Freeze-dried sponge (4.5 kg) was blended and exhaustively extracted 4×16 L acetone. The extracts, after filtration, were concentrated in vacuo until dried. The crude extract (215 g) was chromatographed on Si gel (column: 150×13 cm) with hexane-acetone (9.5:0.5–1:1) and then MeOH to yield five fractions: fr. 1 (hexane–acetone 9.5:0.5) was chromatographed on Si gel (column: 100×6 cm) with a hexane–acetone gradient (9.5:0.5–9: 1) to yield (+)-ircinal A[9] (2.2 g, 4.8×10^{-2}% dry wt), (−)-ircinol A[10] (1.3 g, 2.8×10^{-2}% dry wt). Fr. 2 (hexane–acetone 9.5:0.5–8:2) was chromatographed on alumina (column: 100×6 cm) with a hexane–acetone gradient (9.5:0.5–7:3) to yield (+)-manzamine A[1] (9.0 g, 0.2% dry wt), (+)-8-hydroxy-manzamine A[7] (1.5 g, 3.3×10^{-2}% dry wt), (+)-manzamine E[8] (1.3 g, 2.8×10^{-2}% dry wt), (+)-manzamine F[8] (1.2 g, 2.6×10^{-2}% dry wt). Fr. 3 (hexane–acetone 8:2–6:4) was further purified by RP-HPLC (Prodigy 5 μM ODS 3 100 Å, 10×250 mm Phenomenex) using CH_3CN–H_2O as an eluent (flow rate of 10 mL/min and UV detection at 410 nm) to yield (+)-6-deoxymanzamine X[11] (15 mg, 3.3×10^{-4}% dry wt) along with the new (−)-12,34-oxamanzamine E (**1**) (10.5 mg, 2.3×10^{-4}% dry wt) and (−)-12,34-oxamanzamine F (**2**) (11.6 mg, 2.5×10^{-4}% dry wt).

00IND 76. The sponge (0.8 kg) was initially preserved frozen. The crude extract (31 g) was obtained by extracting the homogenized, freeze-dried sponge with acetone (3×6 L) was combined and concentrated under vacuum. The extract was subjected to Si gel chromatography using a gradient of hexane to acetone and finally MeOH. The manzamine containing fractions were rechromatographed on Si gel using a gradient of hexane–acetone (9.5:0.5–8:2) to afford (+)-manzamine A[1] (990 mg, 1.2×10^{-4}% dry wt), (+)-8-hydroxymanzamine A[7] (1.2 g, 1.5×10^{-1}% dry wt), (+) manzamine F[8] (350 mg, 4×10^{-1}% dry wt), *neo*-kaluamine[4] (40 mg, 5×10^{-3}% dry wt), (+) ircinal A[9] (80 mg, 1×10^{-2}% dry wt) and (−)-12,34-oxamanzamine E (**1**) (4.5 mg, 5.6×10^{-4}% dry wt).

01IND 51. The lyophilized sponge (3.8 kg, dry weight) was blended and exhaustively extracted with hexane and acetone. The combined extract (110 g) was subjected to Si gel vacuum liquid chromatography on Si gel (column: 150×13 cm) with a CH_2Cl_2–acetone gradient (9.9:0.1–1:1) then with $CHCl_3$–MeOH (9.9:0.1–2.5:7.5) and finally with MeOH to yield nine fractions: fr. 5 (CH_2Cl_2–acetone 9.5:0.5) was rechromatographed on Si gel and eluted with chloroform–acetone gradient to yield crude manzamines, which were further purified over alumina (hexane–acetone, 95:5), RP-HPLC (Luna 15 μM C8, 100×250 mm Phenomenex) using CH_3CN–H_2O (0.1% TFA) as an eluent (flow rate of 19.8 mL/min and UV detection at 410 nm) to obtain (+)-8-hydroxymanzamine A (40 mg, 1×10^{-3}% dry wt), (+)-manzamine A (3.2 g, 8.4×10^{-3}% dry wt), (−)-12,34-oxamanzamine E (**1**) (4 mg, 1.1×10^{-4}% dry wt) and (+)-12,34-oxamanzamine A (**4**) (2.2 mg, 3.0×10^{-5}% dry wt).

4.3.1. (−)-12,34-Oxamanzamine E (1). Brown amorphous solid ($CHCl_3$); mp 152°C dec., $[\alpha]_D^{25}=-54.6$ (*c* 0.3, $CHCl_3$); UV λ_{max} (log ε) (MeOH) 252 (3.82), 275 (3.65), 354 (3.41) nm; IR ν_{max} ($CHCl_3$) 3650 (NH), 3001–2818, 1714 (C═O), 1620, 1592, 1533, 1452, 1267, 1144, 1052 cm^{-1}; HRFABMS *m/z* calculated for $C_{36}H_{43}N_4O_2$ $(M+H)^+$ 563.3386, found 563.3414; ^1H and ^{13}C NMR, see Table 1.

4.3.2. (−)-12,34-Oxamanzamine F (2). Yellowish powder (EtOH), mp 158°C dec., $[\alpha]_D^{25}=-49.2$ (*c* 0.10, $CHCl_3$); UV λ_{max} (log ε) (MeOH) 251 (3.83), 273 (3.69), 356 (3.42) nm; IR ν_{max} ($CHCl_3$) 3658 (NH), 3377 (OH), 3002–2822, 1714 (C═O), 1620, 1592, 1533, 1452, 1267, 1144, 1052 cm^{-1}; HRFABMS *m/z* calculated for $C_{36}H_{43}N_4O_3$ $(M+H)^+$ 579.3335, found 579.3313; ^1H and ^{13}C NMR, see Table 1.

4.3.3. (+)-12,34-Oxamanzamine A (4). White powder (MeOH), mp 164°C dec., $[\alpha]_D^{25}=+40.0$ (*c* 0.6, $CHCl_3$); UV λ_{max} (log ε) (MeOH) 252 (3.823), 271 (3.71), 358 (3.41) nm; IR ν_{max} ($CHCl_3$) 3635 (NH), 3368 (OH), 3001–2815, 1715 (C═O), 1625, 1590, 1535, 1451, 1265, 1145, 1050 cm^{-1}; HRFABMS *m/z* calculated for $C_{36}H_{43}N_4O$ $(M+H)^+$ 547.3408, found 547.3458; ^1H and ^{13}C NMR, see Table 1.

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