Naamidines H and I, Cytotoxic Imidazole Alkaloids from the Indonesian Marine Sponge *Leucetta chagosensis*

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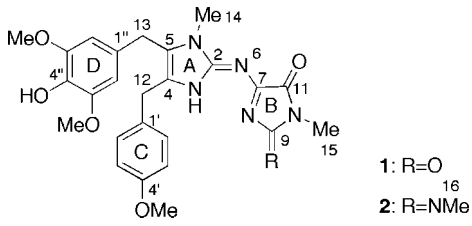
Two new imidazole alkaloids, naamidines H (**1**) and I (**2**), were isolated from the marine sponge *Leucetta chagosensis*, collected in North Sulawesi, Indonesia. The compounds (**1** and **2**) showed cytotoxicity against HeLa cells with IC_{50} values of 5.6 and 15 μg/mL, respectively.

A family of imidazole alkaloids has been reported as biologically active constituents of marine sponges of the genera *Leucetta,*[1–3] *Clathrina,*[4] and *Leucosolenia.*[5] The alkaloids contain a central imidazole ring to which one or two modified benzyl groups are attached at the C-4, C-5, or N-3 positions, and most alkaloids in this group possess a hydantoin or hydantoin derivative at the N-6 position. Among these alkaloids, clathridine was isolated as a very stable zinc complex.[4] So far, the alkaloids in this class have been reported to show cytotoxic,[2] antimicrobial,[2] and leukotriene B_4 receptor antagonist[3] activities. Furthermore, Ireland and co-workers recently revealed that naamidine A potently inhibited epidermal growth factor (EGF)-stimulated DNA synthesis[6] and that molecular targets of naamidine A were extracellular signal-regulated kinase (ERK) 1 and ERK2.[7] Successively, naamidine A[8] and a series of derivatives[9] of naamidine A were synthesized and tested for their ability to inhibit mitogenesis in BaF/ERX cells. Interestingly, these alkaloids were also isolated from *Notodoris*nudibranchs,[10] which are found to feed on *Leucetta* sponges and sequester these alkaloids in their bodies. As part of a continuing study to discover new bioactive metabolites from marine organisms, we examined a bright yellow sponge, *Leucetta chagosensis* (Calcarea sponge), which was collected in North Sulawesi, Indonesia, in September 2006. The EtOH extract showed significant cytotoxicity against HeLa cells. Herein we describe the isolation, structure elucidation, and biological activity of two new alkaloids, naamidines H (**1**) and I (**2**).



The sponge (100 g, wet weight) was extracted with EtOH immediately after collection. The EtOAc-soluble part of the extract was fractionated by SiO_2 column chromatography with *n*-hexane/EtOAc/MeOH. The 50% hexane–EtOAc eluent was purified by ODS column chromatography with 75% MeOH–H_2O to afford naamidine H (**1**, 47.5 mg, 0.047% wet weight). The 90% EtOAc–MeOH eluent was subjected to Sephadex LH-20 with MeOH to afford naamidine I (**2**, 16.2 mg, 0.016%).

FABMS of naamidine H (**1**) showed a quasi molecular ion peak at *m/z* 494 $[M + H]^+$, and the molecular formula was determined to be $C_{25}H_{27}N_5O_6$ on the basis of its HRFABMS; thus **1** must have 15 degrees of unsaturation. The ^{1}H NMR spectrum of **1** (Table 1) showed a singlet aromatic signal at δ 6.12 (2H, s), two doublet aromatic signals at δ 6.79 (2H, d, $J = 9.0$ Hz) and 7.16 (2H, d, $J = 9.0$ Hz), and six singlet signals in the range δ 3–4 [δ 3.15 (3H, s), 3.48 (3H, s), 3.69 (6H, s), 3.74 (3H, s), 3.89 (2H, s), and 3.92 (2H, s)]. Analysis on the basis of NMR data including HMQC and HMBC spectra readily showed that 4-methoxybenzyl (ring C) and 4-hydroxy-3,5-dimethoxybenzyl (ring D) groups were attached to C-4 and C-5 carbons, respectively (Figure 1). The positions of methoxy groups were indicated by HMBC cross-peaks between 4′-OMe (δ 3.74) and C-4′ (δ 158.5) and between 3″-OMe/5″-OMe (δ 3.69) and C-3″/C-5″ (δ 147.4). The positions of the two functionalized benzyl groups were secured by HMBC cross-peaks from H_2-12 (δ 3.92) to C-4 (δ 127.4) and from H_2-13 (δ 3.89) to C-5 (δ 127.7) and by NOE correlations between H_2-13 and H_3-14. These olefinic carbons C-4 and C-5 were incorporated into a 2-imino-1-methylimidazole ring (ring A), which was determined by chemical shifts of *N*-methyl group C-14 (δ_H 3.48 and δ_C 30.6) and guanidine carbon C-2 (δ 143.1) as well as by HMBC cross-peaks from H_3-14 to C-2 and C-5. The presence of a CO-NMe-CO moiety was indicated by the chemical shifts of the high-field methyl group C-15 (δ_H 3.15 and δ_C 25.0) and by HMBC cross-peaks from H_3-15 to C-9 (δ 153.9) and C-11 (δ 161.0). Taking into account the molecular formula, the remaining atoms, one nitrogen and one quaternary carbon (δ 144.9), together with the CO-NMe-CO moiety, were suggestive of a hydantoin derivative (ring B), a moiety previously found in imidazole alkaloids from *Leucetta* sponges;[1,5] therefore, the structure of **1** was established.

Naamidine I (**2**) had a molecular ion peak at *m/z* 507 $[M + H]^+$ by FABMS, which matched the formula $C_{26}H_{31}N_6O_5$. Although ^{1}H and ^{13}C NMR data of **2** (Table 1) were similar to those of **1**, the presence of an additional methyl group C-16 (δ_H 3.08 and δ_C 34.6) was exhibited in **2**. This methyl group showed an HMBC correlation with quaternary carbon C-9 at δ 152.0, which suggested that an oxygen atom at the C-9 position in **1** was replaced by an NMe group in **2** (ring B in Figure 2). The structure of the ring B moiety in **2** was reported to exist in (2*E*,9*E*)-pyronaamidine 9-(*N*-methylimine),[2] and the NMR data of ring B in both compounds

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Table 1. ^{1}H and ^{13}C NMR data[a] for **1**[b] and **2**[c]

	1		**2**	
no.	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)	δ_C
2		143.1 C		147.3 C
4		127.4 C		126.4 C
5		127.7 C		123.7 C
7		144.9 C		155.6 C
9		153.9 C		152.0 C
11		161.0 C		164.2 C
12	3.92 s	30.5 CH_2	3.95 s	29.5 CH_2
13	3.89 s	29.5 CH_2	4.01 s	28.1 CH_2
14	3.48 s	30.6 CH_3	3.40 s	29.3 CH_3
15	3.15 s	25.0 CH_3	2.98 s	25.0 CH_3
16			3.08 s	34.6 CH_3
1′		133.0 C		130.8 C
2′, 6′	7.16 d 9.0	129.7 CH	7.26 d 8.0	129.7 CH
3′, 5′	6.79 d 9.0	114.2 CH	6.86 d 8.0	113.9 CH
4′		158.5 C		157.9 C
1″		120.8 C		127.7 C
2″, 6″	6.12 s	104.7 CH	6.38 s	105.6 CH
3″, 5″		147.4 C		148.1 C
4″		133.9 C		134.1 C
4′-OMe	3.74 s	55.3 CH_3	3.71 s	55.0 CH_3
3″-OMe, 5″-OMe	3.69 s	56.3 CH_3	3.60 s	55.9 CH_3

[a] Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C). [b] Measured in $CDCl_3$. [c] Measured in DMSO-d_6.

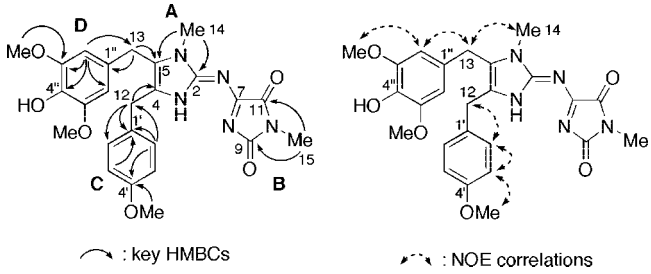


Figure 1. Key HMBCs and NOE correlations for **1**.

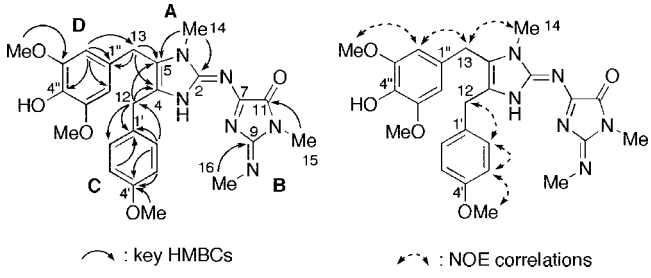
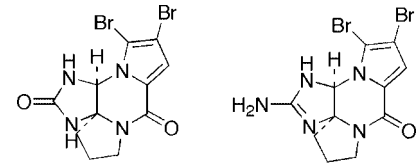


Figure 2. Key HMBCs and NOE correlations for **2**.

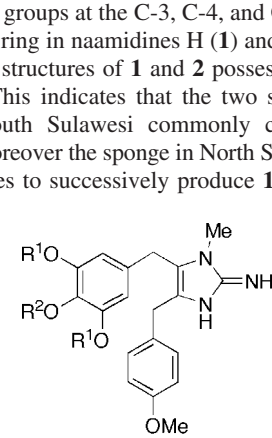
matched very well; thereby, the structure of **2** was established. It is noteworthy that the carbon chemical shifts of C-1′ and C-1″, along with carbons in rings A and B, are different between **1** and **2**, although the structural difference exists only in the C-9 positions; this suggests that the conjugation state of **1** differs from that of **2**, and this effect reaches to C-1′ and C-1″.

Naamidines H (**1**) and I (**2**) were cytotoxic against HeLa cells at IC_{50} values of 5.6 and 15 μg/mL, respectively. Incidentally, the cytotoxicity of dibromophakelstatin (**3**), which contained a urea moiety, was greater than that of dibromophakellin (**4**), in which a guanidine moiety was replaced with the urea moiety in **3**: the IC_{50} values of these compounds against five human cancer cell lines differ by 12.5–182 times.[11] Interestingly, **1** and **2** also showed a similar structure–activity relationship, although the *N*-methyl group was attached to the guanidine moiety in **2**: the IC_{50} of **1** was 3 times greater than that of **2**. These data indicate that the structural difference between urea and guanidine moieties could influence the cytotoxicity. Guanidine-containing natural products show various biological activities and are challenging targets for organic synthesis; therefore the structure–activity relationship between urea and guanidine groups, which was observed for naamidines H (**1**)/I (**2**) and dibromophakelstatin (**3**)/dibromophakellin (**4**), may be valuable information for synthetic chemists. Antibacterial activity was also reported for imidazole alkaloids of this group;[2] however, **1** and **2** were inactive to *E. coli* using the disk assay (50 μg/6 mm disk).



3 **4**

To date, more than 40 imidazole alkaloids, which contain one or two modified benzyl groups and often a hydantoin moiety, have been isolated from *Leucetta* and *Clathrina* sponges. Among them, most alkaloids bear a benzyl group containing hydroxy/methoxy groups at the C-3 and C-4 positions, or a methylenedioxy group, and this corresponds to the same hydroxy pattern with dopamine. Additionally, many alkaloids contain another hydroxy or methoxy group at the C-2 position.[2] On the other hand, only naamines E[12] (**5**) and G[13] (**6**), which were isolated from *L.*cf. *chagosensis* in Fiji Island and *L. chagosensis* in South Sulawesi, respectively, bear hydroxy/methoxy groups at the C-3, C-4, and C-5 positions (Chart 3). Further, the D ring in naamidines H (**1**) and I (**2**) is the same as that in **6**, and the structures of **1** and **2** possess additional B rings compared to **6**. This indicates that the two sponges from North Sulawesi and South Sulawesi commonly contain enzymes to produce **6**, and moreover the sponge in North Sulawesi may contain additional enzymes to successively produce **1** and **2**.



5: R^1=H, R^2=Me
6: R^1=Me, R^2=H

Experimental Section

General Experimental Conditions. UV spectra were measured on a Shimadzu UV-1600 UV–visible spectrophotometer. IR spectra were recorded on a Shimadzu IR-460 infrared spectrophotometer. NMR spectra were recorded on a JEOL GSX500 NMR spectrometer in $CDCl_3$ or DMSO-d_6. Chemical shifts were referenced to the residual solvent peaks (δ_H 7.26 and δ_C 77.0 for $CDCl_3$ and δ_H 2.49 and δ_C 39.5 for DMSO-d_6). Mass spectra were measured on a JEOL SX-102 mass spectrometer.

Animal Material. The marine sponge was collected by scuba diving at a depth of 10 m in North Sulawesi, Indonesia, in September 2006 and soaked in EtOH immediately. The sponge was identified as *Leucetta chagosensis* (Calcarea sponge). A voucher specimen (ZMAPOR19879) was deposited at the Institute for Systematics and Ecology, University of Amsterdam, The Netherlands.

Extraction and Isolation. The sponge (100 g, wet weight) was extracted with EtOH three times. The EtOAc-soluble part of the extract was fractionated by SiO_2 column chromatography with a stepwise gradient of *n*-hexane/EtOAc/MeOH. The 50% *n*-hexane–EtOAc eluent was purified by ODS column chromatography with 75% MeOH–H_2O to afford naamidine H (**1**, 47.5 mg, 0.047% wet weight). The 90% EtOAc–MeOH eluent was subjected to Sephadex LH-20 with MeOH to afford naamidine I (**2**, 16.2 mg, 0.016%).

Naamidine H (1): yellow, amorphous solid; UV (MeOH) λ_{max} (log ε) 225 nm (sh, 4.27), 278 (3.77), 382 (3.97); IR (dry film) ν_{max} 3600, 1750, 1734, 1716, 1699, 1684, 1653, 1558, 1541, 1508, 1489, 1473, 1456 cm^{-1}; NMR data ($CDCl_3$), see Table 1; HMBC H_2-12/C-4, C-1′, C-2′, C-6′; H_2-13/C-5, C-1″, C-2″, C-6″; H_3-14/C-2, C-5; H_3-15/C-9, C-11; H-2′, H-6′/C-12, C-1′, C-2′, C-3′, C-4′, C-5′, C-6′; H-3′, H-5′/C-1′, C-2′, C-3′, C-4′, C-5′, C-6′; H-2″, H-6″/C-13, C-2″, C-3″, C-4″, C-5″, C-6″; 4′-OMe/C-4′; 3″-OMe, 5″-OMe/C-3″, C-5″; FABMS (positive) *m/z* 494 $[M + H]^+$; HRFABMS $[M + H]^+$ *m/z* 494.2035 (calcd for $C_{25}H_{28}N_5O_6$ 494.2040).

Naamidine I (2): yellow, amorphous solid; UV (MeOH) λ_{max} (log ε) 225 nm (sh, 4.29), 275 (3.85), 384 (3.91); IR (dry film) ν_{max} 3600, 1732, 1716, 1684, 1653, 1558, 1539, 1506, 1489, 1471, 1456 cm^{-1}; NMR data (DMSO-d_6), see Table 1; HMBC H_2-12/C-4, C-5, C-1′, C-2′, C-6′; H_2-13/C-4, C-5, C-1″, C-2″, C-6″; H_3-14/C-2, C-5; H_3-15/C-11; H_3-16/C-9; H-2′, H-6′/C-12, C-1′, C-2′, C-4′, C-6′; H-3′, H-5′/C-1′, C-2′, C-3′, C-4′, C-5′, C-6′; 4′-OMe/C-4′; H-2″, H-6″/C-13, C-1″, C-2″, C-4″, C-6″; 3″-OMe, 5″-OMe/C-3″, C-5″; FABMS (positive) *m/z* 507 $[M + H]^+$; HRFABMS $[M + H]^+$ *m/z* 507.2367 (calcd for $C_{26}H_{31}N_6O_5$ 507.2356).

Cytotoxicity Assay. Cytotoxicity was evaluated in HeLa cells. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were seeded into 96-well microplates (3×10^3 cells/well) and precultured for one day. The medium was replaced with that containing test compounds at various concentrations, and the cells were further cultured at 37 °C for 3 days. The medium was then replaced with 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.2 mg/mL in medium), and the cells were incubated under the same conditions for 4 h. After addition of 200 μL of DMSO, the optical density at 570 nm was measured with a microplate reader.

Antimicrobial Test. Growth-inhibitory activity was determined by the paper disk method. A paper disk (6 mm) with each sample was incubated on an agar plate containing the bacterium at 25 °C.

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Note Added after ASAP Publication: The compound names naamidines G and H were changed to naamidines H and I in the version posted on Sept 20, 2007.

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