Lissoclibadins 4−7, Polysulfur Aromatic Alkaloids from the Indonesian Ascidian *Lissoclinum* cf. *badium*[⊥]

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Four new polysulfur aromatic alkaloids, lissoclibadins 4 (**1**), 5 (**2**), 6 (**3**), and 7 (**4**), were isolated from the ascidian *Lissoclinum* cf. *badium* collected in Indonesia, together with seven known alkaloids, lissoclibadins 1 (**5**), 2 (**6**), and 3 (**7**), lissoclinotoxins E (**8**) and F (**9**), 3,4-dimethoxy-6-(2′-*N*,*N*-dimethylaminoethyl)-5-(methylthio)benzotrithiane (**10**), and *N*,*N*-dimethyl-5-(methylthio)varacin (**11**). Compounds **1**−**11** were isolated from the ascidian collected in March (wet season), while **5**−**11** have been obtained previously from the organism collected in September (dry season) at the same site. The structures of the new compounds were assigned on the basis of their spectroscopic data. Lissoclibadins 4−7 (**1**−**4**) inhibited the colony formation of Chinese hamster V79 cells with EC_{50} values of 0.71, 0.06, 0.06, and 0.17 μM, respectively. Compounds **1**−**4** showed also weak antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*.

Ascidians (tunicates) are a prolific bioresouce for bioactive metabolites. Many interesting compounds have been obtained especially from colonial ascidians. In the course of our ongoing studies on bioactive metabolites from marine organisms, we have isolated in the present investigation four new polysulfur aromatic alkaloids, lissoclibadins 4 (**1**), 5 (**2**), 6 (**3**), and 7 (**4**), from the colonial ascidian *Lissoclinum* cf. *badium* collected in Manado, Indonesia, together with seven known alkaloids, lissoclibadins 1 (**5**), 2 (**6**), and 3 (**7**), lissoclinotoxins E (**8**) and F (**9**), 3,4-dimethoxy-6-(2′-*N*,*N*-dimethylaminoethyl)-5-(methylthio)benzotrithiane (**10**), and *N*,*N*-dimethyl-5-(methylthio)varacin (**11**), previously obtained from the same ascidian.[1,2] The related aromatic alkaloids possessing polysulfide structures have been isolated from ascidians of the genera *Lissoclinum*,[3−8] *Eudistoma*,[6] and *Polycitor*.[9] More than 10 monomeric polysulfides,[3−6,8,9] six dimeric polysulfides,[1,2,4,7,10] and a trimeric[1,2] polysulfide have been reported previously. These compounds were reported to have various bioactivities, e.g., antifungal activity,[1−5,9] antibacterial activity,[1,2,4,9] cytotoxicity,[1−3,5,10] antimalarial (*Plasmodium falciparum*) activity,[5] inhibition of protein kinase C,[6,7] and inhibition of IL-8 α and β receptors.[7]

Four new compounds (**1**−**4**) were isolated from an ethanol extract of *L*. cf. *badium* collected in March (the wet season), together with **5**−**11**. On the other hand, compounds **5**−**11** have been obtained from the same ascidian collected in September (the dry season), at the same sampling site. Lissoclibadins 4−7 (**1**−**4**) showed inhibitory activity against the colony formation of Chinese hamster V79 cells and antimicrobial activity against Gram-positive and -negative bacteria and a yeast. We describe herein the isolation, structure elucidation, and bioactivity of these four new compounds (**1**−**4**).

Lissoclinum cf. *badium* was collected at Manado, Indonesia, in March 2006 and extracted with ethanol. The ethanol extract was separated by passage over HP-20 and LH-20 columns followed by HPLC to give the four new lissoclibadins 4−7 (**1**−**4**) and seven known compounds (**5**−**11**). The structures of the seven known compounds (**5**−**11**) were assigned by comparing their spectroscopic data with those of the compounds obtained previously.[2]

Lissoclibadin 4 (**1**) was isolated as a bis-TFA salt. The molecular weight (482 daltons) and formula ($C_{22}H_{30}N_2O_4S_3$) were deduced from HRFABMS and NMR data (Table 1). Two sets of ^{1}H and ^{13}C NMR signals were observed in the NMR spectra of **1** and assigned to two identical aromatic amine moieties by the analysis of its ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra. Each unit had a pentasubstituted benzene ring and two methylenes, an OH, an OMe, and an NMe_2 group. The connectivity of the two methylene groups at the 7 and 8 positions was revealed by the ^{1}H−^{1}H COSY spectrum. Key HMBC correlations were detected from an aromatic proton signal (H-5) to C-1, 3, 4, 6, and 7, H_3-9 to C-4, and H_2-7 to C-1, 5, and 6 (Table 1). NOEs were observed between H-5 and H_3-9 (OMe) and H-5 and H_2-7. These data revealed the positions of the aromatic proton at C-5, the OMe at C-4, and the OH at C-3. Therefore, **1** had two identical aromatic amine units depicted as unit **a** shown in Figure 2.

On subtraction of the sum of two units from the molecular formula of **1**, there remained three sulfur atoms. Therefore, each unit was connected through one disulfide bond and a sulfide bond. As in the case of lissoclibadin 2 (**6**),[2] only antiparallel (*trans*-type) orientation of two identical aromatic units would result in an asymmetric structure. Consequently, the structure of lissoclibadin 4 was assigned as **1** (Figure 1).

Lissoclibadin 5 (**2**) was also obtained as a bis-TFA salt. HRFABMS and NMR data (Table 2) revealed the molecular mass (542 Da) and formula ($C_{24}H_{34}N_2O_4S_4$) of **2**. Compound **2** gave two sets of ^{1}H and ^{13}C NMR signals (Table 2), which showed two different aromatic amine units. One unit in **2** had the same structure (unit **a**) as that in **1**. The other unit had two more methyl singlets at δ_H 2.46 (δ_C 19.2) and 3.93 (61.2) ascribed to SMe and OMe, respectively, but an aromatic proton signal was not detected in the ^{1}H NMR spectrum of **2**. The structure of this hexasubstituted benzene unit was the same as those in **5**−**11**. Therefore, **2** had units **a** and **b** shown in Figure 2, and the two units were connected through a disulfide and a sulfide bond.

An NOE correlation was detected between an OMe at δ 3.93 (H_3-9′ of unit **b**) and NMe_2 at δ 3.01 (H_6-10 of unit **a**) in the

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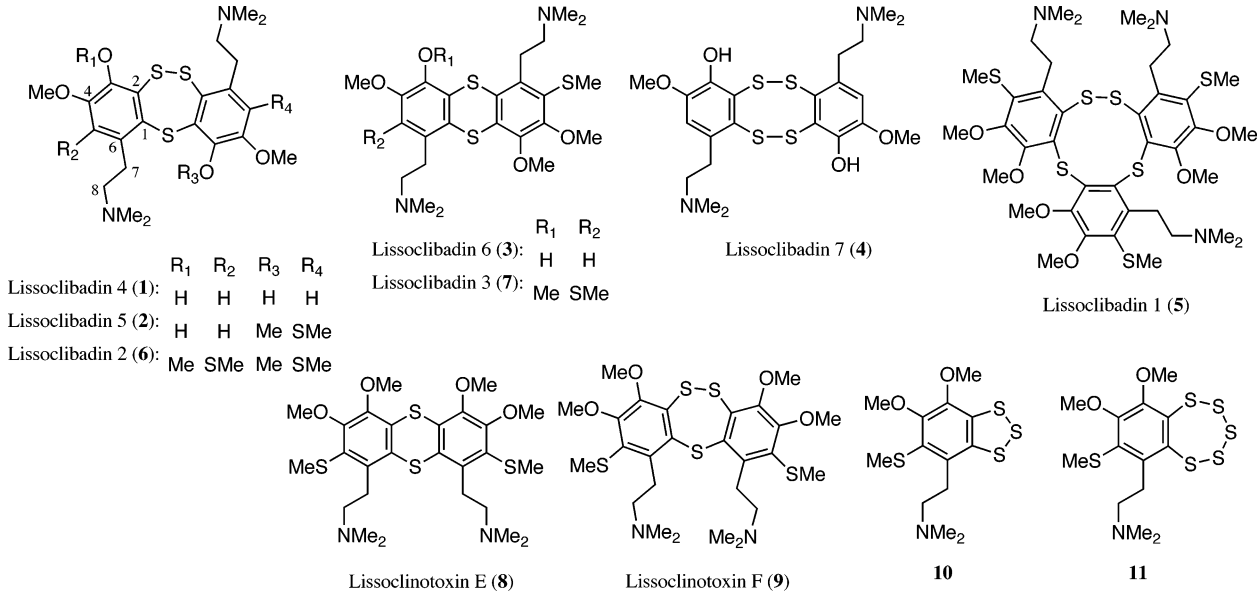

Figure 1. Structures of compounds **1**−**11** isolated from *Lissoclinum* cf. *badium*.

Table 1. ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) Data for Lissoclibadin 4 (**1**) in CD_3OD

position	δ_C	δ_H, mult.	HMBC	NOESY
1	137.1			
2	124.1[a]			
3	149.6			
4	150.7			
5	114.4	7.00, s	1, 3, 4, 6, 7	7, 9
6	129.6			
7	30.9	3.29, m	1, 5, 6	5
8	59.3	3.33, m	6, 7, 10	
9	56.9	3.95, s	4	5
10	43.6[b]	2.98, s	8, 10	
1′	122.2			
2′	124.9[a]			
3′	144.6			
4′	149.0			
5′	113.5	6.83, s	1′, 3′, 4′, 6′	7′, 9′
6′	131.3			
7′	31.2	3.33, m	1′, 5′, 6′	5′
8′	58.8	3.63, m	6′, 7′, 10′	10′
9′	56.7	3.86, s	4′	5′
10′	43.7[b]	3.03, s	8′, 10′	8′

[a,b] Signals are interchangeable for the same letters.

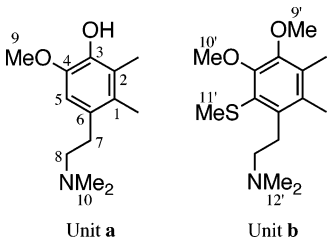

Figure 2. Partial structures of units **a** and **b**.

NOESY spectrum of **2**. Thus, the structure of lissoclibadin 5 was elucidated as **2** (Figure 1).

Lissoclibadin 6 (**3**) was purified as a bis-TFA salt. The molecular mass (510 Da) and formula ($C_{24}H_{34}N_2O_4S_3$) of **3**, assigned from its HRFABMS and NMR data, were 32 Da (S) less than those of **2**. ^{1}H and ^{13}C NMR data for **3** (Table 3) revealed two sets of signals ascribable to units **a** and **b** (Figure 2), as in the case of **2**. An NOE correlation was observed between OMe at δ 3.86 (H_3-9′ of unit **b**) and NMe_2 at δ 3.01 (H_6-10 of unit **a**) in the NOESY spectrum of **3**. Therefore, the two units **a** and **b** were connected through two sulfide bonds with an antiparallel orientation. Accordingly, the structure of lissoclibadin 6 was determined as **3** (Figure 1).

Lissoclibadin 7 (**4**) was isolated as a bis-TFA salt and showed one set of ^{1}H and ^{13}C NMR signals (Table 4). The molecular mass (514 Da) and formula ($C_{22}H_{30}N_2O_4S_4$) showed that **4** has a symmetric structure. The aromatic amine unit assigned from the NMR data of **4** (Table 4) was unit **a** in Figure 2. The sum of the two units was 128 Da (S_4) less than the molecular mass (formula) of **4**. Since **4** has a symmetrical structure, two units would be connected through two disulfide bonds, which was also suggested by a mass spectrometric fragment ion peak at *m*/*z* 258. An example of this kind of dimeric structure has been reported for lissoclinotoxin D (Figure 3).[4] The antiparallel orientation of lissoclinotoxin D was selected on the basis of a computational modeling study.[4,10] Therefore, although an antiparallel structure for **4** is shown in Figure 1, a parallel (*cis*-type) structure cannot be excluded since biosynthetic enzymes may construct a thermodynamically more unfavorable structure.[1,2,10]

Lissoclibadins 4−7 (**1**−**4**) have been isolated from *L.* cf. *badium* collected in March (wet season) in 2006 but were not obtained from the same organism collected in September (dry season) in 2003, 2004, and 2005 at the same sampling site.

The antimicrobial activities of **1**−**4** are listed in Table 4 together with the reported values of **5**−**11**.[2] Interestingly, the new lissoclibadins 4−7 (**1**−**4**) showed weak antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, while the dimeric compounds **6** and **7** did not exhibit such activity. The two new dimeric compounds **2** and **3** also showed modest antiyeast activity against *Saccharomyces cerevisiae*. The phenol group in **1**−**4** may mediate their antimicrobial activity.

Compounds **1**−**4** were also tested for their effects on the rate of colony formation of V79 cells. This bioassay reflects the direct action of compounds on the cells.[11] Compounds **1**−**5** showed EC_{50} values of 0.71, 0.06, 0.06, 0.17, and 0.20 μM, respectively. In previous experiments, the EC_{50} value of **5** was 0.40 μM and those of **6**−**11** were 0.08, 0.34, 0.27, 0.28, 0.19, and 0.15 μM, respectively.[11]

Experimental Section

General Experimental Procedures. UV and IR spectra were recorded on a Hitachi U-3310 spectrophotometer and a Perkin-Elmer Spectrum One FT-IR spectrometer, respectively. NMR spectra were

Table 2. ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) Data for Lissoclibadins 5 (**2**) and 6 (**3**) in CD_3OD

	lissoclibadin 5 (**2**)				lissoclibadin 6 (**3**)			
position	δ_C	δ_H, mult.	HMBC	NOESY	δ_C	δ_H, mult.	HMBC	NOESY
1	124.8				135.5			
2	132.8				140.7[c]			
3	146.0				149.8			
4	149.6				151.2			
5	114.2	6.94, s	1, 3, 4, 6, 7	7, 9	115.2	7.06, s	1, 3, 7	7, 9
6	132.8				136.7			
7	30.9	2.98, m	8	5, 8, 10	32.2	3.25, m	1, 5, 8	5
		3.41, m	1, 5, 6, 8	5, 8, 10		4.57, m	5, 6, 8	
8	59.3	3.41, m	7, 10	7, 10	60.5	3.24, m	7, 10	10
						3.66, m	10	10
9	57.6	3.89, s	4	5	61.0	3.90, s	4	5
10	43.5[a]	3.01, s	8, 10	7, 8, 9′	43.6	3.01, s	8, 10	8, 9′
1′	128.4				141.7			
2′	136.6				141.1[c]			
3′	154.1				158.2			
4′	157.0				157.9			
5′	134.0				136.3			
6′	136.2				132.7			
7′	29.0	2.98, m	8′	8′, 12′	30.9	3.41, m	5′, 8′	
		3.61, m	6′, 8′	8′, 12′		3.59, m	1′, 6′, 8′	8′
8′	58.9	3.19, m	7′, 12′	7′, 12′	58.1	3.14, m	12′	7′, 12′
		3.92, m	7′, 12′	7′, 12′				
9′	61.2[b]	3.93, s	3′	10, 10′	56.9	3.86, s	3′	10, 10′
10′	62.0[b]	3.93, s	4′	9′, 11′	62.0	3.87, s	4′	9′, 11′
11′	19.2	2.46, s	5′	10′	19.0	2.45, s	5′	10′, 12′
12′	43.6[a]	3.01, s	8′, 12′	7′, 8′	43.6	3.01, s	8′, 12′	8′, 11′

[a,b,c] Signals are interchangeable for the same letters.

Table 3. ^{13}C (100 MHz) and ^{1}H NMR (400 MHz) Data for Lissoclibadin 7 (**4**) in CD_3OD

position	δ_C	δ_H, mult.	HMBC	NOESY
1	136.1			
2	129.9			
3	151.7			
4	151.2			
5	115.7	7.03, s	1, 3, 4, 6, 7	7, 9
6	133.1			
7	31.8	3.24, m	1, 5, 6, 8	5
8	59.5	3.26, m	6, 7, 10	10
9	57.0	3.90, s	4	5
10	43.6	2.94, s	8, 10	8

Table 4. Antimicrobial Activity of Compounds **1**−**4** (This Study) and **5**−**11**[2]

	M. hiemalis[a]		*S. cerevisiae*		*S. aureus*		*E. coli*	
compd	50[b]	20	50	20	50	20	50	20
1	*c*				13.1[d]	10.8	15.7	
2			6.2		15.8	9.0	11.6	
3			7.8		13.8	10.0	10.8	
4			16.8		13.1	9.4	9.9	
5								
6	13.8							
7								
8								
9	18.0	10.5						
10	23.0	17.4	11.8		10.3		17.8	14.4
11	26.2	19.6	15.2	10.5	14.2		17.1	13.1

[a] Test microorganisms: see Experimental Section. [b] Amount (μg/disk). [c] Not active. [d] Inhibition zone (mm).

measured on a JEOL AL 400 NMR spectrometer. Mass spectra were obtained by a JEOL JMS-MS 700 mass spectrometer (FAB mode, *m*-nitrobenzyl alcohol or glycerol as the matrix).

Ascidian. *Lissoclinum* cf. *badium* was collected by scuba diving at −7 to −19 m off the coral reef in Manado, Indonesia, in March 2006. The voucher specimen is deposited at the Nagoya University Museum as NUM-Az0391.

Extraction and Isolation. The ascidian (about 600 g) was cut into small pieces and soaked in EtOH on a boat immediately after collection.

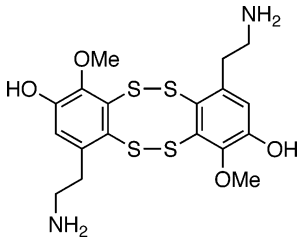

Figure 3. Structure of lissoclinotoxin D.

The ethanol extract was filtered, and the organism was further extracted three times with EtOH for 1 h by sonication. The extract was evaporated (16.0 g), suspended in 1.0% NH_4OH(aq), and applied on an HP-20 column (1 L). The column was washed with 1.0% NH_4OH(aq) and eluted with EtOH. The EtOH eluate was partitioned between *n*-hexane and MeOH−0.1% TFA (4:1), and the lower layer was evaporated to give 6.5 g of an alkaloid fraction. The alkaloid fraction (1.4 g) was separated by LH-20 column chromatography (250 mL) with MeOH into two fractions (1 and 2). Fraction 1 (0.8 g) was subjected to LH-20 column chromatography and HPLC (ODS, linear gradient elution with MeOH−0.1% TFA, 1:4 to 9:1) to give **1** (4.1 mg), **2** (5.1 mg), **3** (5.0 mg), **5** (35.0 mg), **6** (10.0 mg), **7** (2.9 mg), **8** (8.4 mg), **9** (8.5 mg), **10** (10.0 mg), and **11** (2.8 mg). Compound **4** (11.0 mg) was isolated from fraction 2 (0.6 g) by LH-20 column chromatography (twice with MeOH) followed by HPLC (same condition as above).

Lissoclibadin 4 (1): isolated as a bis-TFA salt; UV (MeOH) λ_{max} (log ϵ) 258 (4.06), 311 (3.81) nm; IR (KBr) ν_{max} 2731, 1683, 1466, 1397, 1267, 1204, 1132, 1069, 961 cm^{-1}; ^{1}H and ^{13}C NMR data, listed in Table 1; FABMS *m*/*z* 483 $[M + H]^+$; HRFABMS *m*/*z* 483.1464 (calcd for $C_{22}H_{31}N_2O_4S_3$, 483.1446).

Lissoclibadin 5 (2): obtained as a bis-TFA salt; UV (MeOH) λ_{max} (log ϵ) 255 (4.10), 302 (3.86) nm; IR (KBr) ν_{max} 2934, 2714, 1683, 1459, 1388, 1203, 1132, 1022, 960 cm^{-1}; ^{1}H and ^{13}C NMR data, listed in Table 2; FABMS *m*/*z* 543 $[M + H]^+$; HRFABMS *m*/*z* 543.1495 (calcd for $C_{24}H_{35}N_2O_4S_4$, 543.1480).

Lissoclibadin 6 (3): isolated as a bis-TFA salt; UV (MeOH) λ_{max} (log ϵ) 260 (4.25), 310 (3.93) nm; IR (KBr) ν_{max} 2725, 1680, 1462, 1380, 1267, 1203, 1131, 1064, 1020, 960 cm^{-1}; ^{1}H and ^{13}C NMR data, listed in Table 2; FABMS *m*/*z* 511 $[M + H]^+$; HRFABMS *m*/*z* 511.1753 (calcd for $C_{24}H_{35}N_2O_4S_3$, 511.1759).

Lissoclibadin 7 (4): obtained as a bis-TFA salt; UV (MeOH) λ_{max} (log ϵ) 260 (4.03), 322 (3.88) nm; IR (KBr) ν_{max} 2732, 1682, 1459, 1201, 1129, 1064, 961 cm^{-1}; ^1H and ^{13}C NMR data, listed in Table 3; FABMS *m*/*z* 258, 515 $[M + H]^+$; HRFABMS *m*/*z* 515.1152 (calcd for $C_{22}H_{31}N_2O_4S_4$, 515.1167).

Antimicrobial Activity. The growth inhibitory activity of **1**−**4** was examined by a paper disk method against *Mucor hiemalis* IAM 6088 (fungus), *Saccharomyces cerevisiae* IAM 1438T (yeast), *Staphylococcus aureus* IAM 12544T (Gram-positive bacterium), and *Escherichia coli* IAM 12119T (Gram-negative bacterium) as test microorganisms. The results are listed in Table 4.

Relative Plating Efficiency. Chinese hamster V79 cells were grown as a monolayer in Eagle's MEM (Nissui Seiyaku Co., Ltd., Tokyo, Japan) with 10% heat-inactivated FBS. Two hundred cells were seeded onto a 60/15 mm Petri dish in 4 mL of MEM with 10% FBS and incubated overnight at 37 °C. Samples were dissolved in DMSO, and 4 μL of each sample was added to the dish, which were incubated for another 4 days. The numbers of colonies in the sample dishes were counted and compared with those in the control dishes. The relative plating efficiency of the sample against V79 cells at a given concentration (0.01−10 μM) was described as the ratio of the number of colonies in the sample dish to that in the control culture as described in previous papers.[12,13]

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