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Ingamines A and B, New Cytotoxic Alkaloids from the Marine Sponge *Xestospongia ingens*

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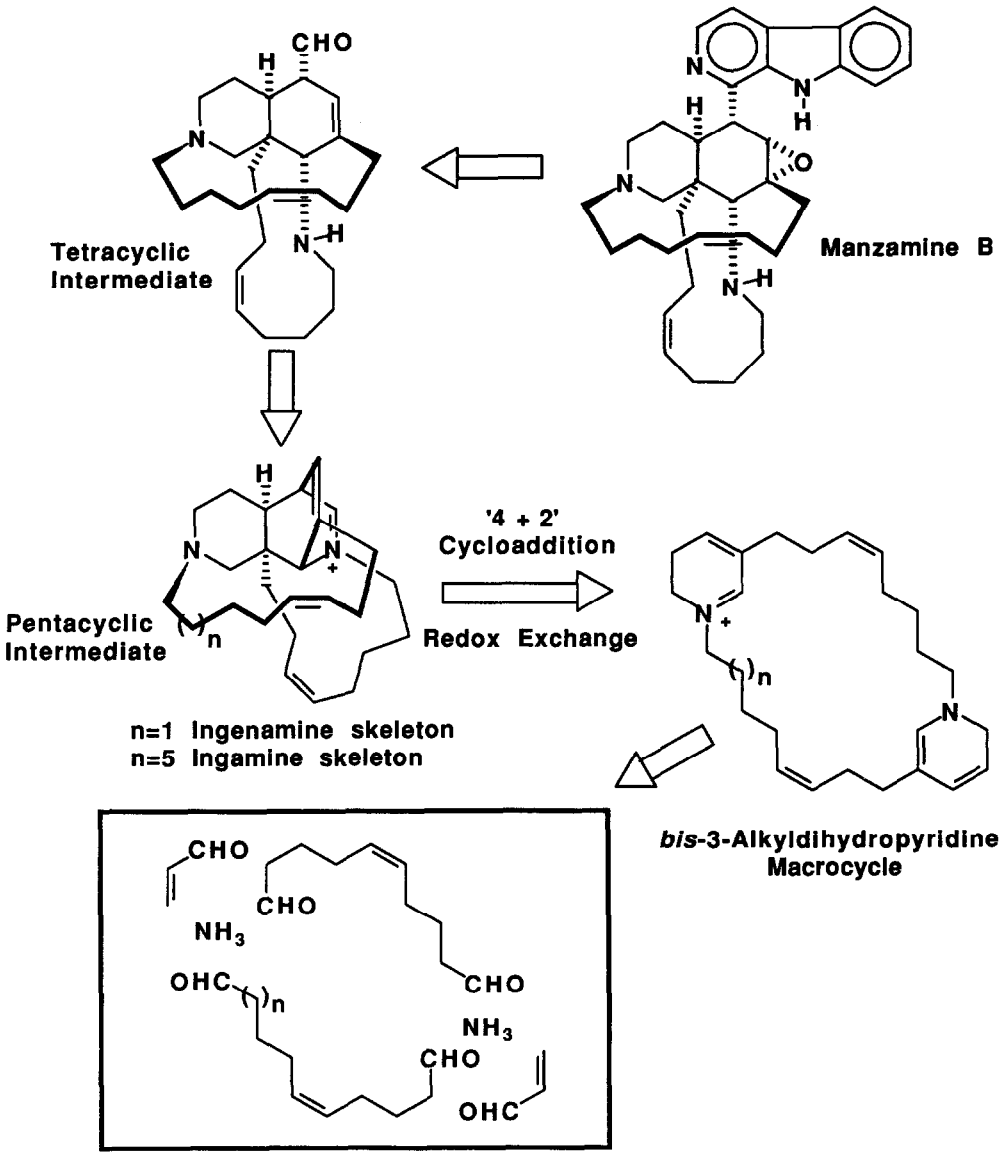
Abstract: Ingamines A (2) and B (3), two novel cytotoxic alkaloids, have been isolated from the marine sponge Xestospongia ingens collected in Papua New Guinea. The structures of 2 and 3 have been elucidated via extensive spectroscopic analysis.

A growing collection of complex and structurally diverse alkaloids, that are related to each other by their apparent biogenetic origin from macrocycles composed of 3-alkylpyridine or reduced 3-alkylpyridine units, have been isolated from marine sponges in recent years. The halitoxins[1] and an epidermal growth factor active constitutent obtained from *Callyspongia fibrosa*[2] are high molecular weight cyclic oligomers (n>4) of 3-alkylpyridine monomers. It has been suggested that the haliclamines,[3] the saraines 1-3,[4] the xestospongins,[5] the petrosins,[6] and saraine-A[7] are all derived from reduced *bis*-3-alkylpyridine macrocycles.[8] Baldwin and Whitehead[9] recently proposed (Scheme 1) that the biosynthesis of the manzamines[10] also proceeds through a *bis*-3-alkyldihydropyridine macrocycle. Their proposal suggested that the macrocycle was initially transformed via the biosynthetic equivalent of an intramolecular '4 + 2' cycloaddition reaction to a pentacyclic intermediate that after redox exchange between the two piperidine rings could be opened via iminium salt hydrolysis to give a tetracyclic aldehye. Condensation of the tetracyclic aldehyde with tryptophan leads to the manzamine skeleton. This elegant proposal suggested the possible occurrence of two new classes of alkaloids represented by their pentacyclic and tetracyclic intermediates, respectively. The subsequent isolation of ircinal B from the sponge *Ircinia* sp.[11] provided the first example of the new type of alkaloid corresponding to the Baldwin and Whitehead tetracyclic intermediate. An interesting parallel to the proposed biogenesis of the manzamines can be found in the suggested biogenesis of xestocyclamine A.[12] It has been proposed that the pentacyclic skeleton of xestocyclamine A, which is isomeric with the skeleton of the Baldwin and Whitehead pentacyclic intermediate, originates from the biosynthetic equivalent of an intermolecular cycloaddition reaction between two monomeric 3-alkyldihyropyridine macrocycles.

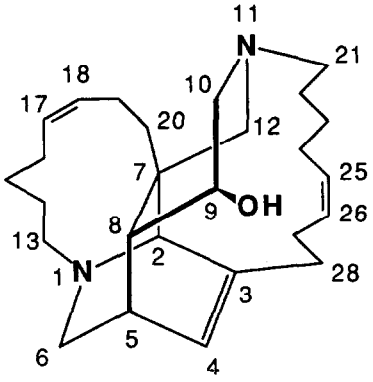
As part of an ongoing search for bioactive metabolites in extracts of tropical marine sponges,[13] it was found that crude extracts of the sponge *Xestospongia ingens*[14] collected in Papua New Guinea exhibited in vitro

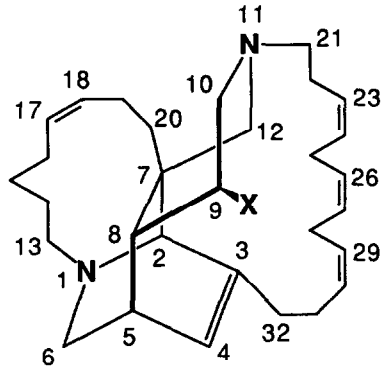


Scheme 1[9]

cytotoxicity against murine leukemia P388. Bioassay guided fractionation of the extracts yielded the cytotoxic alkaloids ingenamine (**1**), ingamine A (**2**), and ingamine B (**3**). Ingenamine (**1**), whose structure has already been reported,[15] represents the first example of a new class of cytotoxic alkaloids that correspond to the pentacyclic intermediate in the Baldwin and Whitehead biogenetic proposal for the manzamines (Scheme 1).

The structures of ingamines A (2) and B (3), two additional new cytotoxic alkaloids that are related to ingenamine (1), are reported below.





1 Ingenamine

2 X= OH Ingamine A

3 X=H Ingamine B

Ingamine A (2) was isolated as an optically active colorless glass that gave a parent ion in the HREIMS at m/z 448.3454 corresponding to a molecular formula of $C_{30}H_{44}N_2O$ (ΔM + 0.1 mmu). The ^{13}C NMR/APT spectrum of ingamine A (2), which showed resolved resonances for all thirty carbon atoms (2 X C; 13 X CH; 15 X CH_2) (Table 1), contained ten deshielded resonances that could be assigned to olefinic carbons. No additional unsaturated functional groups were apparent from the ^{13}C NMR data, indicating that ingamine A was pentacyclic. An IR band at 3307 cm^{-1} and NMR resonances at δ 68.9 (CHOH: C9) and 3.40 (CHOH) were assigned to a secondary alcohol, accounting for the one proton not attached to carbon. The 1D and 2D ^1H and ^{13}C NMR spectra of ingamine A (2) showed strong resemblences to the ^1H and ^{13}C NMR spectra of ingenamine (1) (Table 1). In particular, resonances corresponding to those assigned to the central tricyclic core (N1 - C12) and the linear alkyl bridge connecting N1 and C7 in the spectra of ingenamine (1) could be found in the NMR spectra of ingamine A (2), suggesting that the two molecules shared these structural features.

Detailed analysis of the COSY, HMQC, HMBC and NOE data obtained for ingamine A (2) confirmed the presence of the tricyclic core (N1-C12) and identified the four attached methylene carbons at N1, C3, C7 and N11 also found in ingenamine (1). COSY correlations were observed between H4 (δ 5.94) and H2 (δ 3.03), H5 (δ 2.66), H32 (δ 2.33), and H32' (δ1.98); between H5 (δ 2.66) and H6' (δ 1.76) and H8 (δ 0.97); between H8 (δ 0.97) and H9 (δ 3.40); and between H9 (δ 3.40) and H10' (δ 2.69). HMBC correlations were observed between H4 (δ 5.94) and C2 (δ 64.1), C5 (δ 34.5), C8 (δ 51.9), and C32 (δ 36.2); between H5 (δ 2.66) and C3 (δ 143); between both H6 (δ 2.91) and H6' (δ 1.76) and C4 (δ 121), C5 (δ 34.5), and C8 (δ 51.9); between between H8 (δ 0.97) and C5 (δ 34.5) and C10 (δ 55.9); and between H9 (δ 3.40) and C5 (δ 34.5). A network of HMBC correlations confirmed the placement of the aliphatic quaternary carbon (δ 45.3: C7) identified by the

APT experiment between C2 and C8 and also identified methylene carbons (C12 and C20) as the other two substituents on the quaternary carbon. Thus, H8 (δ 0.97) and H2 (δ 3.03) both showed two bond HMBC correlations to δ 45.3 (C7), and H2 (δ 3.03) was also correlated through three bonds to C8 (δ 51.9). A pair of geminal methylene protons at δ 2.45 (H12) and 2.07 (H12'), that were correlated to a carbon at δ 50.2 in the HMQC spectrum, showed two bond HMBC correlations to the quaternary carbon (δ 45.3: C7) and one of them (H12') showed a three bond correlation to a methylene carbon at δ 41.5 (C20). In addition, H12 (δ 2.45) showed a three bond HMBC correlation to C8 (δ 51.9) and H12' (δ 2.45) showed a three bond correlation to C2 (δ 64.1). The protons (δ 1.98 and 1.74: H20, H20') attached to the methylene carbon at δ 41.5 (C20) showed two bond correlations to the quaternary carbon at δ 45.3 (C7). Furthermore, in the HMBC spectrum H20' (δ 1.74) was correlated to C12 (δ 50.2), H20 (δ 1.98) was correlated to C2 (δ 64.1), and H8 (δ 0.97) was correlated to δ 41.5 (C20).

The ^{13}C NMR chemical shifts of C2 (δ 64.1) C6 (δ 54.2), C10 (δ 55.9) and C12 (δ 50.2) (Table 1) in ingamine A (**2**) were nearly identical to the chemical shifts of the corresponding carbons in ingenamine (**1**) (C2, δ 63.8; C6, 53.8; C10, 52.5; C12, 50.8) indicating that the two nitrogen atoms were also attached to C2 and C6, and C10 and C12, respectively, in **2**. HMBC correlations between H2 (δ 3.03) and C6 (δ 54.2), and between H6 (δ 2.91) and C2 (δ 64.1) were consistent with the nitrogen bridge between C2 and C6, and additional correlations between H6' (δ 1.76) and a methylene carbon at δ 54.3 (C13) and between one of the protons (δ 2.82: H13) attached to the methylene carbon at δ 54.3 and C2 (δ 64.1) and C6 (δ 54.2) identified the methylene carbon (C13) as the third substituent of the tertiary amine. Similarly, HMBC correlations between H12 (δ 2.45) and C10 (δ 55.9) provided evidence for the nitrogen bridge between C10 and C12, and correlations between a methylene carbon at δ 58.3 (C21) and H12' (δ 2.07) identified this methylene carbon as the third substituent of the second tertiary amine. ROESY correlations observed between H9 (δ 3.40) and H12' (δ 2.07), between H9 and H4 (δ 5.94), and between H8 (δ 0.97) and H6 (δ 2.91) confirmed that the central core of ingamine A (**2**) had the same relative stereochemistry as in ingenamine (**1**) and that the C7 to C12 piperidine ring still occupied a boat conformation.

The remaining portion of ingamine A (**2**), consisting of eight aliphatic methylene and eight olefinic methine carbons, had to form a pair of linear bridges between the four methylene appendages at N1, C3, C7 and N11 to complete the final two rings required by the unsaturation number indicated by the molecular formula. Detailed analysis of the COSY, HMQC, and HMBC data for ingamine A (**2**) clearly supported an eight carbon alkyl bridge spanning N1 and C7 that was identical to the N1/C7 alkyl bridge previously identified in ingenamine (**1**). HMQC/COSY correlations identified the chemical shifts of all of the pairs of geminal protons on C13 (δ (^{13}C:^{1}H) 54.3: 2.25, 2.82), C14 (δ 26.0: 1.24, 1.48), C15 (δ 26.3: 1.47, 1.59), C16 (δ 22.9: 1.59, 2.27), C19 (δ 20.8: 1.8, 2.20) and C20 (δ 41.5: 1.74, 1.98) in this bridge. COSY correlations were observed between H13 (δ 2.82) and H14 (δ 1.48) and H14' (δ 1.24); between H14' (δ 1.24) and H15 (δ 1.59); between H15' (δ 1.47) and H16 (δ 2.27) (long range COSY); between H16 (δ 2.27) and H17 (δ 5.63); between H18 (δ 5.65) and H19 (δ 2.20); and between H19 (δ 2.20) and H20 (δ 1.98). In addition, HMBC correlations were observed between H13' (δ 2.25) and C15 (δ 26.3); between H15 (δ 1.59) and C13 (δ 54.3); between H15 (δ 1.59)/ H15' (δ 1.47) and C16 (δ 22.9); between H17 (δ 5.63) and C19 (δ 20.8); between H18 (δ 5.65) and C16 (δ 22.9); between H16 (δ 2.27), H16' (δ 1.59) and both C17 (δ 131.4) and C18 (δ 130.3); and between H19 (δ 2.20) and both C17 (δ 131.4) and C18 (δ 130.3); and between H20 (δ 1.98)/H20' (δ 1.74) and C19 (δ 20.8).

Comparison of the ^{13}C NMR chemical shifts of C16 (δ 22.9) and C19 (δ 20.8) with the data reported for the ircinals and the haliclamines (Z alkenes have allylic carbons at δ < 27 : E alkenes have allylic carbons at δ >30) confirmed the Z geometry for the $\Delta^{17,18}$ alkene.

With the nature of the N1 to C7 alkyl bridge established, it was apparent that the C3 to N11 bridge had to be twelve carbons long and it had to contain three olefins. The absence of a UV chromophore in ingamine A (**2**) indicated that there was no conjugation between the three olefins. In addition, the ^{1}H NMR spectrum contained two pairs of geminal methylene protons (H25 (δ 2.90)/ H25' (δ 2.68): H28 (δ 2.90)/ H28' (δ 2.78)) that had chemical shifts typical of protons on doubly allylic carbons in polyunsaturated fatty acids and these H25/H25' and H28/H28' resonances were all correlated into the cluster of olefinic protons between δ 5.4 to 5.8 in the COSY spectrum (Table 1). These two pieces of evidence, along with the HMBC data (Table 1), were consistent with the methylene interupted triene substructure (C23 to C30) shown in **2**. In addition, the COSY and HMBC data (Table 1) demonstrated that there were two methylene carbons between both N11 and C3 and the respective ends (C23 and C30) of the triene substructure. Thus, COSY correlations were observed between H21/H21' (δ 2.62) and H22/H22' (δ 2.27); between H22/H22' (δ 2.27) and H23 (δ 5.31); between H32' (δ 1.98) and H31' (δ 2.18) and between H31' (δ 2.18) and H30 (δ 5.47). HMBC correlations were observed between H23 (δ 5.31) and C21 (δ 58.3) and between H 32' (δ 1.98) and C30 (δ 128.7). Once again, the chemical shifts of the allylic carbons indicated that the $\Delta^{23,24}$, $\Delta^{26,27}$, and $\Delta^{29,30}$ olefins had the Z geometry. Therefore, the complete the structure of ingamine A (**2**) is as shown.

Ingamine B (**3**) was isolated as an optically active colorless glass that gave a parent ion in the EIHRMS at m/z 432.3504 corresponding to a molecular formula of $C_{30}H_{44}N_2$ (ΔM 0.0 mmu), differing from the molecular formula of ingamine A (**2**) simply by the absence of an oxygen atom. The 1D and 2D ^{1}H and ^{13}C NMR data obtained for ingamine B (**3**) showed a close correspondence to the data obtained for ingamine A (**2**) (Table 1). The only significant differences in the data for the two compounds were the chemical shifts of the resonances assigned to C5, C8, C9 and C10 and their attached protons. Since ingamine A (**2**) contains an alcohol substituent at C9, and the molecular formula of ingamine B (**3**) contains no oxygen atoms, it was apparent that ingamine B (**3**) simply lacked the C9 hydroxyl present in ingamine A (**2**). A detailed analysis showed that the COSY, HMQC, HMBC and ROESY data obtained for ingamine B was completely consistent with the proposed structure **3**.

Ingamine A (**2**) and B (**3**) are new members of the ingenamine (**1**)[15] class of alkaloids. Their skeletons differ from that of ingenamine only by having a twelve carbon alkyl bridge between C3 and N11 instead of the eight carbon alkyl bridge present in ingenamine. The putative biogenetic precursor to the ingamines would be an unsymmetrical *bis*-3-alkyldihydropyridine macrocycle containing one eight carbon and one twelve carbon alkyl chain (See Scheme 1, n=5). Haliclamines A and B, which consist of two tetrahydropyridines linked by C9 and C12 alkyl chains, represent an example of such an unsymmetrical macrocycle.[3] Ingamines A (**2**) and B (**3**) both showed in vitro cytotoxicity against murine leukemia P388 with ED_{50}'s of 1.5 μg/mL.

Table 1. NMR Data (^{1}H: 500 MHz; ^{13}C: 125 MHz; COSY: 400 MHz; HMBC: 500 MHz; ROESY: 500 MHz).

	Ingenamine (1) (MeOH-d_4)		Ingamine A (2) (CDCl 3)			
Carbon no.	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	[a]COSY Correlations	[b]HMBC Correlations
2	63.8	3.30, bs	64.1	3.03	H4	H4, H6, H12', H13, H20, H32
3	143.0		143.8			H2, H5, H32, H32'
4	125.8	6.01, d(6.4)	121.2	5.94, d(6.1)	H2, H5, H32, H32'	H2, H5, H6, H6', H8, H32, H32'
5	34.8	2.75, m	34.5	2.66	H4, H6', H8	H4, H6, H6', H8, H9
6	53.8	2.89, dd(9.3, 1.8)	54.2	2.91	H6',	H2, H8, H13, H13'
6'		1.76, dd(9.3, 2.4)		1.76	H6, H5	
7	45.2		45.3			H2, H5, H8, H12, H12', H20, H20'
8	51.6	0.87, dd(10.5, 1.8)	51.9	0.97, bd(8.9)	H5, H9	H2, H4, H5, H6, H6', H12
9	66.1	3.35, ddd(10.5, 12.1, 4.8)	68.9	3.40, td(9.4, 4.2)	H8, H10'	H8
10	52.5	3.00, dd(12.1, 4.8)	55.9	2.84		H8, H12, H21
10'		2.92, t(12.1)		2.69	H9	
12	50.8	3.10, d(12.3)	50.2	2.45, d(11.0)	H12'	H2, H8, H20', H21
12'		2.22, bd(12.3)		2.07, d(11.0)	H12	
13	55.1	3.02, td(12.7, 4.9)	54.3	2.82	H13', H14, H14'	H2, H15, H6'
13'		2.24, m		2.25	H13, H14, H14'	
14	26.9	1.53	26.0	1.48	H14', H13, H13'	H13
14'		1.31		1.24	H14, H13, H13', H15	
15	27.4	1.62	26.3	1.59	H15', H14'	H13', H16, H16'
15'		1.48		1.47	H15	
16	23.8	2.41	22.9	2.27	H16', H17	H15, H15', H17, H18
16'		1.62		1.59	H16, H17	
17	130.2	5.65, dt(10.3, 5.8)	131.4	5.63	H16, H16'	H16, H16', H19
18	133.3	5.70, dt(10.3, 5.3)	130.3	5.65	H19, H19'	H16, H16', H19
19	21.2	2.40	20.8	2.20	H19', H18, H20, H20'	H17, H18, H20, H20'
19'		1.82, m		1.8	H19, H18, H20, H20'	
20	41.6	1.93	41.5	1.98	H20', H19, H19'	H8, H12', H19
20'		1.71, m		1.74	H20, H19, H19'	
21	56.7	3.49, m	58.3	2.62	H22	H12', H22, H23
21'		2.88		2.62		
22	20.2	1.92	23.0	2.27	H21, H23	H23, H24
22'		1.54		2.27		
23	26.04	1.53	126.2	5.31	H22, H24	H22, H24, H25
24	26.26	2.31	129.3	5.48	H23, H25, H25'	H23, H25
24'		2.07				
25	132.6	5.32, bt(10.5)	26.0	2.90	H25', H24, H26	H23, H24
25'				2.68	H25, H24, H26	
26	133.7	5.45, bt(10.5)	127.9	5.42	H25, H25'	H25, H25', H28, H28'
27	26.3	2.37	127.4	5.40	H28, H28'	H25, H25', H28, H28'
27'		2.15				
28	37.2	2.41	25.9	2.90	H27, H28', H29	H26, H30
28'		2.36		2.78	H27, H28, H29	
29			128.7	5.40	H28, H28'	H28, H28', H31, H31'
30			128.7	5.47	H31, H31'	H28, H28', H31, H31', H32'
31			25.2	2.34	H31', H30	H29, H32, H32'
31'				2.18	H31, H30, H32'	
32			36.2	2.33	H32', H4	H2, H4, H31, H31'
32'				1.98	H32, H4, H31'	

[a] Correlated to proton resonance in δ ^{1}H column. [b] Correlated to carbon resonance in δ ^{13}C column.

Table 1 continued. NMR Data.

	Ingamine A (2)	Ingamine B (3) ($CDCl_3$)				
	ROESY Correlations	δ ^{13}C	δ ^{1}H	[a]COSY Correlations	[b]HMBC Correlations	ROESY Correlations
2	H12', H13, H13', H32, H32'	64.0	2.98, bs	H4	H4, H13, H20, H32, H32'	H13, H31', H32, H32'
3		142.9			H2, H31', H32, H32'	
4	H5, H9, H31, H31'	121.2	5.89, d(6.2)	H2, H5, H32, H32'	H2, H6, H6', H8, H32, H32'	H9', H31, H31'
5		37.7	2.25	H4, H6', H8	H4, H6, H6', H8, H9'	H8, H9'
6	H8	54.0	2.88, dd(9.1, 1.9)	H6'	H2, H4, H8, H13, H13'	H8
6'			1.71, dd(9.1, 2.6)	H6, H5		
7		45.7			H2, H5, H12', H19, H20'	
8	H5, H6, H20, H20'	43.5	0.96, m	H5, H9, H9'	H2, H4, H5, H6, H6', H9', H10'	H5, H6, H20
9	H4, H5, H12'	26.6	1.34	H9', H8, H10'		
9'			1.18, qd(13.3, 4.0)	H9, H8, H10, H10'		H5, H4, H12'
10		49.6	2.95	H10', H9'	H9', H20'	
10'			2.59	H10, H9, H9'		
12	H12'	49.3	2.4 , bd(10.2)	H12'	H20'	
12'	H2, H9, H12		2.03, d(10.2)	H12		
13	H2	54.2	2.83, td(12.7, 5.1)	H13', H14, H14'	H2, H6', H15	H2
13'	H2		2.23	H13, H14, H14'		
14		26.0	1.46	H14', H13, H13'	H13', H15, H15'	
14'			1.23	H14, H13, H13', H15		
15		26.3	1.55	H15', H14'	H13', H16, H16'	
15'			1.47	H15, H16		
16		22.9	2.29	H16', H15', H17	H15, H15', H17, H18	
16'			1.55	H16, H17		
17		131.3	5.62, td(10.3, 5.1)	H16, H16' H18	H15', H16', H19	
18		130.7	5.68, td(10.3, 6.1)	H17, H19, H19'	H16, H16', H19, H20'	
19		21.0	2.23	H19', H18, H20, H20'	H17, H18, H20, H20'	
19'			1.76, m	H19, H18, H20, H20'		
20	H8	40.9	1.90, td(11.9, 7.2)	H20', H19, H19'	H8, H12', H19	H8
20'	H8		1.64	H20, H19, H19'		
21		58.6	2.60, dt(12.4, 3.3)	H21', H22	H12', H22, H23	
21'			2.51, td(12.4, 5.6)	H21, H22		
22		23.4	2.26	H21, H21', H23	H23, H24	
23		126.7	5.32	H22, H24	H24, H25	
24		129.1	5.48	H23, H25, H25'	H23, :H25, H25'	
25		26.0	2.91	H25', H24, H26	H23, H24, H27	
25'			2.69, dt(16.0, 6.0)	H25, H24, H26		
26		128.1	5.43	H25, H25'	H25, H25'	
27		127.4	5.41	H28, H28'	H28, H28	
28		25.9	2.91	H28', H27, H29	H26	
28'			2.78, dt(16.0, 6)	H28, H27, H29		
29		128.9	5.40	H28, H28	H28, H28'	
30		128.6	5.47	H31, H31'	H31, H31', H32'	
31	H4	25.5	2.34	H31, H30'	H29, H32, H32'	H4
31'	H2, H4		2.18	H31, H30, H32'		H2, H4
32	H2	36.1	2.32	H32', H4	H2, H4	H2
32'	H2		1.99	H32, H4, H31'		H2

EXPERIMENTAL

Specimens of *Xestospongia ingens*[14] were collected by hand using SCUBA on reefs at depths of -15 to -20 m near Sek Point off Madang, Papua New Guinea. Freshly collected sponge was frozen on site and transported to Vancouver over dry ice. The frozen sponge (200 g) was thawed, and extracted exhaustively with methanol. The methanol extract was concentrated in vacuo and partitioned successively between an aqueous solution and first hexanes, then ethyl acetate. Repeated fractionation of the hexanes soluble material (280 mg) on a silica gel flash column (gradient elution: EtOAc:Hex:*i*-Pr_2NH 50:50:1 to EtOAc:Hex 50:50) gave pure ingamine B (**3**) (32 mg). The ethyl acetate soluble material was chromatographed on Sephadex LH-20 (eluent: EtOAc:MeOH:H_2O 40:10:4) and a silica gel flash column (eluent: EtOAc) to give crude ingamine A (**2**) (65 mg). Final purification was achieved by preparative silica gel TLC (eluent: EtOAc:*i*-Pr_2NH 92:8) to give ingamine A (**2**) (40mg).

Ingamine A (2): colorless glass; $[\alpha]_D$ +131° (c 0.8; MeOH); IR (film) 3307, 3010, 2943, 2924, 1660, 755, 723 cm^{-1}; LREIMS (m/z, relative intensity) 448 (M^+, 100), 431 (22), 407 (18), 319 (27), 242 (39), 188 (34), 108 (92), 93 (99); HREIMS M^+ m/z 448.3454 ($C_{30}H_{44}N_2O$ ΔM -0.1 mmu), 242.1905 ($C_{17}H_{24}N$, ΔM -0.3 mmu), 188.1444 ($C_{13}H_{18}N$, ΔM + 0.5 mmu) ; ^1H and ^{13}C NMR (see Table 1).

Ingamine B (3):colorless glass; $[\alpha]_D$ +108° (c 0.5; MeOH); IR (film) 3010, 2924, 2852, 1655, 755, 723 cm^{-1}; LREIMS (m/z, relative intensity) 432 (M^+, 100), 244 (26), 206 (77), 188 (17), 149 (36), 110 (81); HREIMS m/z 432.3504 ($C_{30}H_{44}N_2$ ΔM 0.0 mmu), 244.2059 ($C_{17}H_{26}N$ ΔM -0.6 mmu), 188.1443 ($C_{13}H_{18}N$ ΔM +0.3 mmu); ^1H and ^{13}C NMR (see Table 1).

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