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Netamines A–G: seven new tricyclic guanidine alkaloids from the marine sponge *Biemna laboutei*

Hagit Sorek,[a] Amira Rudi,[a] Sophie Gueta,[a] Fernando Reyes,[b] Maria Jesus Martin,[b] Maurice Aknin,[c] Emile Gaydou,[d] Jean Vacelet[e] and Yoel Kashman[a,*]

[a]*School of Chemistry, Tel-Aviv University, Ramat Aviv, 69978, Israel*
[b]*PharmaMar S. A, P. I, La Mina-Norte 28770, Colmenar Viejo, Madrid, Spain*
[c]*Laboratoire de Chimîe des Substances Naturelles et des Aliments, Faculté des Sciences et Techniques, Université de la Réunion, 15 Avenue Rene cassin, B.P. 7151, 97715 Saint Denis, Cedex 9, France*
[d]*Université de Droit, d'Economie et des Sciences d'Aix Marseille III, France*
[e]*Centre d'Oceanologie de Marseille, Staition Marine d'Endoume, Université de la Méditerranée, UMR 6540, 13007 Marseille, France*

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Abstract—In our continuing program to identify bioactive compounds from marine invertebrates, the MeOH/EtOAc (1:1) extract of three collections of the Madagascar sponge, *Biemna laboutei*, was found to be cytotoxic to a series of human tumor cells. From the two sponges, seven new guanidine alkaloids, designated netamines A–G (**1**–**7**), have been isolated and their structures elucidated. Compounds **3** and **4** were found to be cytotoxic against three tumor cells with GI_{50} values in the micromolar range.

1. Introduction

The first report of a complex sponge-derived polycyclic guanidine alkaloid was on the pentacyclic ptilomycalin A, which we isolated from both a Caribbean sample of *Ptilocaulis spiculifer* and a Red Sea collection of *Hemimycale* sp.[1,2] Shortly thereafter, the structure of hydroxylated ptilomycalins, designated crambescidins 800, 816, 830, and 844, isolated from the Mediterranean sponge *Crambe crambe* and other sponges have been reported.[3–8] Pentacyclic guanidine alkaloids have also been reported from Brazilian specimens of *Monanchora unguiculata*[4] and Caribbean collection of *Batzella* spp.[5] Tricyclic guanidine alkaloids bearing the (5,6,8b)-triazaperhydroacenaphthylene skeleton (ptilocaulin derivatives and mirabilins) were reported from *Batzella* spp.,[5,9] *P. spiculifer*,[10] and curiously, from two New Caledonian starfish[11] (probably due to sequestration of these alkaloids from prey-sponges). Due to similar morphological characters and secondary metabolites, it is suggested that the above mentioned sponges should eventually be united in one sponge genus which, for priority reasons, has to be *Crambe*.[4]

Many of the cyclic guanidine derivatives show noteworthy biological activities, e.g., HIV gp120-human CD4-binding inhibition, $p56^{lck}$-CD4 dissociation induction, Ca^{2+} channel blocker activities, cytotoxicity, and antifungal and antimicrobial activities.[2,5,9,11]

As part of a continuing program to discover bioactive compounds from marine invertebrates,[12–14] we found that the extracts of the Poecilosclerida sponge *Biemna laboutei* (Hooper, 1996) to be cytotoxic. The sponge was collected twice near the Sainte-Marie Island on the east coast of Madagascar, in May 2004 and once at Itampule, Madagascar, in January 2005.

2. Results and discussion

The $CHCl_3$/MeOH (1:1) extracts of the frozen *B. laboutei* samples were subjected to solvent-partitioned, i.e., aq MeOH against hexane and CH_2Cl_2 and the CH_2Cl_2 fraction was chromatographed on Sephadex LH-20, eluted with hexane/MeOH/$CHCl_3$ (2:1:1), to afford a complex cytotoxic mixture of nitrogen-atom containing compounds. From the later mixture we isolated upon repeated Sephadex LH-20 and silica gel chromatography, and in several cases also RP-18 HPLC, seven compounds designated netamines A–G (compounds **1**–**7**) (in 9, 0.5, 2.5, 3, 12, 0.6, 2×10^{-3} wt %) thus far (Fig. 1). Additional hydroxylated netamines, which exist in minute quantities, are under investigation.

The molecular formula of netamine A (**1**) was established by HRFABMS to be $C_{19}H_{35}N_3$ *m/z* 306.2903 $[M+H]^+$ (calcd

Keywords: Marine sponges; Guanidine alkaloids; Heterocyclic.
* Corresponding author. Tel.: +972 3 6408419; fax: +972 3 6409293; e-mail: kashman@post.tau.ac.il

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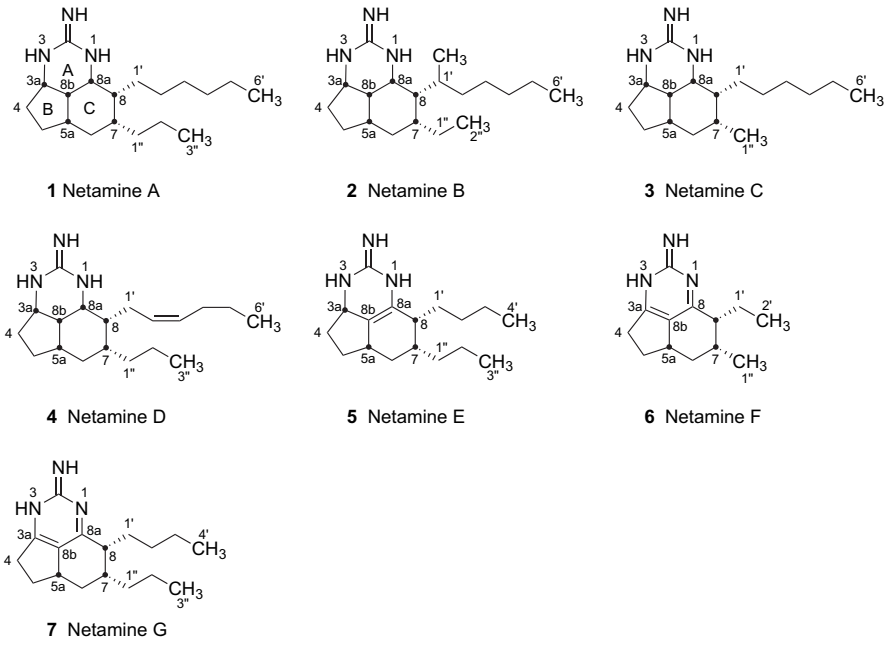


Figure 1. Netamines A–G (**1**–**7**).

306.2901) indicating four degrees of unsaturation. Analysis of the 1D and 2D ^{1}H and ^{13}C NMR data for **1** (Table 1) exhibited a single sp^2 carbon atom (δ 156.0), which together with three NH groups (δ_H 7.80, 7.85, and 6.90), suggested a guanidine moiety. In the absence of a carbon–carbon double bond, besides the guanidine imine, netamine A (**1**) had to be tricyclic to account for the four degrees of unsaturation. Two methines next to nitrogens (C-3a and C-8a, δ_C 53.6, δ_H 3.80 dd and δ_C 49.4, δ_H 3.57 dd, respectively) were a good starting point for the structure elucidation of the ring system. The latter two methines were connected to each other via another methine (C-8b) resonating at δ_C 35.2 and δ_H 2.30. The COSY experiment (Table 1) further connected the latter methine to another CH-group (C-5a, δ_C 35.3, δ_H 2.05 m). The high degree of overlapping of methylene protons made interpretation of the COSY map difficult. Assistance from selective 1D TOCSY experiments, applying different mixing times (10–100 ms), provided several proton sequences (3a, 4, 5, 5a and 5a, 6, 7). CH correlations deduced from an HMBC experiment (Table 1; Fig. 2) gave the crucial information for constructing a (5,6,8b)-triazaperhydroacenaphthylene ring system. The ^{15}NH HMBC agreed well with the suggested heterocyclic ring system namely, $^{3}J_{NH}$ correlations observed between H-8b, H-4 and a nitrogen atom resonating at 84.1 ppm ($^{1}J_{NH}$=93 Hz) established this nitrogen to be N-3. $^{3}J_{NH}$ correlations from H-8b to a second nitrogen atom resonating at 87.5 ppm determined it to be N-1 ($^{1}J_{NH}$=93 Hz). The proton on N-2 (δ_N 65.3 ppm) gave $^{3}J_{NH}$ correlations to both N-1 and N-3 ($^{1}J_{NH}$=93 Hz) (Fig. 3). Essentially, the latter unsaturated ring system is known in ptilocaulin,[15] isoptilocaulin,[3] and several mirabilins,[16] however, the saturated system is new. Indeed, a saturated ring system with unknown stereochemistry of the five chiral centers has been reported

Table 1. 1D and 2D NMR data for netamine A (**1**) in $CDCl_3$

Position	δ_C, ppm[a,b]	δ_H, ppm (mult, *J* Hz)	COSY (^{1}H–^{1}H)[c]	HMBC (H–C)
1	—	7.80 (br s)		—
2	156.0s	6.90 (br s)		3a, 8a, NH-1, NH-2, NH-3
3	—	7.85 (br s)		—
3a	53.6d	3.80 (dd, 6.2, 3.9)	4, 8b	4β, 5a, 5β, 8a, 8b, NH-2
4	33.5t	1.63 (m) 1.93 (m)	3a, 5	3a, 8b
5	30.5t	1.36 (m) 1.95 (m)	4, 5a	3a, 5a
5a	35.3d	2.05 (m)	5, 6, 8b	3a, 6β, 8a, 8b,
6	32.5t	0.98 (q, 12.0) 1.73 (dt, 12.7, 4.8)	5a, 7	8b
7	39.3d	1.13 (m)	6, 8, 1″	6β, 6α, 7, 8, 8a
8	43.4d	1.51 (m)	7, 8a, 1′	1″, 6β, 6α, 7, 8a, NH-1
8a	49.4d	3.57 (dd, 5.0, 1.5)	8, 8b	1′, 5a, 5β, 8, 8b, NH-1
8b	35.2d	2.30 (dt, 11.1, 5.9)	3a, 8a	3a, 4β, 5a, 8a, NH-1
1′	31.7t	1.27 (m)	8, 2″	7
2′	27.7t	1.28 (m)	1′	8
3′	29.6t	1.32 (m)		8
4′	35.1t	1.27 (m)		6′
5′	22.6t	1.28 (m)	6′	6′
6′	14.2q	0.89 (t, 7.0)	5′	
1″	40.2t	1.40 (m) 1.48 (m)	7, 2″	3″, 6α
2″	20.4t	1.28 (m) 1.39 (m)		3″
3″	14.0q	0.90 (t, 7.0)	2″	

[a] $CDCl_3$, using a Bruker ARX-500 instrument.
[b] Multiplicities were determined by DEPT and HSQC experiments.
[c] The CH correlations were assigned by an HSQC experiment.

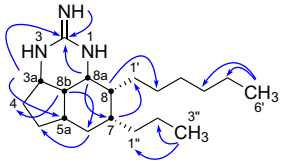


Figure 2. Key HMBC correlations in netamin A (**1**).

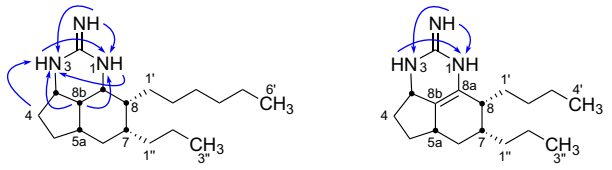


Figure 3. ^{15}N HMBC correlations in netamine A (**1**) and netamine E (**5**).

synthetically.[17] Additional HMBC correlations for **1** clearly indicated that the heterocyclic ring system is substituted at positions C-7 and C-8 by two alkyl groups ($\delta_{CH(7)}$ 39.3 d and $\delta_{CH(8)}$ 43.4 d)—including a combined nine carbon atoms. A fragmentation of 86 m.u. in the MS measurement (*m/z* 220 [M+H$^+$−C_6H_{13}]), demonstrated that one chain is a hexyl group and, therefore, the second chain has to be a propyl substituent. The latter propyl group was confirmed by CH correlations from CH_3 (3″) to CH_2 (1″) and CH_2 (2″). The propyl group was determined to be attached to C-7 on the basis of HMBC correlations of H-6α to C-1″, and H-7 to C-1″, C-2″, and C-1′, and therefore, the hexyl group substitutes the C-8 atom. The stereochemistry of netamine A (**1**) was unequivocally determined by NOE correlations shown in Figure 4 (because of overlapping protons, other NOE's

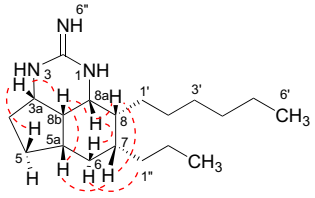


Figure 4. Key NOEs for netamine A–D (**1**–**4**).

are less clear). Namely, an NOE between the center H-8b (δ 2.30) and protons H-3a, H-5a, and H-8a (δ 3.80, 2.05, and 3.57) determined all four to be directed to the same β-side of the tricyclic ring system, thereby forming a twisted 'cap' shape system. Next, the stereochemistry of C-7 was determined from the multiplicity of H-6α, i.e., the latter proton, appearing as a quartet with a coupling constant of ca. 12 Hz, has to be axial and thus H-5a and H-7 also have to be axial. Hence, the propyl chain at C-7 has to be α-equatorial. The latter three axial protons require the cyclohexane ring (C) to be in a slightly twisted chair conformation. Additional NOE's between H-5a and H-7 and between H-6β (equatorial) and H-7 further supported the suggested configuration of C-7. An NOE between H-6α and H-1″ unequivocally confirmed the stereochemistry of C-7. Next, the stereochemistry at C-8 was determined from NOEs from both H-8a(β) and H-7(β) to H-8, namely H-8 also has to be in the β-configuration, and therefore, the two chains are cis to each other.

The second isolated compound, netamine B (**2**) was only obtained in minute amounts (2 mg, 0.5×10^{-3}% dry weight). Its mass spectrum suggested the same formula, $C_{19}H_{35}N_3$, as that of **1**, implying, together with the NMR spectroscopic data, an isomeric structure. The major difference in the ^{1}H NMR spectrum of **2** was an additional methyl group, δ_H 1.01 d (*J*=6.5 Hz). The CH correlations of this methyl to C-1′ and C-8 determined its location on C-1′. As in **1**, the two side chains include nine carbon atoms that differ, however, from the chains of **1**. Namely, C-7 carries an ethyl group (C-1″, -2″) (Table 2) (*m/z* 279.2 [M+H$^+$−C_2H_5]), confirmed by CH correlations from CH_3-2″ to CH_2-1″ and CH-7, and C-8 the rest of the chain-carbons. The above mentioned CH_3 (d), together with the correlations of a second triplet methyl group, CH_3-6′ (δ_H 0.83, δ_C 13.9) to C-5′ and C-4′, established the *iso*-heptyl structure of the C-8 side chain. Similar NOEs to the one measured for **1** suggested the same stereochemistry for **2** (Fig. 4).

From a second Saint-Marie collection of *B. laboutei* were isolated netamines C (**3**) and D (**4**). Their structure determination was done similar to that of compounds **1** and **2**. Netamine C, with two carbon atoms less than **1**, was found to be its C-7-CH_3 lower homolog, and netamine D (**4**), with two protons less, the 2′(3′)-dehydro analog of **1**. In the structure

Table 2. ^{13}C NMR spectral data of netamines A–H (**1**–**8**)[a,b]

C	1	2	3	4	5	6	7	8
2	156.0s	156.0s	154.9s	154.8s	155.0q	163.0s	162.8s	156.0s
3a	53.6d	53.7d	53.7d	53.6d	54.3d	175.0s	174.8s	53.7d
4	33.5t	34.3t	33.4t	33.3t	34.4t	33.6t	34.0t	33.5t
5	30.5t	30.2t	30.4t	30.4t	30.8t	33.1t	32.9t	30.6t
5a	35.3d	34.4d	35.8d	35.4d	38.0d	37.8d	37.3d	35.1d
6	32.5t	33.2t	35.1t	35.1t	36.9t	39.6t	35.9t	32.5t
7	39.3d	35.6d	34.5d	38.9d	38.7d	33.2d	38.5d	39.3d
8	43.4d	45.0d	45.0d	43.6d	42.5d	47.7d	44.0d	43.6d
8a	49.4d	49.8d	49.8d	48.7d	129.2s	166.0s	166.1s	49.4d
8b	35.2d	35.2d	34.8d	34.9d	119.8s	127.0s	126.4s	35.3d
1′	31.7t	31.5d	34.7t	32.2t	29.1t	22.8t	30.5t	34.8t
2′	27.7t	27.4t	27.5t	127.3d	27.8t	9.5q	27.5t	29.9t
3′	29.6t	29.3t	29.4t	132.0d	24.7t	—	23.1t	22.9t
4′	35.1t	34.5t	31.7t	29.5t	14.9q	—	14.1q	14.2q
5′	22.6t	23.1t	22.6t	22.7t	—	—	—	—
6′	14.2q	22.4t	14.0q	13.8q	—	—	—	—
7′	—	13.8q	—	—	—	—	—	—
1″	40.2t	34.8t	23.1q	40.0t	37.7t	20.8q	36.8t	40.2t
2″	20.4t	14.0q	—	20.3q	21.7t	—	20.1t	20.2t
3″	14.0q	—	—	14.1q	15.2q	—	14.3q	14.0q

[a] $CDCl_3$, using a Bruker ARX-500 instrument (except **5** which was in CD_3OD).
[b] Multiplicities were determined by DEPT and HSQC experiments.

elucidation of **4**, due to the double bond label, the identification of the side chains and their locations was more facile. Namely, it was found that a hex-2-enyl group is attached to C-8 and a propyl group to C-7. The *Z*-configuration of the double bond was determined on the basis of the 11.0 Hz coupling constant between the two olefinic protons as well as an NOE between them.

Netamine E (**5**) was isolated from the third, Itampule sponge specimen, and assigned the molecular composition of $C_{17}H_{29}N_3$ (CIMS *m/z* 276.2 $[M+H]^+$). According to the spectral data of compound **5** (Tables 2 and 4), it was determined to possess a 8a(8b)-isomeric ptilocaulin type structure. CH correlations, derived from an HMBC experiment [correlations from H-3a, H-7, and H-8 to C-8a (129.2), and from H-3a, H-8, and H-5a to C-8b (119.8)], confirmed the unsaturated tricyclic ring system. The δ_N values of the three NH groups (δ_N 70.4, 85.3, and 96.0), which agreed well with the nitrogen chemical shifts of **1**, fully supported the suggested structure. The 8a(8b)-double bond assisted with the differentiation of the side chains, as H-8 is in an allylic position resonating at δ_H 1.98 ppm. Again, as with most other netamines, the 'lower' chain was a higher homolog of the C-7 methyl found in ptilocaulin and the mirabilins. The C-7 chain of **5** was determined to be a propyl group, as in **1** and **4**, and the C-8 chain to be a shorter butyl substituent (*m/z* 219.2 $[M+H^+-C_4H_9]$). The stereochemistry elucidation of **5** started from the multiplicity of H-6α, namely, as explained above for **1**–**4**, a quartet with a 12 Hz coupling constant pointed to two vicinal (H-5a and 7) axial proton atoms. The key NOEs, depicted in Figure 5, established the all cis-geometry of the heterocyclic methine protons (H-3a, 5a, 7, and 8).

Hydrogenation of compound **5** over Pd mainly afforded compound **8** (Scheme 1) which, according to the carbon chemical shifts, which is an excellent probe for the ring's stereochemistry (Table 2), has the same stereochemistry as netamines A–D.

Two additional compounds, netamines F and G, were isolated from the latter sponge and differed from netamines A–D by possessing an unsaturated ring system, as in mirabilins A–C,[16] however, with a different stereochemistry. The imine, rather than the tautomeric aminopyrimidine, structure of the latter was recently determined by Hamann via X-ray diffraction analysis of a mixture of two ptilocaulins.[18] Netamine F (**6**) is the *cis*-7-methyl-8-ethyl analog and **7** the 1(8a),3a(8b)-dehydro analog of **5** (Fig. 1). Comparison of the NMR data (Tables 2 and 4) of netamines F and G (**6** and **7**) with compounds **1**–**5** (Tables 2 and 3) and mirabilins A–C[16] clearly pointed to the 7,8-disubstituent-1(8a),3a(8b)-unsaturated tricyclic ring system. Netamine F (**6**), the *cis*-7-methyl-8-ethyl substituted compound (Fig. 6), is the smallest homolog among the netamines while netamine G (**7**) possesses the same skeleton as **5**. Indeed,

Table 3. ^{1}H NMR spectral data of netamines A–D (**1**–**4**)[a]

H	1	2	3	4
1-NH	7.80 (br s)	7.75 (br s)	6.93 (br s)	6.94 (br s)
2-NH	6.90 (br s)	6.90 (br s)	7.53 (br s)	7.55 (br s)
3-NH	7.85 (br s)	7.80 (br s)	7.67 (br s)	7.64 (br s)
3a	3.80 (dd, 6.2, 3.9)	3.80 (m)	3.86 (m)	3.83 (m)
4	1.63 (m)	1.62 (m)	1.63 (m)	1.64 (m)
	1.93 (dd, 13.0, 5.0)	1.85 (m)	1.84 (dd)	1.84 (dd)
5	1.36 (m)	1.28 (m)	1.30 (m)	1.34 (m)
	1.95 (m)	1.95 (m)	1.94 (m)	1.98 (m)
5a	2.05 (m)	2.05 (m)	2.08 (m)	2.07 (m)
6	0.98 (q, 12.0)	0.92 (m)	1.06 (m)	0.94 (m)
	1.73 (dt, 12.7, 4.8)	1.73 (m)	1.63 (m)	1.73 (ddd)
7	1.13 (m)	2.03 (m)	1.21 (m)	1.16 (m)
8	1.51 (m)	1.30 (m)	1.36 (m)	1.55 (m)
8a	3.57 (dd, 5.0, 1.5)	3.48 (br d)	3.51 (br d)	3.55 (br d)
8b	2.30 (dt, 11.1, 5.9)	2.30 (dt)	2.32 (m)	2.32 (m)
1′	1.27 (m)	1.25 (m)	1.28 (m)	2.00 (m)
				2.04 (m)
2′	1.28 (m)	1.30 (m)	1.31 (m)	5.34 (m)
3′	1.32 (m)	1.28 (m)	1.28 (m)	5.47 (dt)
4′	1.27 (m)	1.28 (m)	1.28 (m)	2.00 (m)
5′	1.28 (m)	1.25 (m)	1.27 (m)	1.38 (m)
		1.35 (m)		
6′	0.89 (t, 7.0)	0.82 (t)	0.89 (t)	0.91 (t)
7′	—	1.01 (d)	—	—
1″	1.40 (m)	1.25 (m)	1.02 (d)	1.28 (m)
	1.48 (m)	1.35 (m)		1.41 (m)
2″	1.28 (m)	0.85 (t)	—	1.28 (m)
	1.39 (m)			1.37 (m)
3″	0.90 (t, 7.0)	—	—	0.88 (t)

[a] $CDCl_3$, using a Bruker ARX-500 instrument.

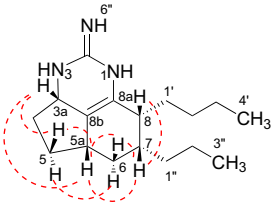


Figure 5. Key NOEs for netamine E (**5**).

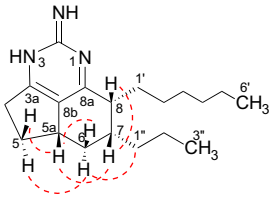
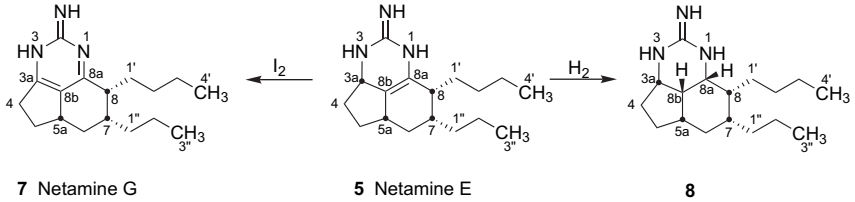


Figure 6. Key NOEs for netamine F–G (**6**–**7**).



Scheme 1. Reduction and oxidation of netamine E (**5**).

Table 4. ^{1}H NMR spectral data of netamines E–H (**5–7**)

H	**5**[a]	**6**[b]	**7**[b]
1-NH	9.82 (br s)	—	—
2-NH	8.65 (br s)	—	—
3-NH	7.43 (br s)	—	—
3a	4.29 (br t, 8.0)	—	—
4	1.70 (m)	2.58 (dd, 17.0, 8.0)	2.65 (dd)
	2.22 (m)	2.90 (m)	2.95 (m)
5	1.30 (m)	1.52 (m)	1.55 (m)
	2.05 (m)	2.35 (dt, 12.0, 7.0)	2.40 (m)
5a	2.45 (m)	2.90 (m)	2.90 (m)
6	0.80 (m)	0.92 (q, 12.0)	0.88 (m)
	2.15 (m)	2.02 (ddd, 12.3, 4.8, 3.2)	2.22 (dt)
7	1.60 (m)	1.86 (m)	1.75 (m)
8	1.98 (m)	2.30 (m)	2.35 (m)
1′	1.70 (m)	1.79 (m)	1.76 (m)
		2.13 (m)	2.04 (m)
2′	1.25 (m)	0.81 (t, 7.3)	1.12 (m)
			1.31 (m)
3′	1.38 (m)	—	1.31 (m)
4′	0.95 (m)	—	0.89 (t)
1″	1.25 (m)	1.08 (d, 6.6)	1.27 (m)
	1.55 (m)		1.58 (m)
2″	1.35 (m)	—	1.31 (m)
	1.50 (m)		1.50 (m)
3″	0.95 (m)	—	0.96 (t)

[a] CD_3OD, using a Bruker ARX-500 instrument (NH values were taken in DMSO-d_6).
[b] $CDCl_3$, using a Bruker Avance-400 instrument.

dehydrogenation of **5** with iodine afforded compound **7** (Scheme 1).

Netamines A–G are presumably derived via intramolecular cyclization of guanidine-substituted polyketides (C_{12} for **6** through C_{16} for **3**, **5**, and **7** to C_{18} for **1**, **2**, and **4**) as earlier suggested by Capon.[16] Outstanding is netamine B (**2**) with the *iso*-heptyl side chain. The netamines add new structures to the group of the tricyclic guanidine alkaloids. Four of the netamines (A–D) possess the unprecedented saturated all cis ring system with six chiral centers. ^{15}N chemical shifts, measured from ^{15}NH HMBC, have been shown to be an additional important tool for the structure elucidation of these guanidines.

3. Biological activity

The in vitro activity of netamines C and D was evaluated against three human tumor cell lines: NSCL (A549), colon (HT29), and breast (MDA-MB-231) (Table 5).[19] All other compounds were less active.

Considering the bioactivities of the guanidine alkaloids all new compounds have to be further examined in a variety of tests.

Table 5. Cytotoxicity data (GI_{50}, μM) for netamines C (**3**) and D (**4**)

Compound	Cell lines/GI_{50} (μM)		
	A549	HT29	MDA-MB-231
3	4.3	2.4	2.6
4	6.6	5.3	6.3

4. Experimental

4.1. General

Optical rotations were obtained with a Jasco P-1010 polarimeter. IR spectra were obtained with a Bruker FTIR Vector 22 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX-500 and Avance-400 spectrometers. ^{1}H, ^{13}C, COSY, HSQC, and HMBC were recorded using standard Bruker pulse sequences. EIMS, CIMS, and HRMS measurements were recorded on a Fisons, Autospec Q instrument.

4.2. Biological material

B. laboutei (Hooper, 1996) was collected twice from the east coast of Madagascar at the Saint-Marie Island, in May 2004 at a depth of 30 m and once from Itampule, 150 km west-south of Tuléar, Madagascar, in January 2005 at a depth of 20 m. The sponge causes dermaties, order Poecilosclerida, family Desmacellidae.

4.3. Extraction and isolation

The frozen sponge *B. labouti* (344 g) was homogenized and extracted with $CHCl_3$/MeOH (2:1). The organic extract was concentrated to yield a crude extract (6.2 g). The crude extract was subjected to partitioning by the Kupchan method.[20] The dichloromethane fraction (640 mg) was repeatedly chromatographed on a Sephadex LH-20 column, eluting with hexane/MeOH/$CHCl_3$ (2:1:1) to obtain compounds **1** (30 mg, 0.009 wt %), **2** (2 mg, 0.0006 wt %), **5** (40 mg, 0.0012 wt %), and **7** (8 mg, 0.002 wt %). A fraction of the Sephadex LH-20 column (containing compound **6**) was subjected to VLC over silica gel, using hexane with increasing proportions of ethyl acetate as eluent. Compound **6** (2 mg, 0.0006 wt %) was afforded by elution with 50% ethyl acetate in hexane.

A frozen second wet sample of *B. labouti* (529 g) was exhaustively extracted with water (3×1 L) and then with CH_2Cl_2/MeOH (1:1) (3×1 L). The organic extract was concentrated to yield a gummy material (5.2 g). This material was subjected to VLC on Lichroprep RP-18 with a stepped gradient from H_2O/MeOH (3:1) to MeOH. Next, one fraction (535 mg), eluted with H_2O/MeOH (1:3) (875 mg), was subjected to preparative HPLC (Symmetry Prep C-18, 19×150 mm, gradient H_2O+0.1% TFA/CH_3CN+0.1% TFA, from 35 to 45% CH_3CN in 20 min, UV detection, 15 ml min^{-1}) to yield **3** (14 mg, 0.0025 wt %) and impure **4** (17 mg, 0.003 wt %) as their TFA salts. The final purification of **4** was achieved by semi preparative HPLC (Symmetry Prep C-18, 7.8×150 mm, isocratic H_2O+0.1% TFA/CH_3CN+0.1% TFA 54:46, UV detection, 2.5 ml min^{-1}), obtaining 8.8 mg of pure compound **4**.

4.3.1. Netamine A (1). Pale yellow oil; $[\alpha]_D^{20}$ +2.2 (*c* 0.65, CH_2Cl_2); IR (CH_2Cl_2) ν_{max} 3244, 3030, 2930, 1667 cm^{-1}; ^{1}H and ^{13}C NMR see Tables 2 and 3; HRFABMS *m/z* $[M+H]^+$ 306.2903 (calcd for $C_{19}H_{35}N_3$, 306.2901), 220.1 (10) ($[M+H]^+-C_6H_{13}$).

4.3.2. Netamine B (2). Pale yellow oil; $[\alpha]_D^{20}$ +5.3 (*c* 0.05, CH_2Cl_2); IR (CH_2Cl_2) ν_{max} 3250, 3930, 2850, 1667 cm^{-1};

^{13}C and ^{1}H NMR see Tables 2 and 3; HRFABMS *m/z* $[M+H]^+$ 306.2893 (calcd for $C_{19}H_{35}N_3$, 306.2901), 279.2 (100) ($[M+H]^+-C_2H_5$).

4.3.3. Netamine C (3). Pale yellow oil; $[\alpha]_D^{25}$ +3.7 (*c* 0.09, MeOH); IR (KBr) ν_{max} 3260, 2931, 2860, 1669 cm^{-1}; ^{13}C and ^{1}H NMR see Tables 2 and 3; (+)-HRESIMS *m/z* $[M+H]^+$ 278.2602 (calcd for $C_{17}H_{32}N_3$, 278.2590).

4.3.4. Netamine D (4). Pale yellow oil; $[\alpha]_D^{25}$ −5.8 (*c* 0.03, MeOH); IR (KBr) ν_{max} 3428, 2932, 2868, 1662 cm^{-1}; ^{13}C and ^{1}H NMR see Tables 2 and 3; (+)-HRESIMS *m/z* $[M+H]^+$ 304.2760 (calcd for $C_{19}H_{34}N_3$, 304.2747).

4.3.5. Netamine E (5). Pale yellow oil; $[\alpha]_D^{21}$ +35.0 (*c* 0.80, CH_2Cl_2); IR (CH_2Cl_2) ν_{max} 3272, 2959, 2872, 1670 cm^{-1}; ^{13}C and ^{1}H NMR see Tables 2 and 4; CIMS *m/z* 276.3 $[M+H]^+$, 233.2 (10) ($[M+H]^+-C_3H_7$), 219.2 (100) ($[M+H]^+-C_4H_9$), 176.1 (70) ($[M+H]^+-C_7H_{16}$); HRCIMS *m/z* 276.2442 (calcd for $C_{17}H_{29}N_3$, 276.2448).

4.3.6. Netamine F (6). Pale yellow oil; $[\alpha]_D^{20}$ +108.0 (*c* 0.05, CH_2Cl_2); IR (CH_2Cl_2) ν_{max} 3852, 3749, 3648, 1698, 1541 cm^{-1}; ^{13}C and ^{1}H NMR see Tables 2 and 4; EIMS *m/z* 217.2 $[M]^+$, 188.2 (100) ($M^+-C_2H_5$), 173.2 (95) ($M^+-C_3H_8$); HREIMS *m/z* 217.1591 (calcd for $C_{13}H_{19}N_3$, 217.1587).

4.3.7. Netamine G (7). Pale yellow oil; $[\alpha]_D^{21}$ +27.0 (*c* 0.20, CH_2Cl_2); IR (CH_2Cl_2) ν_{max} 2959, 2872, 1601 cm^{-1}; ^{13}C and ^{1}H NMR see Tables 2 and 4; EIMS *m/z* 273.2 $[M^+]$, 230.2 (20) ($[M]^+-C_3H_7$), 216.2 (80) ($[M]^+-C_4H_9$), 173.1 (100) ($M^+-C_7H_{16}$); HREIMS *m/z* 273.2219 (calcd for $C_{17}H_{27}N_3$, 273.2213).

4.3.8. Hydrogenation of netamine E (5). Netamine E (8.5 mg) in methanol (10 ml) was hydrogenated over 10% Pd/C (10 mg), for 4 h at 3 atm pressure. The solution was filtered through Celite, then evaporated and chromatographed on Sephadex LH-20 to afford compound **8** (4 mg).

4.3.9. Oxidation of netamine E (5). Netamine E (2 mg) in CH_2Cl_2 (5 ml) was stirred at room temperature with I_2 (10 mg) for half an hour, then washed with a saturated solution of sodium sulphite, dried and evaporated to afford netamine G (**7**) (2 mg).

4.4. Biological activity

A colorimetric type assay, using the sulforhodamine B reaction, was adopted for quantitative measurement of the cell growth and viability, according to the technique described in the literature.[19] The in vitro activity of the compounds was evaluated against three human tumor cell lines: NSCL (A549), colon (HT29), and breast (MDA-MB-231).

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