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New bioactive alkaloids from the marine sponge *Stylissa* sp.

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ABSTRACT

Chemical investigation of the Indonesian marine sponge *Stylissa* species, which was collected at 2008 from Derawan Islands, Berau, NE Kalimantan, Indonesia offered four new brominated alkaloids, including 12-*N*-methyl stevensine (**1**), 12-*N*-methyl-2-debromostevensine (**2**), 3-debromolatonduine B methyl ester (**3**), 3-debromolatonduine A (**4**) together with eight known alkaloids identified as *Z*-hymenialdisine, *Z*-debromohymenialdisine, Stevensine, 2-debromostevensine, 3-bromoaldizine, 3,4-dibromopyrrole-2-carbamide, latonduine A, and latonduine B methyl ester (**5**–**12**), respectively. The structures of all isolated compounds were unambiguously elucidated based on extensive 1D and 2D NMR spectroscopy and HRMS analysis as well as comparison with those reported in literature. All isolated compounds were tested for their cytotoxicity against mouse lymphoma cell line L5187Y. The results showed that only **1**, **5**, **6**, and **11** showed significant in vitro activity with EC_{50} values of 3.5, 1.8, 2.1, and 9.0 μg/mL, respectively.

1. Introduction

The chemistry of marine natural products has grown enormously in the last decade.[1,2] Marine organisms, especially sponges, are prolific producers of structurally novel natural products.[3,4] Sponges of the genus *Stylissa* are considered members of continued interest because they have been the source of highly interesting bioactive compounds, which include dimeric alkaloids such as dibromophakellin,[5] *E*-hymenialdisine,[6] *Z*-3-bromohymenialdisine,[7] and sceptrin[8] in addition to a series of brominated pyrrole alkaloids, which include massadine,[9] stylisins 1 and 2,[10] 2-debromohymenine,[11] stylissadines A and B,[12,13] and other brominated alkaloids. These compounds have attracted particular attention because of their ability to inhibit protein kinases.[14] Several other metabolites, including cyclic heptapeptides named stylissamides E, F[15] have been isolated from the *Stylissa* genus, polyhydroxylated sterols such as stylisterols A–C[16] with cytotoxic activity toward hela cells have been isolated from the Okinawan marine sponge *Stylissa* sp. Finally, a dimeric oroidin derivative carteramine A was isolated as a neutrophil chemotaxis inhibitor from the marine sponge *Stylissa carteri*.[17]

In the course of our investigation of marine sponges, we have examined the chemical composition of the Indonesian marine sponge *Stylissa* species (Demospongiae, order Halichondrida, family Dictyonellidae), which were collected by hand using SCUBA from Derawan Islands, Berau, NE Kalimantan. The bioassay-guided fractionation led to the isolation of four new brominated alkaloids (**1**–**4**), along with eight known metabolites (**5**–**12**). This paper reports on the structure elucidation as well as the cytotoxic activity of the isolated compounds toward the mouse lymphoma cancer cell line L5187Y.

2. Results and discussion

2.1. Structural elucidation

12-*N*-Methyl stevensine (**1**), (Fig. 1) was obtained as pure yellowish-brown residue with an UV (MeOH) λ_{max} at 234 nm. The positive ESIMS showed a cluster at *m*/*z* 400:402:404 $[M+H]^+$ with the ratio 1:2:1 indicating the presence of two bromine atoms in the molecule. The molecular formula of $C_{12}H_{12}Br_2N_5O^+$ was established by HREIMS analysis of **1**, which showed a cluster peak at *m*/*z* 401.9387 $[M+H]^+$, with a 14 mass unit increase (CH_2) compared to stevensine (**7**), also isolated during this study. Comparison of the ^1H NMR spectra of **1** and **7** showed a close relationship between both compounds except for the presence of an additional methyl group at δ 3.01 ppm and the absence of a proton signal resonating between δ 12.50 and 13.50 ppm in **1**. The ^1H NMR spectrum of compound **1** in DMSO-d_6 (Table 1) revealed the presence of eight peaks assigned as, two broad singlet peaks, each one integrated for one proton at δ_H 13.34 and 12.55, assigned to 1N–H and 14N–H, respectively. Another two exchangeable resonances were observed at

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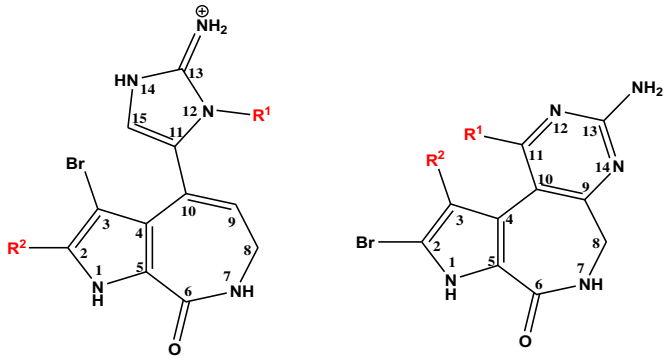
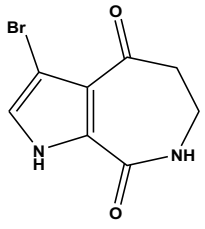


	R^1	R^2
1	CH_3	Br
2	CH_3	H
7	H	Br
8	H	H

	R^1	R^2
3	CO_2CH_3	H
4	H	H
11	H	Br
12	CO_2CH_3	Br



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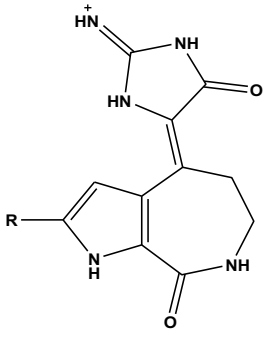

	R
5	Br
6	H

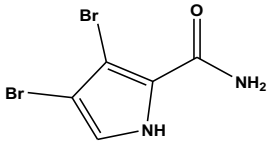

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Fig. 1. Structure of the isolated compounds.

Table 1
^{1}H NMR data for compounds **1**–**4** in DMSO-d_6 (500 MHz)

Position	**1**	**2**	**3**	**4**
	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1-NH	13.34 s	12.45 s	13.12 s	12.55 s
2	—	7.23 br s	—	—
3	—	—	6.85	6.68 s
7- NH	8.11 t (5.1)	8.04 t (5.3)	8.28 t (5.2)	7.88 t (5.1)
8	3.49 t (7.0)	3.49 t (6.6)	4.01 d (5.2)	3.91 d (5.1)
9	6.24 t (7.0)	6.19 t (6.6)	—	—
11	—	—	—	8.54 s
15	7.01 s	7.0 s	—	—
12-NCH_3	3.01 s	3.0 s	—	—
13-NH_2	7.66 s	7.66 br s	7.25 br s	6.83 br s
14-NH	12.55 s	Not observed	—	—
OCH_3	—	—	3.75 s	—

δ_H 8.11 (t, J=5.1 Hz, 1H) assigned to the 7N–H and at δ_H 7.66 (s, 2H) indicating the presence of NH_2 at C-13 as observed for **7**. The remaining peaks of the ^{1}H NMR spectrum of compound **1** showed a methine singlet peak at δ_H 7.01 for H-15, an olefinic proton at δ_H 6.24 (t, J=7.0 Hz, 1H) assigned for H-9, an aliphatic methylene group at δ_H 3.49 (t, J=7.0 Hz, 2H) assigned for two protons at C-8, in addition to a singlet methyl group at δ_H 3.01 ppm. The ^{13}C NMR spectrum (Table 2) of **1** contained 12 well-resolved resonances consistent with the HREIMS data. The DEPT ^{13}C and HMQC spectra

Table 2
^{13}C NMR data of compounds **1** and **3**, DMSO-d_6

Position	**1** (125 MHz) δ_C	**3** (150 MHz) δ_C
2	108.1	108.2
3	97.3	104.7
4	121.0	120.3
5	124.7 s	125.3
6	161.5 s	166.2
8	37.3 t	46.4
9	129.9 d	163.7
10	128.3 s	110.3
11	126.8 s	156.6
13	146.9 s	161.3
15	112.0 d	166.6
N–CH_3	30.0 q	—
OCH_3	—	52.6
C=O	—	166.2

of **1** demonstrated that only four of the protons were attached to carbons, one methyl at δ_C 30.0 (CH_3), one methylene at δ_C 37.3 (C-8), and two methine groups at δ_C 112.0 (C-15) and 129.9 (C-9), while the remaining carbons were assigned as quaternary carbons. The COSY spectrum (Fig. 2) showed only one continuous spin system NH(7)CH_2(8)CH(9). The extra methyl group was found to be attached to 12N because of its downfield chemical shift as well as their HMBC correlations to two quaternary carbons assigned for C-11 and C-13 (Fig. 2). Furthermore, 12*N*-CH_3 showed a cross-peak with 9-CH in the ROESY experiment. Confirmation of the structure of **1** was provided by careful analysis of 2D NMR spectroscopic experiments in association with the published data of compound **7**. Thus, compound **1** was identified as a new natural product, for which the name 12-*N*-methyl stevensine is proposed.

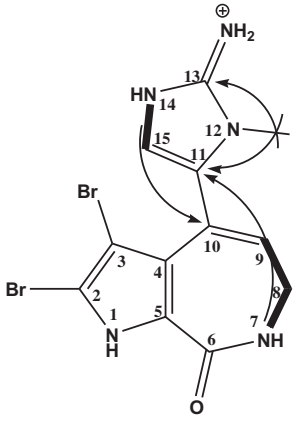

Fig. 2. Key COSY (▬) and HMBC (↷) correlations of **1**.

12-*N*-Methyl-2-debromostevensin (**2**) (Fig. 1) was obtained as yellowish-brown residue with an UV (MeOH) λ_{max} at 224 nm. The positive ESIMS showed a cluster at m/z 322:324 $[M+H]^+$ with the ratio 1:1 indicating the presence of one bromine atom. This was corroborated by HREIMS measurement showing a cluster peak at m/z 322.0298 $[M+H]^+$, indicating a molecular formula of $C_{12}H_{13}BrN_5O^+$. Comparison of the mass and the ^{1}H NMR spectra of **2** (Table 1) with those of **1** and **8** showed a close structural relationship between all compounds except for the presence of an extra proton signal and the absence of a bromine atom (loss of 79 mass unit) compared to **1**, and the presence of one methyl group compared to **8**. This was confirmed by the corresponding signals observed for 2C–H (δ_H 7.23) and 12*N*–CH_3 (δ_H 3.00 ppm). The COSY spectrum of compound **2** (Fig. 3) showed a clear long range cross-peak between the additional aromatic proton resonating at δ_H 7.23 ppm and the exchangeable proton at δ_H 12.45 ppm assigned to

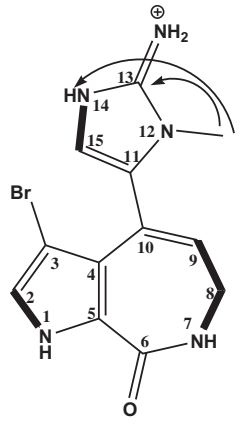

Fig. 3. Key COSY (▬) and ROESY (↷) correlations of **2**.

the pyrrole 1N–H, thus placing the additional proton at C-2 and replacing the bromine atom in compound **2**. Finally, a clear correlation between the methyl protons and both protons of NH_2-13 and NH-14 in the ROESY spectrum (Fig. 3), confirmed the attachment of the methyl group to 12N. On the basis of the 1D and 2D NMR spectra compound **2** was identified as a new natural product for which the name 12-*N*-methyl-2-debromostevensine is proposed.

The ESIMS of compound **3** (Fig. 1) exhibited a cluster at *m*/*z* 353:355 $[M+H]^+$ with the ratio 1:1 in the positive mode indicating the presence of one bromine atom. This was confirmed by HREIMS showing a cluster peak M^+ at *m*/*z* 352.0003, which was appropriate for the molecular formula $C_{12}H_{10}BrN_5O_3$. Comparison of the mass and ^{1}H NMR spectra of **3** and **12** (Table 1) showed high similarity between both compounds except for the presence of an additional proton signal at δ 6.85 ppm and the absence of a bromine atom (79 mass unit decrease). The ^{13}C and DEPT spectra of **3** (Table 2) indicated the presence of 12 carbons, including one methoxy group at δ_C 52.6, one methylene at δ_C 46.8, and one methine at δ_C 104.7, while the rest were quaternary carbons. The ^{1}H NMR spectrum showed a singlet at δ_H 13.12 corresponding to the exchangeable proton of the pyrrole NH, a triplet signal at δ_H 8.28 (t, J=5.2 Hz, 1H) assigned for the 7N–H amide, a broad singlet at δ_H 7.25 (s, 2H) corresponding to the exchangeable resonances of 13N–H_2 group, a doublet peak at δ_H 4.01 (d, J=5.2 Hz, 2H) assigned to the aliphatic methylene protons CH_2-8. The position of the extra proton was established by the COSY spectrum where no correlation between 1N–H and 2-CH was detected. This indicated that C-2 is blocked by a bromine atom as in compound **12**. The complete assignment of the structure was confirmed by the HMBC spectrum and comparison with the NMR spectroscopic data of compound **12**. Therefore, compound **3** was isolated as a new natural product, for which the name 3-debromolatonduine B methyl ester is proposed.

3-Debromolatonduine A (**4**) (Fig. 1) was obtained as white amorphous solid with an UV (MeOH) λ_{max} at 248, 283 nm. The positive ESIMS showed a cluster at *m*/*z* 294:296 $[M+H]^+$ with the ratio 1:1 indicating the presence of one bromine atom. This was corroborated by HREIMS analysis showing a cluster peak at *m*/*z* 294.1077, which is appropriate for the molecular formula $C_{10}H_8BrN_5O$ (calcd 294.1074). The ^{1}H NMR spectrum of compound **4** showed high similarity to those of **3** and **11**, likewise isolated in this study, except for the absence of an extra methyl ester group compared to **3**, and the presence for an extra proton signal at δ_H 6.68 ppm (loss of 79 mass unit) compared to **11**.[18] The attachment of the extra proton appeared at δ_H 6.68 was confirmed by COSY and ROESY spectra. The absence of any COSY correlation between the extra proton signal and the pyrrole NH, and the presence of a ROESY cross-peak with H-11 (δ_H 8.54 ppm) confirmed its attachment at C-3 (Fig. 4). All proton signals were assigned and their attachment

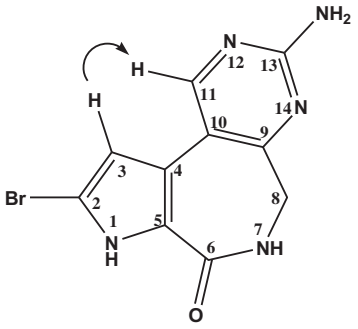

Fig. 4. Important ROESY correlation of **4**.

was confirmed by analysis of COSY and ROESY data as well as comparison with those of compound **11**.[18] Thus, compound **4** was identified as new natural product, for which the name 3-debromolatonduine A is proposed.

2.2. Cytotoxic activity

The cytotoxic activity test of the isolated alkaloids from *Stylissa species* against L5178Y mouse lymphoma cells of the rats using 10 μg/mL as a standard dose for all the tested compounds showed that compounds **1**, **5**, **6**, and **11** have a strong activity, while compound **10** showed weak activity (Table 3). According to these results, it is suggested that the presence of a carbonyl or *keto* group in the imidazole ring of this class of alkaloids may increase the activity against mouse lymphoma cells. Furthermore, it is also suggested that the presence of *N*-methyl group instead of *N*H group in the imidazole ring may also increase the activity of the compounds. On the other hand, it is suggested that the absence or presence of bromine atoms in this class of alkaloids has no effect on the activity of compounds against mouse lymphoma cells.

Table 3
Cytotoxicity data of the isolated compounds toward L5178Y

Compound	L5178Y% of inhibition concentration (10 μg/mL)	EC_{50}
1	86.1	3.5
2	8.1	
3	10.2	
4	6.6	
5	99.6	1.8
6	101.0	2.1
7	7.5	
8	15.1	
9	9.0	
10	33.8	
11	89.3	9.0
12	1.7	
Control (Kahalalide F)	—	6.3

3. Experimental section

3.1. General

The NMR spectra were recorded at 500 MHz (^{1}H, DMSO-d_6) and 125 MHz (^{13}C, DMSO-d_6) on a Bruker Unity spectrometer. Final NMR spectroscopic assignments were based on comparison to previously published data and 2D NMR spectroscopic data derived from HMQC and HMBC. ESIMS were obtained with a Thermofinnigan LCQ DECA mass spectrometer coupled to an Aligent 1100 HPLC system equipped with a photodiode array detector. Sephadex LH20 was used for separation of the crude fractions. HPLC was performed on semi-preparative HPLC equipment (Merck) on a (Eurospher,

250×10 mm) C-18 column (Waters) with a Gynkotek photodiode-array and flow rate of 5 mL/min. Pre-coated silica gel 60 F_{254} plates (E. Merck) were used for TLC.

3.2. Biological material

The sponge sample of *Stylissa* sp. was collected from Derawan island, Indonesia using scuba method. The specimen was preserved in MeOH for 48 h and then transported to the home laboratory at ambient temperature. The frozen organism was soaked three successive times for 24 h in 100% MeOH. The sponge was identified by Prof. Nicole de Voogd, Leiden, Netherlands.

3.3. Extraction and isolation

The frozen sponge was extracted exhaustively with MeOH. The MeOH extract was dried completely and gave a residue. The sponge was powdered and macerated in 70% MeOH (3×5 L) at room temperature. The combined extracts were evaporated in vacuo. The brown viscous residue was dissolved in water (500 mL) and was successively extracted with EtOAc (3×500 mL) (4.65 g), BuOH (3×200 mL) (2.23 g), and finally the aqueous phase (3.43 g). The EtOAc fraction (4.50 g) was subjected to Sephadex LH20 column using DCM/MeOH (1:1) to give six fractions. The different fractions were subjected to further Sephadex LH20 columns chromatography using 100% MeOH as the mobile phase and finally to a semi-preparative C18 HPLC column (Eurospher, 250×10 mm) using gradient elution starting with 25% MeOH in H_2O to 100% MeOH in 20 min with a flow rate of 5 mL/min to give compounds **5**–**12**. The BuOH (2.23 g) fraction was subjected to VLC on a RP column with gradient elution from 20% MeOH in H_2O to 100% MeOH followed by Sephadex LH20 chromatography using 100% MeOH as the mobile phase to give seven fractions. Fractions 2 and 3 were subjected to further purification using a Sephadex LH20 column with 100% MeOH as mobile phase and finally subjected to reversed-phase HPLC with gradient elution starting from 25% MeOH in H_2O to 100% MeOH in 20 min with a flow rate of 5 mL/min to give 12-*N*-methyl stevensine (**1**) (14 mg), 12-*N*-methyl-2-debromstevensine (**2**) (3.7 mg), 3-debromolatonduine B methyl ester (**3**) (6 mg) and 3-debromolatonduine A (**4**) (2.5 mg).

3.4. Cytotoxic activity method

The cytotoxic activity against L5178Y mouse lymphoma cells in rats was determined using the micro-culture tetrazolium (MTT) assay[19] and compared to that of untreated controls. All experiments were carried out in triplicates and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

3.5. Conclusion

*3.5.1. 12-N-Methyl stevensine (**1**).* Yellowish-brown residue (14 mg); IR (KBr film) ν_{max} 3420, 2962, 1641, 1518, 1450 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6), Table 1; ^{13}C NMR (125 MHz, DMSO-d_6), Table 2; HREIMS at *m*/*z* 401.9387 $[M+H]^+$ (calcd for $C_{12}H_{12}Br_2N_5O^+$ 401.9388).

*3.5.2. 12-N-Methyl-2-debromostevensin (**2**).* Yellowish-brown residue (3.7 mg); IR (KBr film) ν_{max} 3418, 2882, 1632, 1532, 1454 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6), Table 1; HREIMS at *m*/*z* 322.0298 $[M+H]^+$ (calcd for $C_{12}H_{13}BrN_5O^+$ 322.0303).

*3.5.3. 3-Debromolatonduine B methyl ester (**3**).* Yellowish-white residue (6 mg); IR (KBr film) ν_{max} 3440, 2942, 1726, 1651, 1514, 1448 cm^{-1}. ^1H NMR and (500 MHz, DMSO-d_6), Table 1; ^{13}C NMR (150 MHz, DMSO-d_6), Table 2; HREIMS at *m*/*z* 352.0003 (calcd for $C_{12}H_{10}BrN_5O_3$ $[M]^+$ 352.0001).

*3.5.4. 3-Debromolatonduine A (**4**).* White amorphous solid (2.5 mg); IR (KBr film) ν_{max} 3432, 2918, 1653, 1510, 1440 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6), Table 1; HREIMS at *m*/*z* 294.1077 $[M+H]^+$ (calcd for $C_{10}H_8BrN_5O$ 294.1074).

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