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From anti-fouling to biofilm inhibition: New cytotoxic secondary metabolites from two Indonesian *Agelas* sponges

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ABSTRACT

Chemical investigation of Indonesian marine sponges *Agelas linnaei* and *A. nakamurai* afforded 24 alkaloid derivatives representing either bromopyrrole or diterpene alkaloids. *A. linnaei* yielded 16 bromopyrrole alkaloids including 11 new natural products with the latter exhibiting unusual functionalities. The new compounds include the first iodinated tyramine-unit bearing pyrrole alkaloids, agelanesins A–D. These compounds exhibited cytotoxic activity against L5178Y mouse lymphoma cells with IC_{50} values between 9.25 and 16.76 μM. Further new compounds include taurine acid substituted bromopyrrole alkaloids and a new dibromophakellin derivative. *A. nakamurai* yielded eight alkaloids among them are three new natural products. The latter include the diterpene alkaloids (−)-agelasine D and its oxime derivative and the new bromopyrrole alkaloid longamide C. (−)-Agelasine D and its oxime derivative exhibited cytotoxicity against L5178Y mouse lymphoma cells (IC_{50} 4.03 and 12.5 μM, respectively). Furthermore, both agelasine derivatives inhibited settling of larvae of *Balanus improvisus* in an anti-fouling bioassay and proved to be toxic to the larvae. (−)-Agelasine D inhibited the growth of planktonic forms of biofilm forming bacteria *S. epidermidis* (MIC < 0.0877 μM) but did not inhibit biofilm formation whereas the oxime derivative showed the opposite activity profile and inhibited only biofilm formation but not bacterial growth. The structures of the isolated secondary metabolites were elucidated based on extensive spectroscopic analysis involving one- and two-dimensional NMR as well as mass spectrometry and comparison with literature data.

1. Introduction

Biofouling organisms such as blue mussels, barnacles, and macroalgae cause serious problems to ships hulls, bridges, cooling systems of power plants, and aquaculture materials[1] causing annual losses to the maritime industry that exceed 200 billion dollars.[2] Long-term use of toxic chemical anti-foulants such as tributyltin (TBT) has led to its accumulation in the food chain as well as to mortality and change of sex of nontarget organisms.[3,4] Consequently, use of TBT has been banned and less toxic alternatives are urgently needed. It is well known that many marine invertebrates such as sponges and corals usually remain remarkably free from settlement by fouling organisms. It has been suggested that they accumulate biologically active compounds that prevent settling by fouling organisms.[5] In fact, several compounds with such activity have been found among marine invertebrates.[6]

In addition to multicellular fouling organisms such as *Balanus larvae*, bacterial biofilms severely contribute to biofouling and are considered to be of particular importance with regard to early stages of fouling. Bacterial biofilms also cause problems in medical health care since they colonize implants such as artificial joints or may clog catheters. Several mechanisms have been proposed to explain why only very few biocides are active against biofilm-embedded bacteria which, in contrast to their planktonic forms, are not undergoing cellular division. When imbedded in biofilms bacteria are less susceptible[7] to antibiotics due to low physicochemical interaction of these compounds with slime[8] thereby causing lower

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diffusion of the antimicrobial agent through the biofilm.[9] In addition, the occurrence in biofilms of high frequency 'persisters' (bacteria that do not grow but do not die in the treatment with antibiotic) might be the cause of these recalcitrant infections.[10] Bacterial biofilms thus have great significance for public health, since biofilm-associated microorganisms exhibit a dramatic decrease in susceptibility to antimicrobial agents.

This susceptibility may be intrinsic as a natural outcome of growth in the biofilm or acquired due to transfer of extrachromosomal elements to susceptible organisms in the biofilm. The susceptibility of biofilms to antimicrobial agents cannot be determined by means of standard microdilution testing, since these tests rely upon the response of planktonic (suspended) rather than biofilm (surface-associated) organisms. Instead, susceptibility must be determined directly against biofilm-associated organisms, preferably under conditions that simulate conditions in vivo.[11]

Sponge-derived anti-fouling molecules have been found to inhibit the settlement of barnacle larvae,[12] inhibit fouling by macroalgae,[5,13] or repel the blue mussel *Mytilus edulis galloprovincialis*.[14] Brominated pyrroles such as pseudoceratidine, oroidin, and mauritiamine and bromotyrosine-derived compounds such as bastadin derivatives, ceratinamide A and B, psammaplin A and moroka'iamine are some examples of sponge-derived secondary metabolites having promising activity as anti-fouling agents but also as inhibitors of bacterial biofilms.[12b–d,15,16,32] These findings led us to investigate two Indonesian *Agelas* sponges that show the occurrence of brominated pyrroles in their chemical profiles. However, isolation of the secondary metabolites showed the predominance of metabolites belonging to diverse chemical groups along with the presence of known brominated pyrroles.

Most members of the sponge genus *Agelas* described so far have been reported from the Caribbean and include 14 species which, are currently under revision.[17] Coral reefs of the Indo-Pacific even though considered as being the most diverse in the oceans have so far yielded only 13 *Agelas* species with a single species reported from Indonesia.[18] Chemically *Agelas* sponges (Agelasidae) are well known for their bromopyrrole alkaloids.[19] These compounds are involved in the sponge's defence mechanism against fishes[20] whereas several pharmacologically important bromopyrrole congeners have been previously described as having cytotoxic,[21] immunosuppressive,[21c] anti-fouling,[12b,22] antifeedant,[20,23] antagonist of serotonergic receptors,[24] or protein kinase inhibitors.[25] Tyrosine-based haloderivatives have also been reported from *Agelas oroides* and include 11-*epi*-fistularin-3 along with two ketone congeners agelorins A and B.[26] Tyrosine-derived halometabolites frequently occur in marine organisms and are known to play an important role for the survival of the organism.[26,27] Bromotyrosine-derived compounds have been observed to show a wide range of interesting biological activities such as antiviral,[28] anti-HIV,[29] antibiotic,[24a,30] Na^+/K^+-ATPase inhibitory,[31] anti-fouling,[22,12c,12d,32] antihistamine-H_3 antagonist,[33] as well as anticancer activities.[34] Besides the brominated pyrrole analogues, *Agelas* sponges are also known to possess bioactive substances derived from diterpene alkaloids which include agelasines,[31d,35] agelasimines,[36] and agelasidines.[37] Unlike the brominated pyrroles that occur also in other sponge genera, diterpene alkaloids seem to be unique to *Agelas* sponges.[35b] The diterpenoid (+)-agelasine D was reported for the first time from an undescribed species of Okinawan *Agelas*.[35a] It has been successfully synthesized from (+)-manool, a commercially available diterpene alcohol.[38]

2. Chemistry

In the cause of this study 24 different alkaloids including 12 new bromopyrrole alkaloids and two new diterpene alkaloids were isolated, structurally identified (Figs. 1 and 2) and investigated for various biological activities. Structure elucidation of the new natural products by spectroscopic methods (mass spectrometry and one- and two-dimensional NMR spectroscopy) and characterization of their bioactivities is reported.

In our search for bioactive secondary metabolites from sponges, a series of brominated pyrrole alkaloids have been isolated from *A. linnaei* (Fig. 1), a new recently described species of the marine sponge genus *Agelas* that was collected from the Thousand Islands, Indonesia on September 2005. Examination of the acetone/methanol extract of the sponge *A. linnaei* resulted in the isolation of 11 new brominated pyrrole derivatives (**5**–**15**) along with the known midpacamide (**1**),[39e] agelongine (**2**),[39b] 4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxylic acid (**3a**),[39e] methyl-4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxylate (**3b**),[36a] and (+)-dibromophakellin HCl (**4**).[39d] The spectral data of the known compounds **1**–**4** are compatible with the data reported in the literature.[39]

A second *Agelas* species collected from Menjangan Island, Bali, Indonesia was taxonomically identified as *A. nakamurai*. The sponge afforded two new diterpenes containing the adenine-related moiety, (−)-agelasine D (**16**) and (−)-ageloxime D (**17**), as well as a new bromopyrrole alkaloid, longamide C (**18**), together with five known metabolites, mukanadin C (**19**),[40] agelasidine C (**20**),[37a] hymenidin (**21**),[24a] bromopyrrole carboxylic acid (**22**),[19b] and bromopyrrole carboxamide (**23**) as shown in Fig. 2.[41]

2.1. Brominated pyrroles

Chemical profiling of the crude organic extract of *A. linnaei* and *A. nakamurai* indicated the presence of several brominated pyrrole derivatives. Peaks observed from the analytical HPLC chromatogram showed UV spectra with absorption maxima at around 270 nm that is characteristic of 2-pyrrole carboxamide substituted chromophores.[42] Some UV absorption pattern modifications were observed for several compounds, such as additional bands or bathochromic shift of the peaks. LC/MS experiment indicated that these substances are all brominated. Pseudo-molecular ion peak cluster having two mass unit differences at a ratio of 1:1 for one bromine or 1:2:1 for two bromines or 1:2:2:1 for three bromines were detected. HRESIMS as well as ^{13}C and DEPT NMR data confirmed the molecular formula for each of the isolated new compounds.

Compound **5** is the new hydroxyl derivative of dibromophakellin (**4**) which exhibited a UV spectrum with λ_{max} at 241.3 and 292.6 was almost identical to that of the isolated known congener **4**. ESIMS pseudo-molecular ion cluster at *m*/*z* 404/406/408 $[M+H]^+$ having an intensity ratio of 1:2:1 indicated the presence of two bromines in the molecule. Accurate mass data established the molecular formula $C_{11}H_{12}Br_2N_5O_2$ for compound **5**. There is an excess of 16 mass units in **5** when compared to **4** indicating the presence of an additional hydroxyl function in the molecule. ^1H and ^{13}C NMR data (Table 2) were very similar to those of the dibromophakellins.[39d,43,44] The numbering scheme followed that of midpacamide (**1**) to indicate the cyclisation of the alkyl chain to form the central aliphatic ring system in the phakellin analogues and to conveniently apply the similar numbering to the other analogues described in this paper. Comparison of the ^1H NMR data to those of the known dibromophakellins helped in assigning the proton signals and in detecting obvious differences such as the emergence of a broad singlet at δ_H 7.19 and the disappearance of the doublet proton at δ_H 6.29 previously assigned to H-12 in compound **4**. In the ^{13}C and DEPT NMR spectra of **5**, the methine carbon signal at δ_C 68.1 which was allocated for C-12 in compound **4** was absent and instead deshielded quaternary carbon signals at δ 89.9 and 91.6 were observed. The attachment of a hydroxyl group at C-12 could account for these deshielded carbon signals. C-12 is directly linked to not only two but three electron-withdrawing groups, the guanidine moiety, pyrrole nitrogen and the hydroxyl group causing

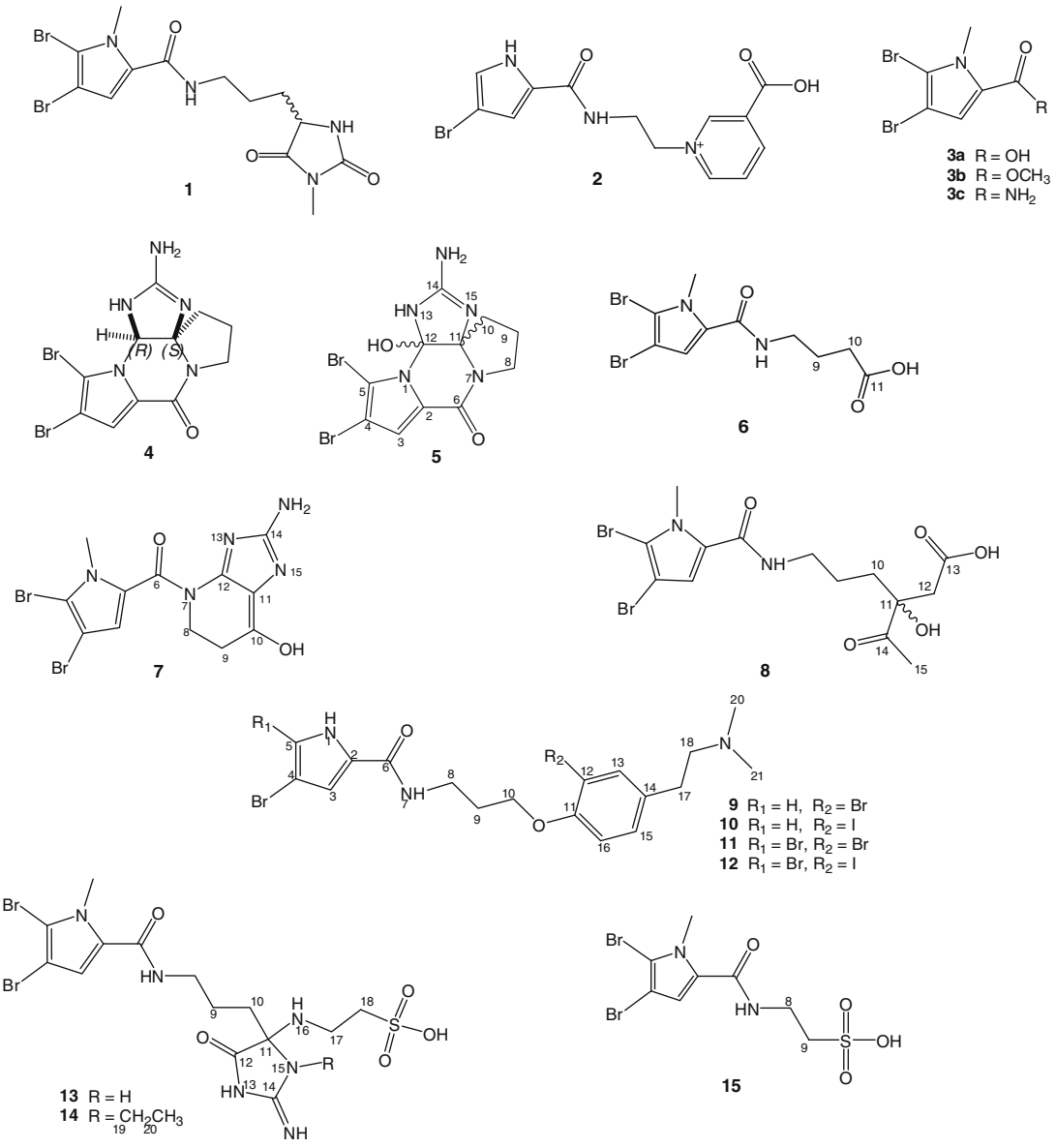

Figure 1. Compounds isolated from *Agelas linnaei*.

the downfield shift to 91.6 ppm. Therefore the broad singlet at δ_H 7.19 was then assigned to the O*H* group and this was confirmed by its HMBC correlation to δ 91.6 (C-12), 89.9 (C-11), and a ^4J correlation with the methylene carbon at 33.0 ppm designated for C-10. On the other hand, the carbon signal at δ 91.6 correlated further with N*H* resonances at positions 13 and 15, proton signals for H-10A and a $^{4(w)}J$ correlation with H-8A. Relative stereochemistry was assigned by using the ROESY data. The ROESY spectrum of **5** showed NOE cross peaks between the hydroxyl proton and H-10A/B suggesting that the O*H* group is *cis* to CH_2-10 (Fig. 3). These results suggest the same configuration as reported previously for compound **4** [(+)-dibromophakellin HCl] which has a 11*S*,12*R* configuration.[45] However, to satisfy the *cis* stereochemistry at C-11/12 in **4**, either 11*R*,12*R* (**5A**) or the 11*S*,12*S* (**5B**) configurations are possible. The isolated (+)-dibromophakellin gave a $[\alpha]_D^{25}$ value of +91.7° ± 1.9° (*c* 0.31, MeOH) while the literature value for its enantiomer is −205° (*c* 2.875, MeOH).[43] The small magnitude of the optical rotation value of **5** at $[\alpha]_D^{25} = -6.6 \pm 0.7$ (*c* 0.23, MeOH) may imply that **5** is a racemic mixture probably with a slight excess of the (−)-enantiomer. After interpretation of the spectral data available, compound **5** was elucidated as dibromohydroxyphakellin HCl, a new member of phakellin group of compound. This is the first described 12-OH analogue of the phakellin family.

Compound **6** is the butanoic acid derivative of the 4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxamide (**3c**), a key building block in this series of bromopyrrole analogues isolated from *A. linnaei*. ESIMS showed a pseudo-molecular ion peak cluster at *m*/*z* 367/369/371 $[M+H]^+$ having a ratio 1:2:1, which indicated the presence of a dibrominated molecule. The molecular formula of $C_{10}H_{13}Br_2N_2O_3$ was deduced by HRESIMS. ^{1}H and ^{13}C NMR spectra (Table 3) of **13** in DMSO-d_6 indicated the presence of a 4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxamide, a propylene chain, and carboxylic acid substructures. ^{13}C NMR spectrum showed two deshielded quaternary carbon at δ_C 159.7 and 174.9, which were, interpreted as carboxamide and carboxylic acid functions, respectively. ^{1}H-^{1}H COSY spectrum revealed one isolated spin system coupling a propylene chain to an amide function (δ_H 8.31, t, *J* = 5.5 Hz, 7-NH; δ_H 3.16, dd, *J* = 5.5 Hz, H_2-8; δ_H 1.68, t, *J* = 5.5 Hz, H_2-9; δ_H 2.21, t, *J* = 5.5 Hz, H_2-10). HMBC $^3J_{C-H}$ cross peaks correlated the carboxylic acid carbon at δ_C 174.9 with CH_2-9 at δ_H 1.68

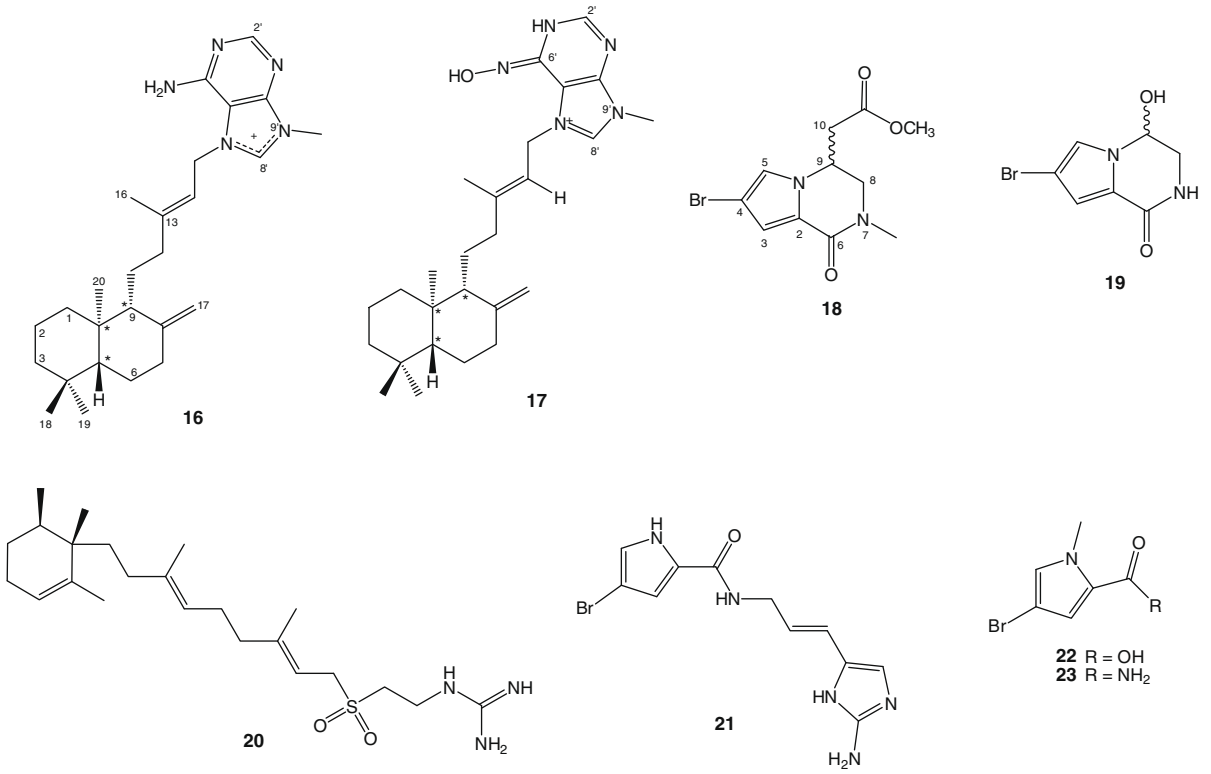

Figure 2. Compounds isolated from *Agelas nakamurai*.

Table 1
^{1}H and ^{13}C NMR data of **5**–**8**[a]

No.	5		6		7		8	
	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT
1			3.85, s	35.3, CH_3	3.64, s	35.2, CH_3	3.85, s	35.3, CH_3
2		126.9, C		128.0, C		129.0, C		128.2, C
3	6.90, s	108.5, CH	6.98, s	113.8, CH	6.62, s	117.0, CH	6.97, s	113.7, CH
4		89.5, C		96.8, C		97.2, C		96.7, C
5		104.1, C		110.3, C		110.2, C		110.1, C
6		156.4, C		159.7, C		159.7, C		159.4, C
7			8.31, t, 4.9				8.17, t, 5.7	
8A	3.86, ddd, 3.1, 9.8, 12.9	35.8, CH_2	3.16, dt, 6.6, 5.0	38.3, CH_2	3.97, dd, 6.0, 6.6	46.3, CH_2	3.06, dt, 7.2, 5.7	38.8, CH_2
8B	3.11, ddd, 5.7, 1.9, 7.9		1.68, dt, 6.9, 7.2	24.7, CH_2	2.47, t, 6.6	36.1, CH_2	1.53, m	24.0, CH_2
9A	1.93, m	16.6, CH_2					1.32, m	
9B	1.49, m		2.20, t, 7.2	31.9, CH_2	11.15, br s (O*H*)	177.5, C	1.53, m	36.8, CH_2
10A	2.06, ddd, 2.8, 14.1, 11.3	33.0, CH_2					1.32, m	
10B	1.67, dt, 2.8, 13.6			174.9, C		110.5, C	7.81, br s (O*H*)	74.9, C
11		89.5, C				152.7, C	2.71, d, 12.9	53.5, CH_2
12	7.19, br s (O*H*)	91.6, C					2.37, d, 12.9	
13	9.82, s (N*H*)							177.4, C
14		156.5, C				153.2, C		207.7, C
15	9.80, s ($N^{+}H$)				6.37, br s (NH_2)			
16							2.02, s	30.9, CH_3

[a] Data were recorded in DMSO-d_6 at 500 MHz (^{1}H) and 125 MHz (^{13}C).

(dt, 6.9, 7.2) while the carboxamide carbon at δ_C 159.7 with CH_2-8 at δ_H 3.16 (dt, 6.6, 5.0). This evidence suggests that the 1*N*-methyl-4,5-dibromo-2-pyrrole carboxamide unit is connected to a carboxylic acid function through the propylene chain.

Compound **7** showed a distinct UV absorption pattern compared to the other bromopyrrole carboxamide congeners isolated from this sponge. The major absorption band was shifted about 70 nm to longer wavelength at 342.9 nm, which might be due to an extended conjugated system and/or to an additional hydroxyl unit in the molecule. ESIMS showed a pseudo-molecular ion peak cluster at m/z 416/418/420 $[M+H]^{+}$ with an intensity ratio of 1:2:1 indicating the presence of two bromines in the molecule. Molecular formula of $C_{12}H_{12}Br_2N_5O_2$ was evident from the HRESIMS. The ^{1}H NMR spectrum exhibited two broad exchangeable downfield signals. The signal at δ_H 11.15 was assigned to a hydroxyl group while the broad singlet at δ_H 6.37 integrating for two protons was allocated to an imidazolamine moiety. ^{1}H NMR signal for an ethylidene unit were observed at δ_H 3.97 (2H, dd, J = 6.0, 6.6 Hz, CH_2-8) and 2.47 (2H, t, J = 6.6 Hz, CH_2-9) which was confirmed by ^{1}H–^{1}H COSY. Their downfield shift may be caused by

Table 2
^{1}H and ^{13}C NMR data of **9**–**12**[a]

No.	9		10		11		12	
	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT
1	11.82, br s (N*H*)		11.80, br s (N*H*)		12.55, br s (N*H*)		12.51, br s (N*H*)	
2		126.9, C		126.9, C		128.3, C		128.3, C
3	6.83, br s	111.3, CH	6.83, dd, 1.6, 0.9	111.3, CH	6.90, d, 0.9	112.5, CH	6.90, d, 0.9	112.5, CH
4		94.8, C		94.8, C		97.6, C		97.6, C
5	6.94, br s	121.0, CH	6.94, dd, 1.6, 0.9	121.0, CH		104.5, C		104.5, C
6		159.6, C		159.6, C		159.0, C		159.0, C
7	8.17, m (N*H*)		8.17, m (N*H*)		8.19, m (N*H*)		8.19, m (N*H*)	
8A	3.40, m	35.4, CH_2	3.40, m	35.4, CH_2	3.39, m	35.7, CH_2	3.39, m	35.7, CH_2
8B								
9A	1.94, m	29.0, CH_2	1.94, m	29.0, CH_2	1.93, m	29.0, CH_2	1.93, m	29.0, CH_2
9B								
10A	4.03, m	66.4, CH_2	4.03, m	66.4, CH_2	4.03, m	66.3, CH_2	4.03, m	66.3, CH_2
10B								
11		152.9, C		155.3, C		152.9, C		155.2, C
12		110.8, C		86.5, C		110.8, C		86.5, C
13	7.42, d, 2.2	132.8, CH	7.61, d, 1.9	138.7, CH	7.42, d, 1.9	132.8, CH	7.61, d, 1.9	138.7, CH
14		134.1, C		134.6, C		134.2, C		134.7, C
15	7.15, dd, 1.9, 8.5	129.0, CH	7.17, dd, 2.2, 8.5	129.8, CH	7.15, dd, 2.2, 8.5	129.0, CH	7.17, dd, 1.9, 8.5	129.8, CH
16	6.99, d, 8.5	113.6, CH	6.88, d, 8.5	112.4, CH	6.98, d, 8.5	113.5, CH	6.88, d, 8.5	112.4, CH
17	2.62, m	31.4, CH_2	2.62, m	31.3, CH_2	2.62, m	31.7, CH_2	2.62, m	31.5, CH_2
18	2.43, m	60.6, CH_2	2.43, m	60.6, CH_2	2.42, m	60.6, CH_2	2.42, m	60.6, CH_2
20/21	2.18, s (6H)	44.9, CH_3	2.18, s (6H)	44.9, CH_3	2.17, s (6H)	44.9, CH_3	2.17, s	44.9, CH_3

[a] Data were recorded in DMSO-d_6 at 500 MHz (^{1}H) and 125 MHz (^{13}C).

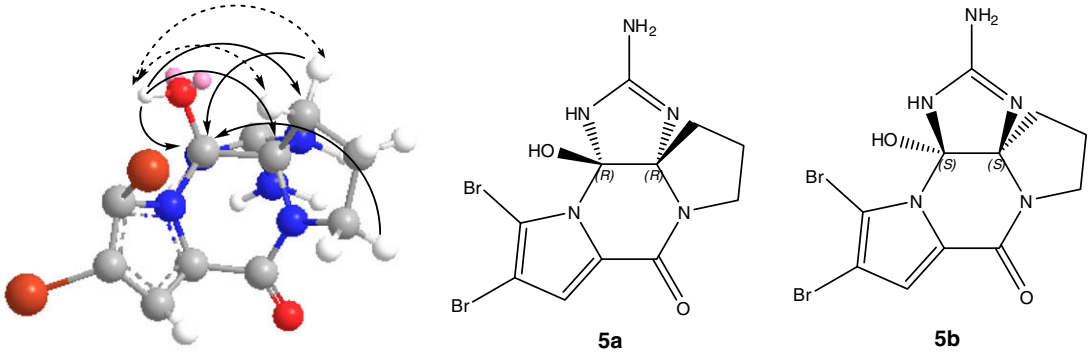

Figure 3. NOE (- - - -) and HMBC (—) correlations of O*H*-12 and C-12 in compound **5**.

their position between two electronegative substituents. The hydroxyl proton showed correlation in the HMBC spectrum to C-12 at δ_C 152.7 (^4J) and C-11 at δ_C 110.5 (^3J). Intense HMBC $^3J_{C-H}$ cross peaks were detected between the methylene proton at δ_H 3.97 (CH_2-8) and the deshielded quaternary carbon signals at δ_C 159.7 (C-6) and 177.5 (C-10), while the methylene proton at δ_H 2.47 (CH_2-9) correlated only with δ_C 177.5 (C-10). At first, this finding led to a dihydropyridone substructure. This was, however, questioned from the chemical shift of a second carbonyl unit, which should be shifted further downfield to ca. 195 ppm as observed in cenocladamide (δ_C 194.1), a dihydropyridone alkaloid isolated from the plant *Piper cenocladum*.[46] The carbon resonance at 177.5 ppm suggested a tetrahydropyridinol function. A related compound, dibromoagelaspongin HCl has been earlier reported from a Tanzanian *Agelas* species.[39a] The structure of compound **7** was unambiguously elucidated as 2-amino-7-hydroxy-5,6-dihydroimidazo[4,5-*b*]pyridin-4-yl)(4,5-dibromo-1-methyl-1*H*-pyrrole-2-yl)methanone and designated the trivial name agelanin A.

Compound **8** is an optically active compound having a $[\alpha]_D^{20}$ value of −18.7 ± 2.8 (*c* 0.06, MeOH). ESIMS experiment showed a pseudo-molecular ion peak cluster at *m*/*z* 453/455/457 $[M+H]^+$ with intensity ratio of 1:2:1 indicative of a dibrominated compound. Molecular formula of $C_{14}H_{19}Br_2N_2O_5$ was inferred by HRESIMS. ^{1}H NMR spectrum of **8** revealed the presence of a 4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxamide substructure. Other signals observed included a broad exchangeable singlet proton of an hydroxyl function at δ_H 7.81, a *N*-propylacetamide chain (δ_H 8.17, t, J = 5.7 Hz, 7-N*H*; δ_H 3.06, dt, J = 7.2, 5.7 Hz, H-8A/B; δ_{H2} 1.53, H-9A and H-10A; δ_{H2} 1.32, m, H-9B and H-10B), a diastereotopic AB methylene proton pair (δ_H 2.71, d, J = 12.9 Hz, H-12A and δ_H 2.37, d, J = 12.9, H-12B), and a methyl ketone singlet at δ_H 2.02. Accordingly, the ^{13}C NMR spectrum of **8** demonstrated three carbonyl resonances at δ_C 159.4 207.7 and 177.4 for a carboxamide, ketone and carboxylic acid functions, respectively. The presence of the ketone unit is supported by the co-occurrence of a keto-methyl signal at δ_C 30.9 and δ_H 2.02. A quaternary carbon signal at δ_C 74.9 was assigned to a hydroxyl bearing sp^3 carbon (C-11).

2D NMR experiments provided data to assemble the different substructures obtained from the 1D spectra (Fig. 4). ^{1}H-^{1}H COSY spectrum of **8** confirmed two spin systems comprising the propylcarboxamidyl moiety and the diastereotopic geminal proton pair. In addition, the HMQC spectrum of **8** revealed the CH direct correlation of the geminal protons with the deshielded tertiary carbon signal at δ_C 53.5 (C-12). Further, the HMBC data indicated $^3J_{C-H}$ correlations of these methylene protons to the ketone carbon (C-14) and to the terminal methylene carbon (C-10) of the propylene

Table 3
^{1}H and ^{13}C NMR data of **13**–**15**[a]

No.	13		14		15	
	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT	^{1}H δ, mult., J in Hz	^{13}C δ, DEPT
CH_3-1	3.86, s	35.3, CH_3	3.86, s	33.4, CH_3	3.86, s	34.8, CH_3
2		127.9, C		127.9, C		127.8, C
3	6.99, s	113.9, CH	7.00, s	114.0, CH	6.83, s	113.0, CH
4		96.8, C		96.8, C		96.3, C
5		110.4, C		110.4, C		110.3, C
6		159.7, C		159.7, C		158.8, C
7	8.20, t, 5.4		8.19, t, 5.4		8.17, t, 5.2	
8	3.14, m	38.1, CH_2	3.14, m	38.0, CH_2	3.44, dd, 6.2, 13.6	35.1, CH_2
9A	1.27, m	22.8, CH_2	1.30, m	22.5, CH_2	2.62, dd, 7.6, 7.0	49.7, CH_2
9B						
10A	1.84, m	33.9, CH_2	1.90, m	33.4, CH_2		
10B						
11		91.5, C		95.7, C		
12A		179.6, C		177.1, C		
12B						
13	8.93, br s[b]		9.07, br s[b]			
14		166.7, C		167.2, C		
14a	8.36, br s[b]		8.88, br s[b]			
15	7.13, br s[b]					
16	9.51, br s		9.73, br s			
17	3.60, m	39.7, CH_2	3.63, m	39.7, CH_2		
18	2.74, t, 7.2	49.2, CH_2	2.75, t, 7.2	49.0, CH_2		
19A			3.28, m	58.7, CH_2		
19B			3.11, m			
20			1.09, t, 6.9	14.9, CH_3		

[a] Data were recorded in DMSO-d_6 at 500 MHz (^{1}H) and 125 MHz (^{13}C).
[b] Signals are interchangeable.

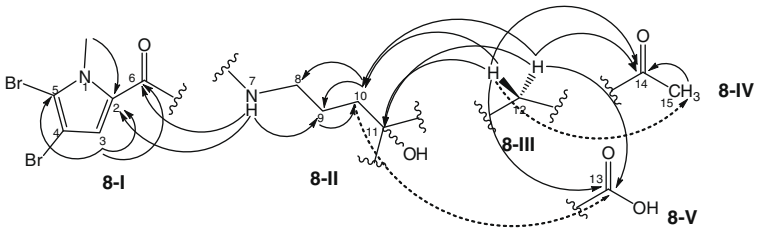

Figure 4. Substructures of **8** as indicated by its 1D NMR spectra and HMBC correlations show the connectivity of the substructures through arrows. $^4J_{C-H}$ correlations are illustrated by dashed lines.

chain, whereas $^2J_{C-H}$ correlations were observed to the carboxylic carbon (C-13) and to C-11. These findings support the presence of a 3-hydroxy-4-oxo-pentanoic acid moiety to which the propylcarboxamidyl chain is attached. The HMBC spectrum also exhibited $^4J_{C-H}$ correlations from H-10B to C-13, H-12B to C-15, and H-15 to C-12 (Fig. 4). We were not able to determine the stereochemistry at position 11. The structure of **8** was 3-acetyl-6-(4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxamido)-3-hydroxyhexanoic acid and assigned the trivial name agelanin B (Fig. 1).

Compound **18** was obtained from the organic extract of *A. nakamurai* as a white amorphous residue. It exhibited a UV spectrum which is comparable to that of mukanadin C (**19**), suggesting a similar chromophoric moiety. ESIMS pseudo-molecular ion peak cluster at m/z 301/303 $[M+H]^+$ at an intensity ratio of 1:1 indicated the presence of two bromine atoms in the molecule. Molecular formula of $C_{11}H_{14}BrN_2O_3$ was deduced by HRESIMS, which was in accordance with six degrees of unsaturation. Literature survey of the molecular weight and formula implied a closely related substance to longamide B methyl ester previously described from *Homoaxinella* sp.,[47] which was obtained as a racemate mixture of 9*S* and 9*R* enantiomers. Whereas, (*S*)-(−)-longamide B methyl ester was later synthetically produced.[48] Longamide B methyl ester has two bromine atoms.[47] However, the molecular weight of **18** is 64 mass units greater than that of longamide B methyl ester. Considering that the isotopic pattern of **18** indicated only one bromine atom in its molecule, a 15 mass units difference plausibly referred to an additional methyl group substituent in **18**. The ^{1}H NMR data is comparable to that of longamide B methyl ester[47] except for the absence of an amide proton signal and presence of an *N*-methyl singlet at δ 3.10 suggesting that the amide function is methylated. ^{1}H NMR spectrum of **18** in $CDCl_3$ revealed two coupling sp^2 proton signals at δ_H 6.89 (1H, s, H-3) and 6.79 (1H, s, H-5) suggesting bromination at position 4. Due to the small amount available, an HMBC spectrum could not be obtained.

The relative stereochemistry of **18** was assigned based on its ROESY spectrum. NOEs were detected between H-9 to H_2-10, H-8A and H-8B; H-9 and H_2-10 to H-5; and H_2 to the *N*-methyl, as well as from H-8B to H_2-10 (Fig. 5). These results indicated a half chair conformation of the six-membered ring where the bulky substitution at C-9 is quasi-axial oriented and H-9 is quasi-equatorial. In this conformation, the *N*-methyl only shows a NOE with H-8A and H-8B, but not with H-9 nor with H_2-10. The coupling patterns observed for H-9 (m); H-8A (dd, J = 4.2, 12.9 Hz); H-8B (dd, J = 3.2, 12.9 Hz) and H_2-10 (dd, J = 7.3, 3.2 Hz) supported the proposed conformation. If H-9 was oriented axially, the coupling constant between H-9 and H-8A would be ca. 9–12 Hz which was not the

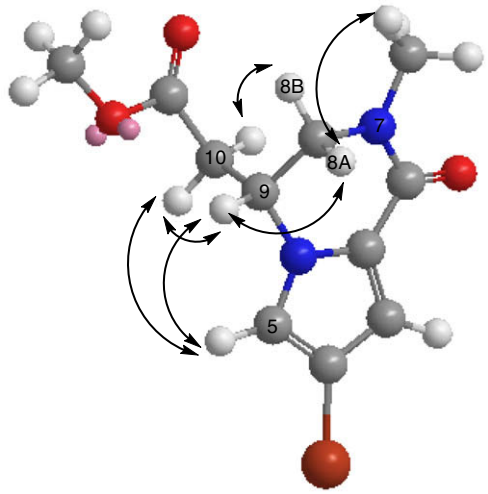

Figure 5. Relative stereochemistry of longamide C (**18**) based on its ROESY spectrum.

case for compound **18** ($^3J_{H8A/H9}$ = 4.2 Hz). Hence, the relative stereochemistry of **18** could be determined. The optical rotation was $[\alpha]_D^{25} = -1.0 \pm 0.4$ (*c* 0.1, MeOH), which was very near zero indicating that compound **18** is a racemic mixture. In parallel we also isolated mukanadin C (**19**) with an optical rotation of $[\alpha]_D^{25} = 0$, which was also a racemic mixture that implied the presence of both *R* and *S* precursors in the biosynthesis of compounds **18** and **19**. Compound **18** was elucidated as the *N*-methyl-3-debromo congener of longamide B methyl ester and was assigned the trivial name longamide C.

2.2. Tyramine analogues

Agelanesins A–D (**9–12**) proved to be new tyramine containing haloderivatives, which so far have only been described from *A. oroides*.[26] It is noteworthy that constituents featuring a halogenated tyramine unit linked to a brominated pyrrole unit as found in the new agelanesins have never before been reported from nature. Replacement of the bromine functionality by an iodine residue in the tyramine moiety as found in **10** and **12** makes this group of compounds even more attractive. Iodo-alkaloids are known as very rare natural products which to date have only been isolated from a few marine algae and sponges.[27,49,50]

Compound **9** was obtained as a yellow oily substance. An intensive ESIMS pseudo-molecular ion peak cluster at *m*/*z* 472/474/476 $[M+H]^+$ having an intensity ratio of 1:2:1 indicated the presence of a dibrominated compound. HRESIMS confirmed the molecular formula $C_{18}H_{24}Br_2N_3O_2$ for **9**. The ^{1}H NMR spectrum of **9** indicated the presence of pyrrole methine proton signals at δ_H 6.83 (1H, dd, *J* = 1.9, 0.6 Hz, H-3) and 6.94 (1H, dd, *J* = 1.9, 0.6 Hz, H-5) which coupled to the N*H*-pyrrole broad singlet proton at δ_H 11.82. Detection of a 1,2,4-trisubstituted benzene ring was based on the ABX coupled signals at δ_H 7.42 (1H, d, *J* = 2.2 Hz, H-13), 7.15 (1H, dd, *J* = 1.9, 8.5 Hz, H-15), and 6.99 (1H, d, *J* = 8.5 Hz, H-16). Six well separated methylene multiplets were detected in the high field region. A signal at δ_H 4.03 was indicative of methylene protons adjacent to an oxygen atom while a methylene unit next to a carboxamide group was present at δ_H 3.40. Resonances at δ_H 2.62 and 2.43 were indicative of their attachment to a benzyl or ammonium moiety. A dimethylammonium unit was evidenced by the methyl singlet at δ_H 2.18 that integrated for six protons. Resonances observed in the ^{13}C NMR spectrum suggested the presence of a brominated phenol through the highly shielded quaternary carbon signal at δ 110.8 (C-12) and the deshielded carbon signal at around δ_C 152.9 (C-11). These characteristic chemical shifts were previously reported in known bromotyrosine derivatives such as purealidin A[51] and purpuramine J.[34e]

In addition to the monobrominated pyrrole and the brominated phenolic ABX ring systems identified from the ^{1}H spectrum of **9**, two further spin systems were confirmed by ^{1}H-^{1}H COSY (Fig. 5). One spin system connected an –O–C*H*$_2$ to a N*H*–C*H*$_2$ through one methylene unit suggesting an oxypropylamide chain while another consisted of a dimethylammonium unit coupled to an ethylidene chain. HMBC correlations provided information needed to assemble the substructures (Fig. 6) obtained from the COSY spectrum of **9**. Cross peaks correlating C*H*$_2$-10 to C-11 indicated the connectivity of the oxypropylamide to the brominated phenolic moiety while HMBC correlations of the aromatic methine protons of the tyrosine unit to the *N*,*N*-dimethylethanaminium carbon chain confirmed the tyramine structure. This indicated that **9** is a monobrominated pyrrole connected to a trisubstituted tyramine through an oxypropylamide chain. Furthermore, this unambiguously identified **1** as 2-(3-bromo-4-(3-(4-bromo-1*H*-pyrrole-2-carboxamido)propoxy)phenyl)-*N*,*N*-dimethylethanaminium which was assigned the trivial name agelanesin A.

Compound **10** was obtained as a yellow oily substance and eluted after compound **9** during HPLC analysis on a reversed phase column. ESIMS showed a pseudo-molecular ion peak cluster at *m*/*z* 519/521 $[M+H]^+$ having an intensity ratio of 1:1 implying the presence of only one bromine atom in the molecule. HRESIMS indicated the molecular formula $C_{18}H_{24}BrIN_3O_2$ for congener **10**. NMR spectral data of **10**, presented in Table 2, indicated a close structural relationship to **9**. However, remarkable differences were found in the proton chemical shifts for the tyrosine ring system. H-13 and H-15 resonances were shifted downfield at δ 7.70 (1H, d, *J* = 1.9 Hz) and 7.23 (1H, dd, *J* = 2.2, 8.5 Hz), respectively, while H-16 was shifted upfield at δ_H 6.88 (1H, d, *J* = 8.5 Hz, H-16). The signal multiplicities still were compatible with a 1,2,4-trisubstituted benzene substructure as found in **9.** ^{13}C NMR experiment was performed with a mixture of compounds **9** and **10** (as free bases) at a ratio of 1:1 in DMSO-d_6. The obtained signals confirmed the similarity of both compounds with differences found in the phenolic moieties. C-11 and C-13 of compound **10** were deshielded and shifted to δ_C 155.3 and 138.7, respectively. On the other hand, C-16 as well as C-12 was shifted upfield to δ_C 112.4, and 86.5, respectively. The NMR data for positions 1–10 and 17–21 in both compounds were identical, it can be deduced that the single bromine atom in the molecule can only be situated on the pyrrole ring, which suggested the occurrence of another substituent on the phenolic ring. Compounds **9** and **10** have an equivalent number of protons and carbons. Instead of two bromine atoms, there is only one bromine unit in **10** as indicated by its mass spectral isotopic pattern. Taking this into account the expected molecular weight for **10** should be 393, which differs by 126 amu when compared to the measured molecular weight of 519. This can be accounted for by the presence of iodine as a substituent of compound **10**, which was confirmed by HRMS. Further support was drawn from observation of the significant high field shift of the quaternary carbon C-12 that clearly indicated the presence of an sp^2 carbon atom linked to iodine. This phenomenon had been reported previously for iodotyrosine compounds such as dakaramine obtained from the sponge *Ptilocaulis spiculifer*.[52] Hence, structure of **10** was elucidated as the iodinated tyramine congener of **9** and named agelanesin B.

Compound **11** was obtained as a yellow oily residue. Its UV spectrum as well as NMR data indicated that it is a close structure analogue of compounds **9** and **10**. The presence of three bromine atoms in the molecule is exhibited by an ESIMS pseudo-molecular ion cluster at *m*/*z* 550/552/554/556 $[M+H]^+$ having an intensity ratio of 1:2.2:1. The molecular formula $C_{18}H_{23}Br_3N_3O_2$ was

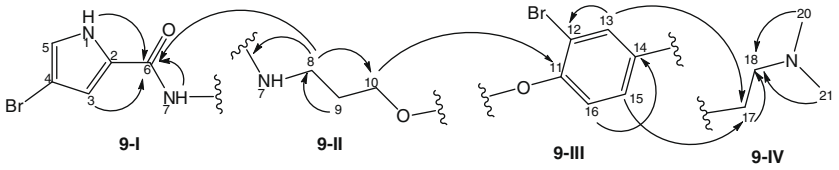

Figure 6. Substructures of **9** as proposed by ^{1}H–^{1}H COSY and HMBC correlations show the connectivity of the substructures through arrows.

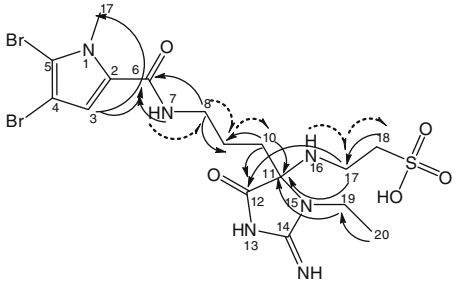

Figure 7. Important HMBC (—) and COSY (- - - -) correlations of **14** in DMSO-d_6.

confirmed by accurate mass measurement. ^{1}H and ^{13}C NMR spectra of **11** were comparable to those of compound **9**. The NMR data for positions 7–21 for both compounds were identical. The only difference to the previously described agelanesin congeners is that in **11**, only one pyrrole methine proton was observed suggesting a dibrominated pyrrole in the structure. Similar to compound **9**, C-12 quaternary carbon signal at δ_C 110.8 indicated that the tyramine unit is brominated. The 2D NMR data of compound **11** were analogous to those of agelanesins A and B. Therefore, **11** was unambiguously identified as the dibrominated pyrrole congener of **9** and assigned the trivial name agelanesin C.

Compound **12** was obtained as a white powder. It is the most lipophilic agelanesin congener identified in this study as evident by its late retention time in the RP-HPLC analysis. The presence of two bromine atoms was evident from the ESIMS pseudo-molecular ion peak cluster at *m*/*z* 598/600/602 $[M+H]^+$ with an intensity ratio of 1:2:1. The molecular formula of $C_{18}H_{23}Br_2IN_3O_2$ was confirmed by HRESIMS. NMR spectral data of **12**, presented in Table 2, are comparable to those of congeners **9**–**11** particularly to that of analogue **11**. The C-12 resonance at an extremely high field chemical shift of δ_C 86.5 was again indicative of an iodine substitution. Following the same rationale as that for derivatives **9** and **11**, **12** was identified as the dibrominated congener of **10** and the iodinated tyramine derivative of **11**. Analogue **12** was named agelanesin D.

2.3. Taurine analogues

Agelas linnaei also yielded three new sulfonic acid congeners **13**–**15** that are structurally related to mauritamide A, a bromopyrrole derivative containing a taurine methyl ester moiety that was first reported from the Fijian sponge *A. mauritiana*.[53] Taurine unit containing brominated pyrroles are quite rare in marine sponges. To date, these compounds have only been described from *A. mauritiana* (mauritamide A)[53] and two protein kinase inhibitors, tauroacidins A and B from the marine sponge *Hymeniacidon* sp.[54]

HRESIMS confirmed the molecular formula $C_{14}H_{20}Br_2N_6O_5S$ and $C_{16}H_{24}Br_2N_6O_5S$ for **13** and **14**, respectively. Their mass spectra indicated the presence of two bromine atoms in both molecules as shown by the intense pseudo-molecular ion peak cluster at *m*/*z* 543/545/547 $[M+H]^+$ for **13** and *m*/*z* 571/573/575 $[M+H]^+$ for **14** with intensity ratios of 1:2:1. Compound **13** was isolated as brown oil (88 mg), which was the major metabolite (yield 0.04% of sponge dry weight). ^{1}H and ^{13}C NMR data (see Table 3) of **13** are reminiscent of the known mauritamide A.[53] The 2D NMR data of **13** were also identical to that of the known congener. The difference of 14 mass units in the molecular weight and the concurrent lack of the methyl ester signal in the NMR spectra of **13** indicated a demethylation of the taurine methyl ester function found in mauritamide A. Compound **13** was designated the trivial name mauritamide B.

The molecular weight of **14** is 28 mass units larger than that of **13** suggesting the presence of an additional ethyl unit in its structure. Comparison of the ^{1}H and ^{13}C NMR data of **13** and **14** accordingly revealed the additional ethyl moiety in **14** as indicated by the proton signals at δ_H 3.13 (m, H-19A), 3.28 (m, H-19B) and 1.09 (t, J = 6.9 Hz, CH_3-20) as well as carbon resonances at δ_C 58.7 (CH_2-19) and 14.9 (CH_3-20). The deshielded chemical shift of the methylene unit indicated that the ethyl unit is adjacent to an oxygen atom or a tertiary amine group. The presence of this ethyl moiety was confirmed by the additional spin system observed in the ^{1}H–^{1}H COSY spectrum. Since there were no significant changes in chemical shifts observed for the taurine unit signals or in the propylamide chain, this additional ethyl chain was attached to the aminoimidazolone ring. The proposed structure was corroborated by the HMBC spectrum of **14**, which was substantiated by the $^{3}J_{C-H}$ cross peaks between methylene protons of C-19 and the quaternary carbon at position 11 (Fig. 7). Attachment of the ethyl function on N-15 was further confirmed by the COSY spectrum, which revealed that N*H*-16 is part of the taurine spin system. The HMBC data of **14** are in accordance with that of **13** and the known congener mauritamide A. Hence **14** was 2-(4-(3-(4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxamido)propyl)-3-ethyl-2-imino-5-oxoimidazo-lidin-4-yl-amino)ethanesulfonic acid and assigned the trivial name mauritamide C.

Compound **15** proved to be a further new taurine-bearing analogue. The presence of two bromine atoms in the molecule was detected from the ESIMS pseudo-molecular ion peak cluster at *m*/*z* 389/391/393 $[M+H]^+$ with an intensity ratio of 1:2.1. HRESIMS verified the molecular formula $C_8H_{11}Br_2N_2O_4S$ for compound **15**. The number of ^{1}H and ^{13}C NMR resonances was in accordance with the molecular formula. The ^{1}H and ^{13}C NMR spectra of **15** exhibited resonances for both the pyrrole and taurine moieties that were previously observed in mauritamides B (**13**) and C (**14**). In comparison to the latter analogues, signals belonging to the propylamide chain and aminoimidazolone ring were absent. The spin systems were confirmed by COSY while connectivity of the taurine unit to the brominated pyrrole ring was corroborated by the HMBC correlation of the methylene proton signals at δ_H 3.44 with the quaternary carbon at δ_H 158.8 of the keto amide function. The HMBC spectrum confirmed the proposed structure of **12** as 2-(4,5-dibromo-1-methyl-1*H*-pyrrole-2-carboxamido)ethanesulfonic acid and designated the trivial name mauritamide D.

2.4. Diterpene alkaloids

Agelasine is a diterpenoid bearing a quaternary 9′-methyladenine moiety in its molecule. To date there are at least 10 agelasine

congeners reported in the literature isolated from *Agelas* sponges.[35] Compound **16** was observed to be the major compound from the sponge specimen we investigated. It was obtained as a white amorphous substance (1.95 g, 0.78% of the sponge dry weight). The molecular formula of $C_{26}H_{41}N_5$ is compatible with the HRESIMS pseudo-molecular ion peak at *m*/*z* 422.3284 $[M]^+$. ^{13}C and ^1H NMR data were identical to that of (+)-agelasine D,[35a,55] the absolute configuration of which has been confirmed by synthetic studies.[56] The ROESY spectrum of **16** showed a similar relative stereochemistry to that found for (+)-agelasine D.[35a] An optical rotation measurement of **16** yielded a value of $[\alpha]_D^{25}$ = −19.8 ± 0.4 (*c* 0.5, MeOH), while the reported $[\alpha]_D^{25}$ value of (+) agelasine D in the literature[35a] was +10.4 (*c* 1.1, MeOH). CD spectra were measured for both compounds[55] but proved to be nonconclusive as the chromophore is not in vicinity of the chiral centers. Co-elution experiments of both compounds in a HPLC system resulted in a single peak. Considering that, both compounds differ only in the $[\alpha]_D^{25}$ value, suggested **16** was the (−)-enantiomer of (+)-agelasine D.

Similarity of the UV absorption pattern to that of **16** suggested that **17** was a related compound. ESIMS data exhibited an intense pseudo-molecular ion peak at *m*/*z* 440 $[M+H]^+$. An 18 mass unit increase in molecular weight of **17** compared to **16** suggested the presence of an additional hydroxyl group and is compatible with the molecular formula of $C_{26}H_{42}N_5O$ from HRESIMS data. Similar to that of agelasine D[35a] and compound **16**, the ^1H and ^{13}C NMR data of **17** were obtained in $CDCl_3$ and in MeOD, respectively. The NMR data of **17** is comparable to that of compound **16** (Table 4) and to that reported in the literature.[35a] Major differences were observed for protons of the adeninium moiety. The N*H*$_2$ resonance at 7.20 disappeared while the signal of H-8′ was observed upfield at δ_H 7.86 compared to 10.94 in agelasine D whereas the H-2′ at δ_H 8.42 in agelasine D was shifted to 8.05 in compound **17**. Proton NCH_3 is shifted upfield to δ_H 2.90 and appears as a doublet having a coupling constant of 4.7 Hz. Compared to agelasine D, signals for H_2-15 and H-14 were shifted upfield to δ_H 5.25 and 4.19, respectively. Comparison of the ^{13}C NMR data to agelasine D showed that C-8′ and C-6′ were the most affected resonances. C-6′ and C-8′ were shifted from 10 to 20 ppm downfield to δ_C 160 and 166 in compound **17**, respectively. The signal for the vinyl methyl group C*H*$_3$-16 also exhibited an upfield shift to δ_C 16.0.

Interestingly, tautomerism in the adeninium moiety of **17** between its amino (tautomer I) and imino (tautomer II) form was observed only in MeOH where doubling of peaks was exhibited in its HPLC chromatogram while two sets of resonances were detected in its ^1H and ^{13}C NMR spectrum. The affected protons were H_2-15 at δ 4.12 and 4.22; H-14 at δ_H 5.20 and 5.28; H-16 at δ 1.54 and 1.48; and H-8′ at δ 7.90 and 8.20 for tautomers I and II, respectively. However, using $CDCl_3$ as a solvent which has an acidic pH, the duplication of the signals disappeared but protonation occurred at N-9′.

The COSY spectrum of **17** in $CDCl_3$ revealed a similar spin system as in **16** for the diterpene unit which was further connected to the adenine moiety through allylic coupling of H-8′ with C*H*$_2$-15. Due

Table 4
NMR data of compounds **16** and **17**

No.	**16**			**17**		
	^1H NMR[a]		^{13}C NMR[b]	^1H NMR[a]		^{13}C NMR[b]
	δ	Integration, multiplicity, *J* in Hz	δ, DEPT	δ	Integration, multiplicity, *J* in Hz	δ, DEPT
1	0.87	2H, m	39.4, CH_2	0.84	2H, m	40.7, CH_2
2	1.42	2H, m	20.4, CH_2	1.10	2H, m	20.4, CH_2
3A	1.34	1H, br d, 12.9	43.3, CH_2	1.34	1H, br d, 12.9	43.3, CH_2
3B	1.22	1H, dd, 4.1, 12.6		1.10	1H, dd, 4.1, 12.6	
5	0.96	1H, m	57.4, CH	1.00	1H, dd, 12.0, 1.6	56.8, CH
6	1.68	2H, m	25.6, CH_2	1.25	2H, m	25.6, CH_2
7A	2.31	1H, dq, 2.5, 12.6	39.4, CH_2	2.31	1H, br d, 12.6	39.4, CH_2
7B	1.84	1H, dd, 5.0, 12.9		1.87	1H, dt, 4.7, 12.9	
9	1.49	1H, br d, 11.3	56.9, CH	1.52	1H, m	58.3, CH
11A	1.66	1H, m	40.3, CH_2	1.65	1H, m	41.6, CH_2
11B	1.10	1H, m		1.45	1H, m	
12A	2.16	1H, m	22.5, CH_2	2.04	1H, m	25.6, CH_2
12B	1.90	1H, m		1.92	1H, m	
4			40.7, C			40.2, C
8			149.5, C			149.8, C
10			34.5, C			34.5, C
13			145.7, C			145.4/144.4, C
14	5.40	1H, t, 7.2	115.7, CH	5.30	2H, t, 6.6	117.9/118.4, CH
15	5.20	2H, d, 7.2	48.6, CH_2	4.12	1H, d, 6.6	45.9/41.6, CH_2
16	1.77	3H, s	17.0, CH_3	1.53	3H, d, 2.84	16.0/16.2, CH_3
17A	4.75	1H, s	107.0, CH_2	4.78	1H, s	107.0, CH_2
17B	4.40	1H, s		4.38	1H, s	
18	0.83	4H, s	22.1, CH_3	0.81	3H, s	34.1, CH_3
19	0.75	3H, s	34.0, CH_3	0.73	3H, s	20.4, CH_3
20	0.60	3H, s	15.0, CH_3	0.59	3H, s	15.1, CH_3
1′-N*H*				4.95	1H, br s[c]	
2′	8.42	1H, s	157.1, CH	8.05	1H, s	157.7/157.3, CH
4′			150.9, C			161.5/160.6, C
5′			111.2, C			97.2/99.2, C
6′-NH_2	7.20	2H, br s[c]	154.1, C			
6′-NOH				4.95	1H, br s[c]	162.0/160.6, C
8′	10.94	1H, s	142.0, CH	7.95	1H, s	165.9/166.5, CH
9′-CH_3	4.05	3H, s	32.0, CH_3	2.90	3H, d, 4.7	28.2, CH_3
9′-N^+H				4.99	1H, d, 4.7	

[a] ^1H NMR data was obtained in $CDCl_3$ at 500 MHz.
[b] ^{13}C NMR data was obtained in CD_3OD at 125 MHz.
[c] Signals are interchangeable.

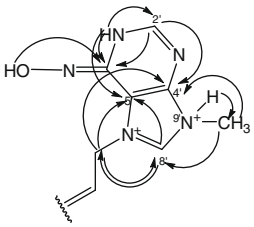

Figure 8. Important HMBC correlations observed in the adeninium moiety of **17** in $CDCl_3$.

to protonation of N-9′ in $CDCl_3$, an exchangeable doublet resonance at δ_H 4.99 (d, J = 4.7 Hz) coupled to the CH_3 proton doublet at δ_H 2.90. In the HMBC spectrum of **17**, another exchangeable signal integrating for two protons was observed at δ_H 4.95 which could be assigned to NH and oxime protons at positions 1 and 6, correlated with C-2′, C-5′, and C-6′, respectively (Fig. 8). A cross peak between the NCH_3 proton and C-8′ in its HMBC spectrum indicated the methyl group was still attached to N-9′ as found in **17**.

The ROESY spectrum of **17** exhibited identical NOE correlations to those found in **1**. Its optical rotation was found to be $[\alpha]_D^{25} = -6.3 \pm 0.6$ (c 0.5, MeOH) and suggested that the relative configuration of **17** is similar to **16**. Compound **17** was elucidated as the oxime derivative of (−)-agelasine D which we named ageloxime D.

3. Discussion and results

Pyrrole imidazole alkaloids are exclusively found in marine sponges, mainly of the families Agelasidae, Axinellidae, and Halichondridae. The underlying $C_{11}N_5$ building block consists of a pyrrolyl-2-carbonyl unit connected via an amide linkage to a 2-amino-5-(3-amino)propylimidazole partial structure.[19a,57] The pyrrole-2-carbonyl unit can be non-, mono-, or dibrominated in the 4- and 5-positions but bromination of the pyrrole 3-position or the imidazole moiety has not been encountered.[58] In some metabolites, the linear chain is cyclised to form an AB core of a pyrrolopyrazinone bicyclic system such as in dibromophakellin, agelastatin A, pala'uamine, longamide A, and cyclooroidin.[59] Although pyrrole imidazole alkaloids are important for chemotaxonomic considerations,[19b] the biosynthetic pathway for these metabolites remains unclear.[60]

Sixteen different brominated pyrrole derivatives (compounds **1**–**16**) were obtained from *A. linnaei*, whereas only five congeners were obtained from *A. nakamurai* (compounds **19** and **21**–**24**). Twelve of the isolated compounds are new pyrrole imidazole derivatives. Interestingly, the *A. nakamurai* secondary metabolites are all derived from monobrominated pyrrole, while in most of the *A. linnaei* metabolites the pyrrole rings are dibrominated with the exception of agelanesin A (**9**), agelanesin B (**10**), and agelongine (**2**) which are monobrominated pyrroles. Another difference observed is that all metabolites of *A. nakamurai* found in this study have a free NH pyrrole while in most of the *A. linnaei* metabolites the NH pyrrole is methylated. A clear example is provided by the presence of 4-bromo-1H-pyrrole-2-carboxylic acid (**23**) in *A. nakamurai*, and its dibrominated *N*-methyl pyrrole congener (**3a**) in *A. linnaei* Furthermore, an analogue of the new *A. linnaei* metabolite, 4-(4,5-dibromo-1-methyl-1H-pyrrole-2-carboxamido)-butanoic acid (**6**) was previously reported from *A. nakamurai* as 4-bromo-2-carboxamido-butanoic acid.[61]

The pyrrole imidazole compounds in *A. linnaei* are highly diverse. Some new functionalities are evident. Agelanin B (**8**), a new midpacamide related compound, displays a very unusual open ring system of 3-hydroxyl-4-oxopentanoic acid to replace the hydantoin ring. Moreover, a novel mode of intramolecular cyclisation found in agelanin A (**7**) introduces a unique functionality in which the propylamide chain is cyclised to form a dihydroimidazopyridinol ring. Despite its very rare mode of cyclisation, a related structure was previously reported as dibromoagelaspongin, a phakellin related compound isolated from Tanzanian *Agelas* sponge species.[39a] The phakellins, one of the unique members of pyrrole imidazole metabolites (**4** and **5**), exhibit a unique array of functionalities including a cyclic guanidine, a pyrrole carboxylic acid, a pyrrolidine, and a congener with vicinal diaminal stereocenters.[62] Oroidin is structurally related to (−)-dibromophakellin by a complex cyclisation of oroidin that formally connects the pyrrole nitrogen atom (N-1) to the unalkylated aminoimidazole carbon (C-12) and the amino nitrogen atom (N-7). Whereas in dibromohydroxyphakellin (**5**) and dibromophakellin HCl (**4**), the linear chain is cyclised to form a pyrrolopyrazinone ring, in agelanesins, the imidazole ring is replaced with a halogenated tyramine. New taurine related compounds were also encountered in two mauritamide A congeners, mauritamide B (**13**) and C (**14**), along with a quite simple compound 2-(4,5-dibromo-1-methyl-1H-pyrrole-2-carboxamido)ethanesulfonic acid (**15**). Moreover, a co-occurrence of the known serotonergic agent, agelongine (**2**) with its pyridinium ring in the structure to replace the imidazole nucleus and an ester linkage to take the place of the amidic bond[39b] was also isolated from this *A. linnaei*.

Four brominated pyrrole derivatives (**9**–**12**) connected to a halogenated tyramine unit were obtained from *A. linnaei*. Interestingly, halogen-bearing tyrosine-derived metabolites are more likely to be found in Verongid sponges, although they were also reported from several other marine sponges.[26,27] It is challenging to find out why this *Agelas* sponge incorporated iodine into the agelanesins instead of bromine. This may be due to the iodide present in seawater, which is far below other halogens such as bromide and chloride. Despite its low concentration, unlike chloride, all known haloperoxidases are effective in oxidizing iodide.[50] Biosynthesis of iodinated metabolites seems to be related to the capability of organisms to concentrate iodine from seawater, rather than to the presence of a specific peroxidase.[27] This has been proposed, as most iodo-metabolites have been isolated from red algae, which are known to contain iodine concentrations as high as 0.5% of their weight.[27] Interestingly, one iodotyrosine alkaloids sponge producer, *Iotrochota birotulata* was reported to contain significant amounts of iodine (0.12–1.21%),[63] together with comparable quantities of bromine (0.16–2.66%).[64] Hence, this supports the association of the presence of iodo-metabolites with high iodine amounts in the sponge tissue.[27] Another interesting query arising from the agelanesins unique structure is their biosynthesis. Pyrrole imidazole compounds such as oroidin and sceptrin are predicted to be produced by sponge cells and not by the associated bacteria.[65] Subsequently it was proposed that brominated tyrosine metabolites might actually be derived from the biosynthetic pathway of microorganisms living in association with sponges[66] because these kinds of metabolites, such as psammaplins, have been isolated from a diverse range of sponge families and that brominated aromatic amino acid derivatives are common metabolites of marine bacteria.[66] This finding suggested the brominated pyrrole 2-carboxylic acids are produced by the sponge cell and associated bacteria that then provide halogenated tyrosine units for agelanesins biosynthesis.

In addition to its unique chemical structure, this group of compounds also shows interesting bioactivity. All alkaloids isolated in this study were assayed for cytotoxicity against the murine L1578Y mouse lymphoma cell line. From the bromopyrrole alkaloids isolated (**1**–**15**, **18**, **19**, and **21**–**23**) only the agelanesins (**9**–**12**) showed prominent activity while others were only moderately active or inactive (Table 5). IC_{50} values of the agelanesins were 9.55 (**9**), 9.25 (**10**), 16.76 (**11**), and 13.06 μM (**12**), respectively. Compounds **9** and **10** exhibited the lowest IC_{50} values. This implied that cytotoxicity of the agelanesins is related to the degree of bromina-

Table 5
Bioactivity of the isolated compounds from *Agelas linnaei* and *Agelas nakamurai*

Compound	Name of compounds	Sponge source	Cytoxicity assay against L5178Y		Antimicrobial assay against *S. epidermidis*		
			Growth in %	IC_{50} (μM)	MIC (μM)	Growth inhibition	Biofilm inhibition
1	Midpacamide	*Agelas linnaei*	46.7	n.t.	>46	n.i.	n.i.
2	Agelongine	*Agelas linnaei*	99.1	n.t.	>59	n.i.	n.i.
3a	Methyl-4,5-dibromocarboxylic acid	*Agelas linnaei*	87.8	n.t.	>71	n.i.	n.i.
3b	Methyl-4,5-dibromocarboxylic acid methyl ester	*Agelas linnaei*	83.4	n.t.	>67	n.i.	n.i.
4a	Dibromphakellin HCl	*Agelas linnaei*	108.8	n.t.	>47	n.i.	n.i.
4b	Dibromphakellin	*Agelas linnaei*	83.0	n.t.	>51	n.i.	n.i.
5	Dibromohydroxyphakellin HCl	*Agelas linnaei*	112.8	n.t.	>45	n.i.	n.i.
6	4-(4,5-Dibromo-1-methyl-pyrrole-2-carboxamido)-butanoic acid	*Agelas linnaei*	107.5	n.t.	>54	n.i.	n.i.
7	Agelanin A	*Agelas linnaei*	98.9	n.t.	>48	n.i.	n.i.
8	Agelanin B	*Agelas linnaei*	92.8	n.t.	>44	n.i.	n.i.
9	Agelanesin A	*Agelas linnaei*	−0.5	9.55	>42	n.i.	n.i.
10	Agelanesin B	*Agelas linnaei*	2.3	9.25	>38	n.i.	n.i.
11	Agelanesin C	*Agelas linnaei*	0.8	16.76	>36	n.i.	n.i.
12	Agelanesin D	*Agelas linnaei*	−2.3	13.06	>33	n.i.	n.i.
13	Mauritamide B	*Agelas linnaei*	92.5	n.t.	>37	n.i.	n.i.
14	Mauritamide C	*Agelas linnaei*	98.6	n.t.	>35	n.i.	n.i.
15	Mauritamide D	*Agelas linnaei*	117.6	n.t.	>52	n.i.	n.i.
16	Agelasine D	*Agelas nakamurai*	1.9	4.03	<0.0877	n.i.	n.i.
17	Ageloxime-D	*Agelas nakamurai*	4.2	12.5	>45	No	Yes
19	Mukanadin-C	*Agelas nakamurai*	78.5	>43.2	>87	n.i.	n.i.
20	Agelasidine C	*Agelas nakamurai*	37.4	n.t.	5.9	n.i.	n.i.

n.i., no inhibition; n.t., not tested.

tion of the pyrrole ring. Increase in bromination decreases the activity as observed for **11** and **12** compared to **9** and **10**. While the presence of an iodide substituent on the tyramine moiety only causes small differences in activity as **9** and **11** have similar activity compared to **10** and **12**.

The relatively high structure diversity and predominance of the pyrrole imidazole alkaloids in *A. linnaei* may be due to their function in chemical defence for the sponge in order to provide a broader spectrum of protection. *A. linnaei* afforded midpacamide as its major metabolite, which was previously reported as a strong feeding deterrent chemical.[19a] However, it displays weak cytotoxicity against the mouse lymphoma cell L5178Y (46.7% inhibition in 23 mM sample concentration) and weak antibacterial activity against *Bacillus subtilis*. The role of cytotoxicity is presumably taken by other metabolites such as the agelanesins.

In contrast to *A. linnaei*, *A. nakamurai* afforded diterpene alkaloids (**16**, **17**, and **20**) as its major metabolites while pyrrole imidazole alkaloids (**18**, **19**, **22**, and **23**) were only minor natural products. Beside the oroidin family of compounds, sponges of the genus *Agelas* also produce unique adenine and hypotaurocyamine terpenoids.[19b] Unlike the oroidin derivatives, *Agelas* terpenoids have not been reported from other sponge families but, as they were not found in every *Agelas* species, they cannot be used as a chemotaxomic marker.[19b] The same phenomenon was also observed in the two *Agelas* sponges examined in this study. Together with the brominated pyrrole derivatives, *A. nakamurai* produced two new adenine diterpenoid derivatives and one diterpene bearing a hypotaurocyamine functionality.

(+)-Agelasine D was previously reported to display powerful inhibitory effect on Na^+, K^+-ATPase as well as antimicrobial activity.[35a] It was also reported to show prominent cytotoxicity against several cancer cells including multidrug-resistant cell lines and also antibacterial activity against *M. tuberculosis* as well as both aerobes and anaerobes Gram-positive and Gram-negative bacteria.[38a] The diterpenoid alkaloids (−)-agelasine D (**16**) and (−)-ageloxime D (**17**) showed IC_{50} value of 4.03 and 12.5 μM in the cytotoxicity assay, respectively, indicating that presence of the oxime function decreases activity towards lymphoma cells.

In addition to evaluating their cytotoxicity all alkaloids isolated in this study were also assayed for anti-fouling activity against larvae of *Balanus improvisus* which are an established model organism for anti-fouling bioassays.[32] Only the diterpenoid alkaloids showed significant activity in the Balanus biassay. Both compounds inhibited settling in a dose dependent manner but proved to be toxic to the nauplii (Figs. 9a and 9b). Anti-fouling assay on the cyprids larvae of *Balanus improvisus* showed that both compounds were toxic to the larvae rather than just inhibiting settlement (Figs. 9a and 9b), where **17** was about 10 times more toxic to the larvae than its congener **16** indicating the importance of the oxime group for anti-fouling activity of the diterpene alkaloids. Similar results had previously been obtained for bromotyrosine alkaloids of the bastadine type. Only bastadin derivatives featuring an oxime function inhibited settling of *Balanus improvisus* larvae whereas replacement of the oxime group against an amino function resulted in a complete loss of anti-fouling activity.[32]

Compounds **16** and **17** were also assayed for biofilm inhibition against *Staphylococcus epidermidis*. Biofilms are formed by colonization on solid supports (bone, implants and catheters) by adher-

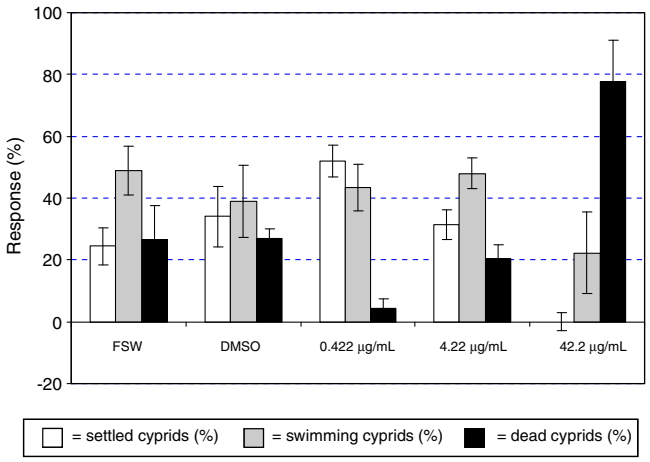

Figure 9a. Result of anti-fouling assay of **16** at concentrations between 100 (42.2 μg/mL) and 0.10 μM (0.422 μg/mL) (data are presented as means percentages ± SE (n = 4); FSE, filtered seawater),

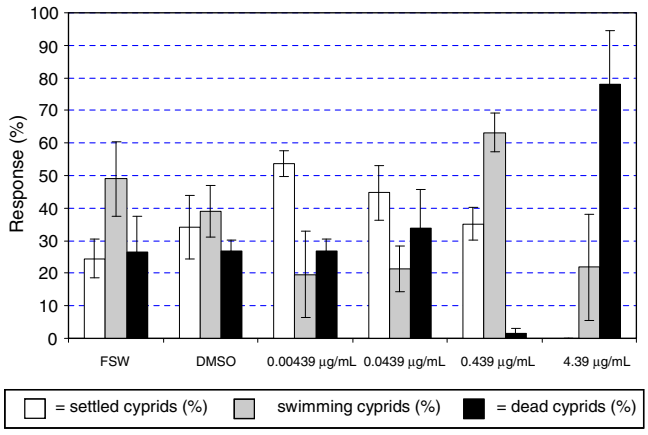

Figure 9b. Result of anti-fouling assay of **17** at concentrations between 100 (42.2 µg/mL) and 0.10 µM (0.422 µg/mL) (data are presented as means percentages ± SE (n = 4); FSE, filtered seawater).

ent bacteria.[10] The ability to form a biofilm on a plastic surface (e.g., catheters) requires at least two properties, the adherence of cells to a surface and accumulation to form multilayered cellular clusters.[67] The second phase of biofilm formation requires the polysaccharide intercellular adhesion (PIA) located at the cell surface,[68] in which the cells are embedded and protected against the host's immune defence and antibiotic treatment.[67] Compound **16** showed antibacterial active against the planktonic form of *S. epidermidis* (MIC < 0.0877 µM) but did not inhibit biofilm formation. On the contrary, **17** which differs from **16** only in the substituent on C-6′, showed biofilm formation inhibition but did not inhibit the growth of the planktonic bacteria. Therefore it can be concluded that the oxime substituent on C-6′ is important for the activity.

It is worth noting that the presence of biofilm inhibitors such as ageloxime D (**17**) and antibacterial agents such as agelasine D (**16**), which are both toxic to the cyprids larvae of *Balanus improvisus* may play an important role in the sponge's chemical defence against biofouling. On the other hand, the brominated pyrroles of *A. nakamurai* such as hymenidin (**21**), which are not active either against the barnacles or against the biofilm formation, have been previously reported as feeding deterrents against fishes[19a] which are important predators of marine invertebrates. It is possible that some of the other bromopyrrole alkaloids isolated in this study are also feeding deterrents and protect *Agelas* sponges against feeding by fishes even though this aspect was not investigated further by us.

Following the ecological role of (−)-agelasine D and (−)-ageloxime D as part of *A. nakamurai* chemical defences, it can be suggested that employment of adenine-related diterpene alkaloids in combination may serve as one promising strategy against biofilm-associated bacteria such as *S. epidermidis*. While (−)-ageloxime D prevent bacteria from forming the biofilm, (−)-agelasine D can inhibit the growth of the nonadherent bacteria.

4. Conclusion

Investigation of two Indonesian *Agelas* sponges that exhibited brominated pyrroles in their chemical profiles, showed a predominance of metabolites belonging to diverse chemical groups along with the occurrence of brominated pyrroles. These new metabolites displayed specificity and selectivity in their biological activity as well as discriminating anti-fouling activity from cytotoxicity. This corroborates the 'job description' of each of the metabolites in the respective sponges.

5. Experimental

5.1. General experimental procedure

Optical rotations were measured on a Perkin-Elmer Model 341 LC polarimeter. ^{1}H and ^{13}C NMR experiments were performed on Bruker AVANCE DRX500 and AVANCE DMX600 NMR spectrometers using $CDCl_3$, MeOD, and DMSO-d_6 as solvent. ESI mass spectra were obtained on a ThermoFinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. HRESIMS were determined on a Micromass Q-Tof 2 mass spectrometer. Column chromatography was performed on silica gel (0.040–0.063 mm; Merck, Darmstad, Germany) or by Sephadex LH20. Low pressure column chromatography was performed on LiChroprep® Si 60 (40–63 µm) size A (240-10) and B (310-25), and LiChroprep® RP-18 (40–63 µm) size A (240-10) and B (310-25) (Merck, Darmstadt, Germany) equipped with pulsatic pump (Duramat, Heidelberg, Germany). For HPLC analysis, samples were injected into an HPLC system equipped with a photodiode array detector (Dionex, München, Germany). Routine detection was at 235, 254, and 340 nm. The separation column (125 × 4 mm i.d.) was prefilled with Eurospher 100-C18, 5 µm (Knauer, Berlin, Germany). Separation was achieved by applying a linear gradient from 90% H_2O (pH 2.0) to 100% MeOH over 40 min. TLC analysis was carried out using aluminium sheet precoated silica gel 60 F254 and on glass precoated RP-18 F254 (Merck, Darmstadt, Germany).

5.2. Animal material

The first *Agelas* sponge investigated in this paper was externally bright orange to cream-orange internally. Its surface texture was very soft and spongy. The sponge was collected in September 2005 by N.J. de Voogd near Peniki E Island, Seribu Islands (also known as Thousands Islands), in Northwest-Java, Indonesia at 15 m by using SCUBA. A specimen was deposited in the sponge collection of the National Museum of Natural History (RMNH Porifera) under Reg. No. RMNH POR. 2109 and was described as *A. linnaei* spec. nov.[69] The new species differs from other *Agelas* species by the overall morphology and the size of the verticillated acanthostyles. Surprisingly, this is only the second *Agelas* species ever described from Indonesia. The species is named to honour of Carolus Linnaeus, or Carl von Linné, to celebrate 250 years of binomial nomenclature.

The second *Agelas* specimen investigated in this study was also a bright orange sponge collected by Susilo Hadi (Department of Zoology, Faculty of Biology, GMU, Indonesia) at a depth of 12 m off the coast of Menjangan Island (North of Bali Island), Indonesia on October 2003. A voucher specimen has been deposited in the Zoological Museum Amsterdam under Reg. No. ZMAPOR18297. The sponge was taxonomically described as *A. nakamurai* (van Soest, 2002). Both sponges were immediately immersed in ethanol after collection and transported to Heinrich-Heine-University Düsseldorf, Germany for further isolation work.

5.3. Extraction and isolation

Lyophilized sponge tissue (198 g) of the *A. linnaei* was ground and extracted exhaustively with acetone followed by methanol. After removing solvent under reduced pressure, the resulting methanol extract was partitioned between n-hexane and 90% MeOH–water to yield the hexane fraction. The residue was partitioned further between ethyl acetate and H_2O to obtain the ethyl acetate fraction (4.3 g). The water phase was partitioned against n-BuOH to yield the butanol (3.5 g) and the water fractions.

The ethyl acetate fraction was subjected to silica gel G60 vacuum liquid chromatography (VLC) using mobile phase with

increasing polarity from 100% *n*-hexane to 100% ethyl acetate followed by 100% dichloromethane to 100% MeOH. This method yielded 21 fractions. Fraction 12 afforded 2.0 g of midpacamide (**1**, 1.01%) as the major metabolite. Other VLC fractions were further subjected to chromatographic separations. Fraction 10 was purified by using a silica gel column G60 (DCM–MeOH, 95:5) to obtain **6** (190 mg, 0.10%). Fraction 14 was chromatographed over a LoBar® silica G60 column (DCM–MeOH, 95:5) and further purified by using Sephadex column LH20 (MeOH) to yield the new agelanin A (**7**, 8 mg, 0.008%). Agelanin B (**8**, 25 mg, 0.026%) was obtained through purification of fraction 17 with LoBar® silica G60 (DCM–MeOH, 98:2) followed by Sephadex LH20 with MeOH as eluent. Fraction 16 was separated with a LoBar® Silica G60 column (DCM–MeOH, 95:5) to afford the known compound **3b** (21 mg, 0.011%). Sub-fraction 8 from fraction 16 was purified further over a LoBar® RP-18 column (MeOH–H_2O, 3:7) to afford the known compound dibromophakellin HCl (**4**, 17 mg, 0.008%), as well as its new hydroxyl analogue (**5**, 47 mg, 0.024%), and the new halogenated tyrosine congeners, agelanesin A (**9**, 20 mg, 0.01%), agelanesin B (**10**, 24 mg, 0.012%), agelanesin C (**11**, 6 mg, 0.003%), and agelanesin D (**12**, 7 mg, 0.003%).

Agelongine (**2**), the new mauritamide congeners, B (**13**), C (**14**), and D (**15**) were isolated from the butanol fraction (3.5 g). Sephadex LH20 was used to purify the butanol fraction resulting in 12 fractions. Fraction 11 is identified as **15** (6 mg, 0.003%), while fraction 1was chromatographed further over LoBar® RP-18 (MeOH–H_2O, 4:6) to give the known agelongine (**2**, 20 mg, 0.01%), mauritamide B (**13**, 88 mg, 0.04%) and mauritamide C (**14**, 33 mg, 0.016%). Acetone extract (6.7 g) was chromatographed over a Sephadex LH20 column (MeOH) to yield compound **3a** (100 mg, 0.051%).

Compound **5**: Obtained as a crystalline residue; $[\alpha]_D^{25} = -6.6 \pm 0.7$ (*c* 0.23, MeOH); LRESIMS (+ mode): 404/406/408 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{11}H_{12}Br_2N_5O_2$ ($[M+H]^+$): 403.9352; found: 403.9360; UV λ_{max} (MeOH) 241.3, 292.6 nm. ^1H and ^{13}C NMR data in DMSO-d_6, see Table 1.

Compound **6**: Obtained as brown oil; LRESIMS (+ mode): 367/369/371 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{10}H_{13}Br_2N_2O_3$ ($[M+H]^+$): 366.9293; found: 366.930; UV λ_{max} (MeOH) 202, 277 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 1. *Compound* **7**. Obtained as brown amorphous substance; LRESIMS (+ mode): 416/418/420 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{12}H_{12}Br_2N_5O_2$ ($[M+H]^+$): 415.9358; found: 415.9370; UV λ_{max} (MeOH) 210.4, 246.9, 342.9 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 1.

Compound **8**: Obtained as dark brown oil; $[\alpha]_D^{20} = -18.7 \pm 2.8$ (*c* 0.06, MeOH); LRESIMS (+ mode): 453/455/457 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{14}H_{19}Br_2N_2O_5$ ($[M+H]^+$): 452.9661; found: 452.9680; UV λ_{max} (MeOH) 208.8, 215.5, 275.6 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 1.

Compound **9**: Obtained as yellow oil; LRESIMS (+ mode): 472/474/476 $[M+H]^+$ (intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{18}H_{23}Br_2N_3O_2$ ($[M+H]^+$): 472.0230; found: 472.0250; UV λ_{max} (MeOH) 206, 271 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 2.

Compound **10**: Obtained as yellow oil; LRESIMS (+ mode): 519/521 ($[M+H]^+$, intensity 1:1); HRESIMS (+ mode): calculated for $C_{10}H_{13}BrIN_2O_3$ ($[M+H]^+$): 520.0091; found: 520.0097; UV λ_{max} (MeOH) 208, 271 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 2.

Compound **11**: Obtained as yellow oil; LRESIMS (+ mode): 550/552/554/556 ($[M+H]^+$, intensity 1:2:2:1); HRESIMS (+ mode): calculated for $C_{18}H_{23}Br_3N_3O_2$ ($[M+H]^+$): 549.9335; found: 549.9350; UV λ_{max} (MeOH) 209, 278 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 2.

Compound **12**: Obtained as white amorphous residue; LRESIMS (+ mode): 598/600/602 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{18}H_{23}Br_2IN_3O_2$ ($[M+H]^+$): 597.9202; found: 597.9210; UV λ_{max} (MeOH) 208, 271 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 2.

Compound **13**: Obtained as brown oil; $[\alpha]_D^{20} = -5.5 \pm 0.8$ (*c* 0.57, MeOH); LRESIMS (+ mode): 543/545/547 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{14}H_{21}Br_2N_6O_5S$ ($[M+H]^+$): 542.9661; found: 542.9660; UV λ_{max} (MeOH) 225, 250, 274 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 3.

Compound **14**: Obtained as brown oil; $[\alpha]_D^{20} = -2.1 \pm 0.4$ (*c* 0.59, MeOH); LRESIMS (+ mode): 571/573/575 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_{16}H_{25}Br_2N_6O_5S$ ($[M+H]^+$): 570.9974; found: 570.9980; UV λ_{max} (MeOH) 212, 252 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 3.

Compound **15**: Obtained as brown oil; LRESIMS (+ mode): 389/391/393 ($[M+H]^+$, intensity 1:2:1); HRESIMS (+ mode): calculated for $C_8H_{11}Br_2N_2O_4S$ ($[M+H]^+$): 388.8806; found: 388.8820; UV λ_{max} (MeOH), 237, 278 nm; ^1H and ^{13}C NMR data in DMSO-d_6, see Table 3.

Sponge tissue of *A. nakamurai* was separated from the ethanolic supernatant and dried at room temperature. Dried sponge tissue (250.7 g) was ground and extracted exhaustively with methanol. After removing the solvent under reduced pressure, the resulting extract was combined with the ethanolic extract to yield a total weight of 37.9 g. Total crude extract was solvent partitioned between *n*-hexane and 90% MeOH–water to obtain the hexane fraction (516 mg). The aqueous methanolic residue was partitioned further between ethyl acetate and H_2O to obtain the ethyl acetate fraction (3.8 g). The resulting water phase was partitioned against *n*-BuOH to afford the butanol extract (12.6 g).

The ethyl acetate fraction (3 g) was subjected to a silica gel G60 vacuum liquid chromatography (VLC) by using eluent with increasing polarity from 100% *n*-hexane to 100% ethyl acetate followed by 100% dichloromethane to 100% MeOH, resulting in 21 fractions. Fraction 3 was chromatographed over a Sephadex LH20 column by using MeOH as eluent resulting in 12 sub-fractions. Sub-fraction 3.4 was subjected to semi preparative HPLC RP18 using gradient composition of methanol–water as mobile phase to give two new compounds, ageloxime D (**17**, 110 mg, 0.044%) and longamide C (**18**, 1.13 mg, 4×10^{-4}%). The program used for each injection is as follows, 0–3 min: MeOH 80%; 3–5 min: 80–100% MeOH; 5–13 min: MeOH 100%; 13–15 min: MeOH 80%. Sub-fraction 3.5 was later shown to contain 4-bromo-1*H*-pyrrole-2-carboxylic acid (**22**). Fraction 4 was subjected to a Sephadex LH20 column using MeOH as eluent to yield sub-fraction 4.3. This fraction was then purified using a silica gel G60 column with a dichloromethane and methanol solvent mixture in the ratio 95:5, yielding (−)-agelasine D (**16**, 1.95 g, 0.77%) and (+)-agelasidine C (**20**, 67 mg, 0.027%). Fraction 6 was purified over Sephadex LH20 using methanol to give hymenidin (**21**, 41 mg, 0.016%), and 4-bromo-1*H*-pyrrole-2-carboxamide (**23**). Compound **19** (mukanadin C, 11.8 mg, 0.004%) was obtained by chromatography of the of ethyl acetate fraction over a silica gel G60 column using dichloromethane and methanol (95:5) as eluent.

Compound **16**: Obtained as white amorphous powder; $[\alpha]_D^{25} = -19.8 \pm 0.4$ (*c* 0.5, MeOH); LRESIMS (+ mode): 422 $[M]^+$; HRESIMS (+ mode): calculated for $C_{26}H_{41}N_5$ ($[M]^+$): 422.3280; found: 422.3284; UV λ_{max} (MeOH) 208.5, 225.5, 269.7 nm; ^1H and ^{13}C NMR data, see Table 4.

Compound **17**: Obtained as yellow-brownish oily substance; $[\alpha]_D^{25} = -6.3 \pm 0.6$ (*c* 0.5, MeOH); LREIMS (+ mode): 440 $[M+]^+$; HRESIMS (+ mode): calculated for $C_{26}H_{42}N_5O$ ($[M]^+$): 440.3389; found: 440.3390; UV λ_{max} (MeOH) 214.2, 271.7 nm; ^1H and ^{13}C NMR data, see Table 4.

Compound **18**: Obtained as white amorphous residue; $[\alpha]_D^{25} = -1.0 \pm 0.4$ (*c* 0.1, MeOH); LREIMS (+ mode): 301:303 ($[M+H]^+$, intensity 1:1); HRESIMS (+ mode): calculated for $C_{11}H_{14}BrN_2O_3$ ($[M+H]^+$): 301.0188; found: 301.0180; UV λ_{max} (MeOH) 202.1,

234.8, 281.1 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.10 (3H, s, NCH_3), 2.80 (dd, J = 3.2, 7.3 Hz, H-10), 3.42 (1H, dd, J = 3.2, 12.9 Hz, H-8B), 3.72 (3H, s, OCH_3), 3.95 (1H, dd, J = 4.2, 12.9 Hz, H-8A), 4.64 (1H, m, H-9), 6.79 (1H, br s, H-5), 6.89 (1H, br s Hz, H-3).

5.4. Cytotoxicity assay

The cytotoxicity against L5178Y mouse lymphoma cells was determined using the microculture tetrazolium (MTT) assay and compared to that of untreated controls.[70] Stock solutions of test samples were prepared in EtOH 96% (v/v). Exponentially growing cells were harvested, counted, and diluted appropriately. Fifty microliters cell suspension containing 3750 cells was pipetted into 96-well microtiter plates. Subsequently, 50 μL of the test samples solution containing the appropriate concentration were added to each well. The concentration range was 3–10 μg/mL. The small amount of EtOH present in the wells did not affect the experiments. The test plates were incubated at 37 °C with 5% CO_2 for 72 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate-buffered saline (PBS: 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4), and from this solution, 20 μL was pipetted into each well. The yellow MTT penetrates healthy living cells, and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3 h 45 min at 37 °C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min, 20 °C, 210g) with 200 μL of DMSO, and the cells were lysed to liberate to formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiter-well-spectrometer. The colour intensity is correlated with the number of healthy living cells.

All experiments were carried out in triplicate and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

5.5. Biofilm inhibition assay

Biofilm inhibition was investigated using an adherent-assay in a polystyrene microtiter culture plate. The culture was diluted with fresh TSB (Tryptone Soya Broth) in a ratio of 1:99 (20 μL of culture + 1980 μL of medium). Every 200 μL of this suspension was pipetted into each of the wells of a 96-well tissue culture plate where every strain was added eightfold. *S. epidermidis* RP62A (wild type) serves as a positive control while *S. carnosus* TM 300 was used as a negative control. The plates were incubated at 37 °C for 18 h. Then the cultural vessels were carefully emptied. The plates were washed three times with PBS buffer. The adhering bacteria were then fixed by heating at approx. 60 °C and coloured with crystal violet for 5 min.[71] The excess colouring material was washed off with water. After drying, the density of the biofilm was determined with an ELISA reader at a wavelength of 490 nm. Absorbance values less than 0.120 were classified as negative, values between 0.120 and 0.240 as weak adherence and results greater than 0.240 as strong adherence. The value 0.120 is equivalent to the triple average value of the negative control.

5.6. Anti-fouling activity

The experiment for evaluating the effects of the substances on settlement and mortality was run using polystyrene petri dishes (diameter 48 mm) to which 10 mL of the substances dissolved in different concentrations in filtered seawater (0.2 μm) with 0.1% DMSO were added. Competent cyprids (16 ± 2 individuals) were added to each dish run in four replicates. Dishes containing filtered seawater and dishes containing filtered seawater with 0.1% DMSO served as controls. Dishes were maintained for 3–4 days at room temperature and then examined under a stereomicroscope for attached and metamorphosed individuals as well as for dead cyprids. The substances were tested at concentrations of 0.10, 1.0, 10.0, and 100 μM dissolved in 0.10% DMSO. A solvent control with 0.10% DMSO was run parallel to the sample.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.028.

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