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Three new sulfur-containing alkaloids, polycarpaurines A, B, and C, from an Indonesian ascidian *Polycarpa aurata*

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Abstract—Three new sulfur-containing alkaloids, polycarpaurines A (**1**), B (**2**), and C (**3**) were isolated from the tropical ascidian *Polycarpa aurata* collected in Indonesia, together with six known compounds (**4**–**9**). The structures of new compounds were assigned on the basis of their spectral data. Compounds **1**, **3**, **4**, and **8** inhibited colony formation of Chinese hamster V79 cells with EC_{50} values of 6.8, 8.6, 3.8, and 10 μM, respectively. Compounds **2** and **7** showed modest activity against V79 cells (EC_{50}>10 μM).

1. Introduction

Many interesting sulfur-containing alkaloids have been isolated from ascidians (tunicates). From ascidians of the genus *Polycarpa*, disulfide alkaloids, polycarpamines A–E,[1] a dimeric disulfide alkaloid, polycarpine (**4**),[2] and its derivatives[3,4] have been reported. These compounds presented various biological activities, such as antifungal activity,[1] cytotoxicity,[3] inhibition of inosine monophosphate dehydrogenase (IMPDH),[2] and induction of apoptosis in JB6 cells through p53- and caspase 3-dependent pathways.[5]

In the search for bioactive metabolites from marine organisms, we found that the ethanol extract of the ascidian *Polycarpa aurata*, collected in North Sulawesi, Indonesia, exhibited notable cytotoxicity against a murine leukemia cell line L1210 and inhibitory activity against the colony formation of Chinese hamster V79 cells. Bioassay-guided separation gave three new alkaloids, named polycarpaurines A (**1**), B (**2**), and C (**3**), together with six known compounds, which were assigned as polycarpine (**4**) and its presumed decomposed compound (**5**) and 2-(4-methoxyphenyl)-*N*-methyl-2-oxoacetamide (**6**), *N*-(4-methoxybenzoyl)-*N*′-methylguanidine (**7**), *N*,*N*-didesmethylgrossularine-1 (**8**), and *p*-methoxybenzoic acid (**9**).[1–4] In this paper we describe the isolation, structure elucidation, and bioactivity of three new compounds **1**–**3**.

2. Results and discussion

Polycarpaurine A (**1**) was obtained as a bis-TFA salt, because the purification was performed by HPLC using a solvent mixture containing 0.1% trifluoroacetic acid (TFA). HRFABMS of **1** showed a pseudomolecular ion $[M+H]^+$ at *m*/*z* 437.1727 and the molecular formula of **1** was deduced as $C_{22}H_{24}N_6O_2S$, which showed 14 degrees of unsaturation. The ^1H and ^{13}C NMR signals of **1** (Table 1) were assigned by HMQC and HMBC spectra. The ^1H NMR spectrum of **1** gave two aromatic proton doublets at δ 7.62 and 7.10, a methoxy singlet at δ 3.88 and an *N*-CH_3 singlet at δ 2.93. HMBC correlations from H-7 to three aromatic carbons C-5, -9, and -11, from H-8 to C-6, -9, and -10, and from H_3-12 (OCH_3) to C-9 revealed the presence of a *p*-methoxyphenyl group. HMBC correlations were also detected from H_3-13 (*N*-CH_3) to C-1 and -3. The above spectral characteristics of **1** were very similar to those of **4**,[2,3,6] an alkaloid possessing two identical phenylimidazole moieties linked by a disulfide bridge. Therefore, **1** was revealed to have the same phenylimidazole unit as **4**. Since only 11 carbon signals were observed in the ^{13}C NMR spectrum of **1**, a symmetric element must be present in the structure of **1** to meet the molecular formula. Subtraction of the sum of two

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Table 1. ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR data for compounds **1–3**

Position	**1** (CD_3OD)			**2** (DMSO-d_6)			**3** (DMSO-d_6)		
	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC
1	113.1			127.5			114.9		
3	149.2			145.8			146.9		
4					12.47, br s			12.87, br s	
5	134.7			123.2			131.8		
6	120.3			120.2			119.7		
7 (11)	131.3	7.62, d (8.8)	5, 9, 11 (7)	131.3	7.47, d (8.4)	5, 9, 11 (7)	129.3	7.87, d (8.8)	5, 9, 11 (7)
8 (10)	115.6	7.10, d (8.8)	6, 9, 10 (8)	112.9	6.95, d (8.4)	6, 9, 10 (8)	113.9	7.03, d (8.8)	6, 9, 10 (8)
9	162.7			159.5			160.0		
12	56.0	3.88, s	9	55.2	3.78, s	9	55.3	3.79, s	9
13	30.3	2.93, s	1, 3	30.9	3.60, s	1, 3	29.7	3.49, s	1, 3
NH_2					7.52, s			7.71, s	

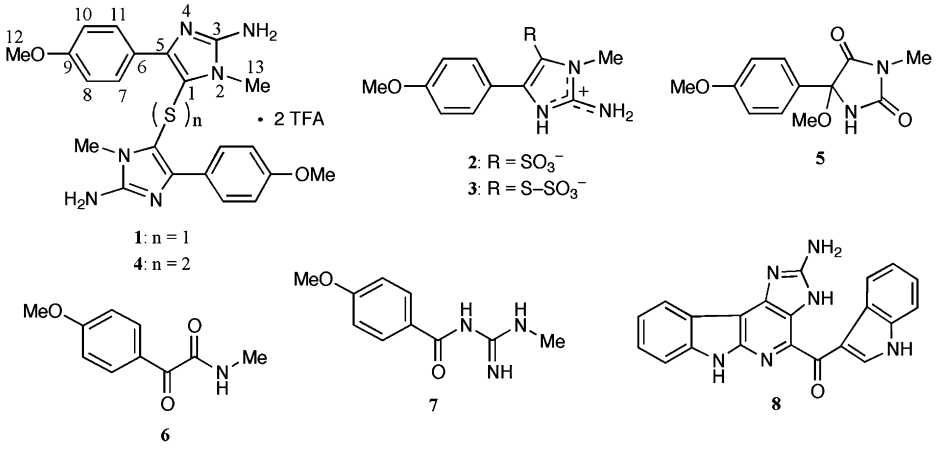
phenylimidazole units from the molecular formula of **1** left one sulfur atom; therefore, the structure of **1** was deduced as a dimeric monosulfide alkaloid.

The molecular formula of compound **2** was determined as $C_{11}H_{13}N_3O_4S$ from the HRFABMS and NMR spectra. ^{1}H and ^{13}C NMR data for **2** (Table 1) resembled those for **1** and **4** except for two ^{13}C signals at δ 127.5 and 123.2, which were assigned as C-1 and -5, respectively, according to the HMBC correlations from H_3-13 (N-CH_3) to δ 127.5 and from H-7 to δ 123.2. The ^{1}H NMR spectrum of **2** showed two exchangeable broad singlets at δ 12.47 (1H) and 7.52 (2H), which were ascribed to the equilibrium of the tautomeric forms of a guanidine moiety. Strong absorption at 1040 cm^{-1} in the IR spectrum of **2** suggested the presence of a sulfate group, which was confirmed by the negative FABMS fragment ion at m/z 80 (SO_3) and a difference of 80 Da between $[M+H]^+$ at m/z 284 and a fragment ion at m/z 204 in the positive FABMS (Fig. 1). The position of the sulfate group was assigned at C-1 from the comparison of ^{13}C NMR data for **2** with those for **1** and **4**.

Compound **3** had the molecular formula of $C_{11}H_{13}N_3O_4S_2$, which was deduced from the HRFABMS and NMR spectra. ^{1}H and ^{13}C NMR data (Table 1) for **3** suggested the presence of the same phenylimidazole moiety as that of **1** and **4**. Subtraction of the phenylimidazole unit, $C_{11}H_{13}N_3O$, from the molecular formula of **3** left S_2O_3. The negative FABMS of **3** showed ion peaks at m/z 80 (SO_3) and 112 (S–SO_3). In the positive FABMS of **3**, the fragment ion peaks at m/z 236 and 204, corresponding to the loss of 80 (SO_3) and 112 Da (S–SO_3) from the $[M+H]^+$ ion peak at m/z 316, respectively, were observed. These data suggested the presence of a thiosulfate group in the structure of **3**. The position of the thiosulfate group was assigned at C-1 by comparison of ^{13}C NMR data for **3** with those for **1**, **2**, and **4**.

A few natural sodium alkyl and alkenyl thiosulfates, such as *n*-propyl, *trans*-1-propenyl, *cis*-1-propenyl, and 2-propenyl thiosulfates, have been reported from onion and garlic as the causative agents of hemolytic anemia in dogs.[7] This is the first report of the isolation of a thiosulfate alkaloid from marine organisms.

The purified compounds (**1–8**) were examined for the effect on the rate of colony formation utilizing Chinese hamster V79 cells. This bioassay reflects the direct action of compounds on the cells. Compounds **1**, **3**, **4**, and **8** inhibited the colony formation of V79 cells with EC_{50} values of 6.8, 8.6, 3.8, and 10 μM, respectively. Compounds **2** and **7** showed modest activity against V79 cells (EC_{50}>10 μM).



3. Experimental

3.1. General

UV and IR spectra were recorded on a Hitachi U-3310 and a Perkin–Elmer Spectrum One FTIR spectrometer, respectively. NMR spectra were measured on a JEOL AL 400 NMR spectrometer. Mass spectra were obtained by a JEOL

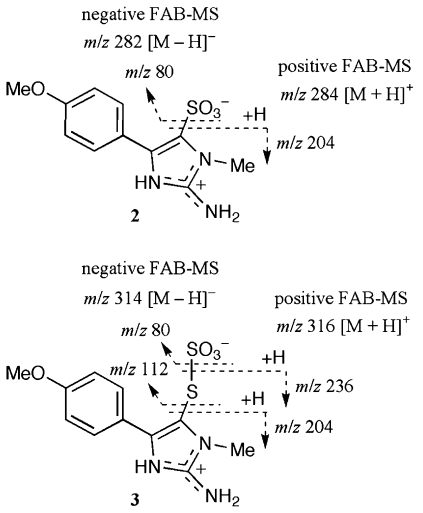


Figure 1. Positive and negative FABMS data for **2** and **3**.

JMS-MS 700 mass spectrometer (FAB mode, *m*-nitrobenzyl alcohol (*m*NBA) or glycerol as the matrix).

3.2. Ascidian

P. aurata was collected by scuba diving in the Lembeh Strait, Indonesia, on September 2004. A voucher specimen is deposited at the Department of Natural Products Chemistry, Tohoku Pharmaceutical University as 04-09-30=1-1a. Ascidian was collected again at the same site in September 2005.

3.3. Extraction and isolation

The ascidian was immediately cut into small pieces and soaked in ethanol on the boat. The ethanol extract (7.0 g) was partitioned between MeOH–H_2O (9/1, 150 mL) and *n*-hexane. The aqueous MeOH layer was evaporated, and the residue was dissolved in water and extracted with EtOAc and water-saturated *n*-BuOH, successively. The EtOAc layer was chromatographed on a SiO_2 column with $CHCl_3$–MeOH (gradient elution) to give fractions E-1–E-14, and the bioactivities against L1210 and V79 cells were detected in E-2 and E-4–E-13. Fractions E-6 and E-2 were separated by HPLC (ODS, 60% MeOH/H_2O) to afford **7** (1.7 mg) and a mixture of **5** and **6** (2:1, 2.0 mg), respectively. Compound **8** (6 mg) was obtained by HPLC separation from E-9 (ODS, 70% MeOH/H_2O containing 0.1% TFA). Compounds **1** (5 mg) and **4** (20 mg) were isolated from E-11 by HPLC (ODS, 60% MeOH/H_2O containing 0.1% TFA). Compound **2** (8 mg) was separated by LH-20 column chromatography (*n*-hexane/$CHCl_3$/MeOH=2:1:1 and then $CHCl_3$/MeOH=2:1) from E-13. The *n*-BuOH extract also showed activity against L1210 and V79 cells and was separated into five fractions (B-1–B-5) by an ODS column (MeOH/H_2O, gradient elution). Compound **3** (5 mg) was obtained from B-3 by HPLC (10–40% CH_3CN/H_2O containing 0.02% TFA, gradient elution).

3.3.1. Polycarpaurine A (1). Compound **1** was obtained as a yellow amorphous powder. UV (MeOH): λ_{max} (log ε) 228 (4.59), 281 (4.52) nm; IR (KBr): ν_{max} 3302, 3120, 1682, 1610, 1509, 1253, 1202, 1188, 1135, 836 cm^{-1}; ^1H and ^{13}C NMR data (CD_3OD) see Table 1; FABMS (glycerol): *m/z* 437 $[M+H]^+$, 235, 204; HRFABMS: *m/z* 437.1727 (calcd for $C_{22}H_{25}N_6O_2S$, 437.1759).

3.3.2. Polycarpaurine B (2). Compound **2** was obtained as a white powder. UV (MeOH): λ_{max} (log ε) 222 (4.51), 261 (4.38) nm; IR (KBr): ν_{max} 3539, 3472, 3415, 3191, 1683, 1510, 1214, 1048, 836 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6) see Table 1; FABMS (negative mode, glycerol): *m/z* 565 $[2M-H]^-$, 282 $[M-H]^-$, 80 $[SO_3]^-$; FABMS (positive mode, mNBA): *m/z* 567 $[2M+H]^+$, 284 $[M+H]^+$, 204 $[M-SO_3+H]^+$; HRFABMS: *m/z* 284.0690 (calcd for $C_{11}H_{14}N_3O_4S$, 284.0705).

3.3.3. Polycarpaurine C (3). Compound **3** was obtained as a pale yellow powder. UV (MeOH): λ_{max} (log ε) 242 (4.27), 300 (4.12) nm; IR (KBr): ν_{max} 3313, 3177, 1677, 1510, 1257, 1226, 1185, 1024, 835 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) see Table 1; FABMS (negative mode, glycerol): *m/z* 629 $[2M-H]^-$, 314 $[M-H]^-$, 234 $[M-SO_3-H]^-$, 112 $[S-SO_3]^-$, 80 $[SO_3]^-$; FABMS (positive mode, glycerol): *m/z* 631 $[2M+H]^+$, 316 $[M+H]^+$, 236 $[M-SO_3+H]^+$, 204 $[M-S-SO_3+H]^+$; HRFABMS: *m/z* 316.0435 (calcd for $C_{11}H_{14}N_3O_4S_2$, 316.0426).

3.4. Bioassay

The L1210 cell line was incubated in RPMI 1640 using 96-well assay plates. Samples were dissolved in MeOH, and 10 μL of each sample was poured into a well and the solvent evaporated. The suspension (0.1 mL, 4×10^4 cells/mL) of L1210 was added to each well and incubated at 37 °C for 72 h in a CO_2 incubator. The shape of the cells was observed after 72 h under an inverted microscope. The number of vital cells in the sample wells was compared with those in the control wells 24 h after adding XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] (cell proliferation kit II®).

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 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 and incubated for another four days. The number of colonies in the sample dishes was 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.[8,9]

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