substrate's binding order.

For further discussion let us return to Scheme I and use the ordered Bi Bi mechanism, where apparent REO binding occurs in the reverse direction, as an example to examine the basis for observing REO kinetic patterns. To reduce eq 1 to eq 2 requires not only $K_{ip} \gg P$ but also $K_p \simeq P$ to retain the main characteristics of the REO mechanism. This means that $K_{ip} \gg K_p$, i.e., $k_4(k_{-2} + k_3)/k_{-2}k_{-3} \gg k_{-1}(k_{-2} + k_3)/k_{-3}(k_{-1} + k_{-2})$; upon simplification, $k_4 \gg k_r$ or $k_4 \gg k_{-1}$ or k_{-2}. Likewise, examination of eq 3a shows that to observe uncompetitive inhibition of P versus both A and B the condition $K_{ip} \gg K_p'$ must be satisfied:

$$k_4(k_{-2} + k_3)/k_{-2}k_{-3} \gg (k_3 + k_4)/k_{-3} \text{ or } k_4 \gg k_{-2}$$

Thus, observation of apparent REO kinetic patterns expected from both eq 2 and 3b requires that the off-rate constant for the first substrate Q (in the reverse reaction), k_4, be large relative to the constant of the rate-limiting step. This requirement can be shown to be also true for other ordered cases such as Theorell–Chance, iso-Theorell–Chance, and iso-ordered mechanisms.

As has been shown previously, the condition $K_{iq} \gg Q$ also leads to the REO type of kinetic patterns. This condition further requires $K_{iq} \gg K_q$, i.e.

$$k_4/k_{-4} \gg k_{-1}k_{-2}/k_{-4}(k_{-1} + k_2) \text{ or } k_4 \gg k_r$$

Clearly, apparent REO phenomena, whether they arise from $K_{ip} \gg P$ or $K_{iq} \gg Q$, all depend on the off-rate constant for the first substrate being much larger than the catalytic rate constant. This common requirement is evident from the relationship $K_{ip}K_q = K_{iq}K_p$; if $K_{ip} \gg K_p$, then $K_{iq} \gg K_q$ must be true. However, the conditions $k_{-4}Q > k_r$ (if $K_{ip} \gg P$) and $k_{-3}P > k_r$ (if $K_{iq} \gg Q$) are necessary to retain REO kinetic patterns since, to keep the term $K_{ip}K_q/PQ$ in proper size, $K_q < Q$ is needed if $K_{ip} \gg P$ and $K_p < P$ if $K_{iq} \gg Q$. It is interesting to note that in the case of CaM kinase II, given the very slow backward reaction ($k_r = k_{-2} = 0.026\ s^{-1}$), one is almost certain to observe apparent REO kinetics because the condition $k_4 \gg k_r$ can be easily fulfilled.

For a rapid-equilibrium random mechanism the condition $K_{ip} \gg P$ means that the initial dissociation constant of P from enzyme is large relative to the concentration of P. Schimerlik and Cleland (1973) reported observing REO substrate addition in the reverse direction of creatine kinase reaction at pH 7. They considered the case to be a degenerate rapid-equilibrium random mechanism and assigned $K_{ip} = \infty$ and $K_q = 0$. In view of the fact that $K_{ip}K_q$ must equal $K_{iq}K_p$ and that $\infty \cdot 0$ is indeterminate, it is preferred to view the apparent REO cases in terms of the relative sizes of Michaelis or dissociation constants for a substrate to its concentration.

Table II summarizes the product inhibition patterns for five sequential mechanisms displaying apparent REO features in the reverse reaction. Predicted inhibition patterns in the forward and reverse direction of the reaction are listed to facilitate the differentiation of mechanisms, including those containing "true" REO mechanisms in the reverse reaction not listed in Table II. For example, in several cases in Table II, P or Q has no inhibitory effect, making the mechanism appear to be true REO. In some cases (see footnotes to Table II), techniques other than product inhibition are needed to make a distinction between mechanisms.

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Halocyamines: Novel Antimicrobial Tetrapeptide-like Substances Isolated from the Hemocytes of the Solitary Ascidian *Halocynthia roretzi*†

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ABSTRACT: Two novel antimicrobial tetrapeptide-like substances, halocyamine A and B, were isolated from the solitary ascidian *Halocynthia roretzi* by a procedure including extraction steps, chromatographies on coarse and fine HP-20 columns, and preparative reversed-phase high-performance liquid chromatography. The structures of halocyamine A and B were determined to be L-histidyl-L-6,7-dihydroxyphenylalanyl-glycyl-6-bromo-8,9-didehydrotryptamine and L-threonyl-L-6,7-dihydroxyphenylalanyl-L-histidyl-6-bromo-8,9-didehydrotryptamine, respectively, by spectral analyses and degradation studies. Besides antimicrobial activities against several kinds of bacteria and yeasts, both of them showed cytotoxic activities against neuronal cells cultured from rat fetal brain, mouse neuroblastoma N-18 cells, and human hepatoma Hep-G2 cells. They were only detected in the "morula"-like cells, which are of the most abundant cell type among the hemocytes of *H. roretzi*.

It has been proposed that antimicrobial substances function as humoral factors in defense mechanisms of invertebrates which lack humoral immunoglobulins (Boman & Hultmark, 1981). Among invertebrates, ascidians are noticeable animals from a viewpoint of the evolution of immune systems, because they are protochordata which occupy the phylogenetical position between vertebrates and true invertebrates. Several antimicrobial substances have been reported to be present in colonial ascidians and shown also to exhibit antiviral and antitumor activities (Rinehart et al., 1981, 1984; Ireland et al., 1982; Kobayashi et al., 1984, 1988; Ishibashi et al., 1987). We cannot define the tissue where they exist, however, because all of them were extracted from the whole animal bodies.

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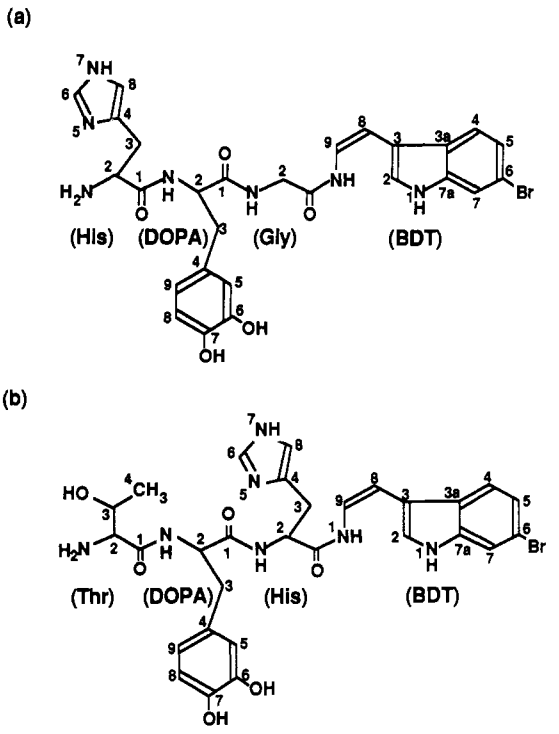

FIGURE 1: Structures of halocyamine A (a) and B (b).

Therefore, we could not discuss their function in relation to the immune systems.

In the course of our studies on the defense mechanisms of the solitary ascidian *Halocynthia roretzi* (Yokosawa et al., 1984, 1986; Harada-Azumi et al., 1987), we surveyed the antimicrobial activity of extracts obtained from various organs of the ascidian and found the presence of antimicrobial substances only in hemocytes. Ascidian hemocytes consisting of several different cell types have been proposed to be involved in the immune response (Wright, 1981). We succeeded in isolating from the extract of hemocytes two novel tetrapeptide-like substances, which we designated as halocyamine A and B (Figure 1), possessing both antimicrobial activity against several kinds of bacteria and yeasts and cytotoxic activity against some cultured cells (Azumi et al., 1987). This paper deals with the isolation, structure determination, biological properties, and distribution of halocyamines.

Materials and Methods

Collection of Hemocytes. Solitary ascidians, *H. roretzi*, type C, were harvested in Mutsu Bay, Japan. The hemolymph was collected from individual animals by cutting their tunic matrix without injuring the internal organs. Hemocytes were obtained by centrifugation (1000*g*, 7 min) and were frozen (−20 °C) until used. The hemolymph from different individuals must not be mixed before centrifugation, because the mixing results in lysis of the hemocytes.

Isolation of Halocyamine A and B. Antimicrobial activity against *Bacillus subtilis* NIHJ PCI 219 was monitored by the paper disk method during each step of the isolation. Alternatively, analytical high-performance liquid chromatography (HPLC)[1] on a reversed-phase column of YMC pack A312 (Yamamura Chem. Lab., Japan) was used for monitoring the antimicrobial components, after their retention times in HPLC were determined.

The hemocytes (107 individuals, 100 g wet wt) were extracted with 120 mL of acetone. After filtration, the acetone extract was concentrated. The aqueous concentrate was extracted twice with 70 mL of 1-butanol at pH 9, and the extract was evaporated. The resultant residue was chromatographed on a column (100 mL) of Diaion HP-20 (<50 mesh, Mitsubishi Chemical Ind., Japan) in 0.1 M phosphate buffer (pH 3.0). Active fractions eluted with 70% acetone–water were pooled, concentrated, and freeze-dried. The crude powder thus obtained was subjected to chromatography on a column (100 mL) of Diaion HP-20 (100–200 mesh) in 0.1 M phosphate buffer (pH 3.0). Stepwise elution was carried out with 20, 40, 60, and 80% methanol–water and then with 70% acetone–water. Fractions showing antimicrobial activity were pooled, concentrated, and applied to preparative HPLC on a reversed-phase column (15 μm, 2 × 25 cm) of YMC pack SH343 (Yamamura Chem. Lab.). The two active substances were separately eluted from the column with 24% acetonitrile in 0.02 M phosphate buffer (pH 4.0). The major substance eluted later was designated as halocyamine A, while the minor one eluted early was designated as B. Each of them was desalted by adsorption on a Diaion HP-20 column and elution with 80% methanol. The effluent was concentrated and freeze-dried to afford halocyamine A (82 mg) or B (22 mg) as a white powder.

Hydrolysis of Halocyamine A and Fractionation of Constituents. A solution of halocyamine A (27.5 mg) in 6 M HCl (16 mL) was heated in an evacuated and sealed glass tube for 24 h at 110 °C. The reaction mixture was diluted with water and concentrated under reduced pressure. After the procedure of dilution and concentration was repeated three times, the concentrate was subjected to preparative HPLC on a column of YMC pack SH343 with 10% methanol in 0.01 M phosphate buffer (pH 3.0) as the eluent. The fraction containing D_2, which was a major constituent except for glycine and histidine, was again chromatographed after removal of methanol on a column (10 mL) of Diaion SP-207 (Mitsubishi Chem. Ind.) with water. The effluent containing purified D_2 was concentrated and freeze-dried to give a white powder (5.0 mg). Component D_2 was similarly isolated from a hydrolysate of halocyamine B.

The optical configuration of each constituent isolated by preparative HPLC was determined according to Weinstein et al. (1982).

Amino Acid Composition of Halocyamine A and B. The composition analyses were carried out on a Hitachi 835 amino acid analyzer after hydrolysis with 6 M HCl at 110 °C for 24 h.

Amino Acid Sequence Determination. The sequences of halocyamine A and B were determined on an Applied Biosystems 477A automatic protein sequencer and a 120A PTH analyzer.

N-Acetylation of Halocyamine A. To a solution of halocyamine A (16 mg) in 2% $NaHCO_3$ (18 mL) and tetrahydrofuran (5 mL) was added acetic anhydride (0.031 mL, 10 equiv.), and the reaction was allowed to proceed under stirrng at room temperature for 1.3 h. The reaction mixture was concentrated and extracted with ethyl acetate. After being washed with aqueous $NaHCO_3$ and water, the extract was concentrated to dryness. The crude product was purified by preparative HPLC on a YMC pack SH343 column with 30% acetonitrile in 0.01 M phosphate buffer (pH 3.0) as a mobile

[1] Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; BDT, 6-bromo-8,9-didehydrotryptamine; DEPT, distortionless enhancement by polarization transfer; COSY, correlated spectroscopy; COLOC, correlation spectroscopy via long-range couplings; NOESY, nuclear Overhauser and exchange spectroscopy; HPLC, high-performance liquid chromatography; MIC, minimum inhibition concentration; PTH, phenylthiohydantoin; TLC, thin-layer chromatography.

phase. The main fractions in chromatography were pooled and extracted with ethyl acetate, and the extract was concentrated to afford *N*-acetylhalocyamine A as a white powder (5 mg).

Mass Spectroscopy. Secondary ionization (SI) mass spectra of halocyamines were obtained on a Hitachi M-80 mass spectrometer with a xenon ion beam source.

NMR Spectroscopy. ^{13}C and ^{1}H NMR spectra of halocyamines in dimethyl-d_6 sulfoxide were obtained on a Bruker AC-300 spectrometer. The δ values were recorded in ppm downfield from tetramethylsilane.

Antimicrobial Activity. Various bacteria and yeasts were allowed to grow at 37 °C for 10 h, at 28 °C for 2 days, and at 28 °C for 3 days in a series of plates of Penassay agar, Trypticase soy agar, and Sabouraud dextrose agar, respectively, each of which contained a different concentration of 2-fold serially diluted halocyamine A or B. The minimum inhibition concentrations (MIC) of halocyamines were determined by measuring the lowest concentrations required for complete inhibition of the growth of the test bacteria and yeasts.

Cytotoxic Activity against Mammalian Cultured Cells. The neuroblastoma N-18 cells (Amano et al., 1972) derived from mouse neuroblastoma C-1300 were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 2% (v/v) newborn calf serum (Nakashibetu Serum Center, Japan) and 2% horse serum (Gibco) at 37 °C under 10% CO_2–90% air in a Petri dish (Falcon) for 6–8 days. After addition of halocyamine A at a concentration of 160 μM, the cell culture was allowed to incubate at 37 °C under 10% CO_2–90% air for 24 h.

The Hep-G2 cells (Knowless et al., 1980) were subcultured in RPMI-1640 medium (Nisui Seiyaku, Japan) supplemented wth 10% fetal calf serum (Hyclone Lab.) at 37 °C under 5% CO_2–95% air. After 2 days, 200 μM halocyamine A was added, and the cells were cultured under the same conditions for 24 h.

Dissociated cells of embryonic (16th day) brain of Wistar rats were prepared and cultured at 37 °C under 10% CO_2–90% air in a poly(D-Lys)-coated plastic dish in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Gibco), 5% horse serum (Gibco), 100 μg/mL penicillin G (Meiji Seika, Japan), and 100 μg/mL streptomycin sulfate (Meiji Seika) (Matsuoka et al., 1987). The neuronal cells were prepared at the fourth day in the culture after treatment with 10 μM cytosine β-D-arabinofuranoside (*ara*-C) for 24 h to suppress multiplication of glial cells and cultured in the presence of 100 μM halocyamine A for 24 h under the same conditions as described above. Halocyamine A (10 mM) dissolved in dimethyl sulfoxide (DMSO) was used as the stock solution to be added to the culture.

Distribution among Hemocytes. Hemocytes obtained from the individual animals as described above were suspended in 21 mL of 66% Percoll solution containing 0.55 M NaCl and 0.1% EDTA, and the hemocyte suspension was centrifuged at 10000*g* for 15 min with a 35° angle rotor. Hemocytes were separated into several bands in the upper Percoll gradient (designated as the U fraction) and also recovered in the precipitate fraction (designated as the P fraction). The hemocytes in the upper part (U-1) and the lower part (U-2) of the U fraction and the P fraction were separately washed by centrifugation and extracted with 1 mL of acetone as described above. After filtration, the acetone extracts of the U-1, U-2, and P fractions were subjected to analytical HPLC as described above.

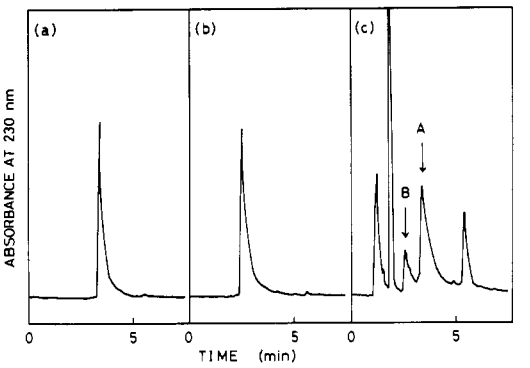

FIGURE 2: Analytical HPLC of (a) purified halocyamine A, (b) purified halocyamine B, and (c) acetone extract on a column (0.6 × 15 cm) of YMC pack A312. Isocratic elution was carried out with 34% acetonitrile in 0.01 M phosphate buffer (pH 4.0) as a mobile phase. The flow rate was 2 mL/min.

Table I: Physicochemical Properties of Halocyamine A and B

property	halocyamine A	halocyamine B
TLC,[a] R_f	0.52	0.49
HPLC,[b] R_t (min)	3.3	2.4
optical rotation (methanol)	$[\alpha]^{26}_D$ +5.2° (*c* 0.50)	$[\alpha]^{25}_D$ + 63.1° (*c* 0.498)
SI-MS, *m/z*	610, 612 (MH^+)	654, 656 (MH^+)
molecular formula	$C_{27}H_{28}H_7O_5Br\cdot {}^1/_2H_3PO_4\cdot {}^1/_2H_2O$	$C_{29}H_{32}N_7O_6Br\cdot {}^1/_2H_3PO_4\cdot 3H_2O$
UV (methanol) (nm) [(ϵ)]	203 (65 900), 232 (36 700), 282 (24 000)	203 (59 500), 232 (32 400), 282 (20 900)
IR (KBr) (cm^{-1})	1660, 1510, 1260, 1110, 1040, 800	1660, 1540, 1260, 1120, 1040, 810

	halocyamine A		halocyamine B	
elemental anal.	found	calcd	found	calcd
C	48.49	48.51	46.04	45.98
H	4.71	4.60	4.97	5.26
N	14.19	14.67	12.37	12.94
O		17.95		23.23
Br	11.14	11.95	10.79	10.55
P	2.04	2.32	2.01	2.04

[a] Adsorbent, silica gel plate 60 F-254 (Merck AG); solvent system, butanol–acetic acid–water (2:1:1). [b] Equipment, Waters 600 delivery system; column, YMC pack A312; mobile phase, 34% acetonitrile–0.01 M phosphate buffer (pH 3.0); flow rate, 2 mL/min.

Results

Isolation of Halocyamines. Halocyamines A and B were extracted with acetone from *H. roretzi* hemocytes and purified by three steps of chromatographies including reversed-phase HPLC. Elution profiles of halocyamine A and B in the crude and purified forms from a reversed-phase column of analytical HPLC are shown in Figure 2. Halocyamine A and B emerged at 3.3 and 2.4 min, respectively, under the HPLC conditions used. In the crude acetone extract, the ratio of halocyamine A to B was approximately 4:1, which corresponded with the ratio of yields for isolated halocyamines (as powder). From 100 g wet wt of the hemocytes obtained from 107 animals were isolated 82 mg of halocyamine A and 22 mg of B, with yields of 23% and 22%, respectively, the values of which can be estimated on the basis of the halocyamine content in the acetone extract. Since hemocytes weighing 100 g in wet weight have a packed volume of less than 80 mL, halocyamines (A plus B) seem to be present in hemocytes at a total concentration of higher than 5 mg/mL.

Physicochemical Properties of Halocyamine A and B. Table I shows physicochemical properties of halocyamine A and B. Both of them were water-soluble basic substances. Halocyamine A gave an $[\alpha]^{26}_D$ of +5.2° (*c* 0.50, methanol),

and its UV spectrum in methanol showed absorption maxima at 203 (ϵ 65900), 232 (36700), and 282 nm (24000). The IR spectrum in KBr indicated peptide-like patterns at 1660, 1510, 1260, 1110, 1040, and 800 cm^{-1}. Halocyamine B gave an $[\alpha]^{25}_D$ of +63.1° (*c* 0.498, methanol). Its UV and IR spectra showed absorption maxima similar to those of A. Both halocyamines showed positive Ehrlich, Pauly, and ninhydrin reactions and negative Dragendorff and Greig–Leaback reactions. The ^{13}C NMR spectra showed 27 carbon atoms for halocyamine A and 29 carbon atoms for halocyamine B (Table II). Molecular formulas of halocyamine A and B were determined to be $C_{27}H_{28}N_7O_5Br\cdot{}^1/_2H_3PO_4\cdot{}^1/_2H_2O$ and $C_{29}H_{32}N_7O_6Br\cdot{}^1/_2H_3PO_4\cdot 3H_2O$, respectively, by elemental analyses and SI-MS data [*m/z* 610 and 612 (MH^+) for halocyamine A and *m/z* 654 and 656 (MH^+) for halocyamine B].

Determination of Structure for Halocyamine A and B. Halocyamines gave mono-*N*-acetyl derivatives by acetylation as revealed by the NMR spectra. Examination by an amino acid analyzer showed that the 6 M HCl hydrolysate of halocyamine A contained histidine, glycine, and one unknown ninhydrin-positive component (D_2) in a molar ratio of 1.0:0.94:0.68 (see Table III) and that the hydrolysate of halocyamine B contained histidine, threonine, and component D_2 in a molar ratio of 1.0:1.22:0.82 (see Table III). Furthermore, the presence of an indole ring and an aromatic ring as constituents was suggested from the UV (Table I) and ^1H NMR spectra of halocyamines (Table II). In the carbonyl regions of ^{13}C NMR spectra, three signals due to acid amide groups were observed at 171.96, 170.27, and 167.39 ppm in halocyamine A and at 171.34, 168.52, and 166.85 ppm in halocyamine B (Table II).

The unknown component D_2 isolated from the acid hydrolysates of halocyamines showed absorption maxima at 218 (shoulder, ϵ 7290) and 278 (3150) nm in the UV spectrum. In the ^1H NMR spectrum of D_2, the signals were observed at 3.93 ppm (dd, J = 7.8, 5.1 Hz) for a methine proton, 3.16 (dd, J = 5.1, 14.7 Hz) and 3.00 ppm (dd, J = 7.8, 14.7 Hz) for a methylene proton, and 6.90 (d, J = 8.1 Hz), 6.83 (d, J = 2.0 Hz), and 6.74 ppm (dd, J = 8.1, 2.0 Hz) for a trisubstituted aromatic ring. These data, as well as the EI-MS data showing the parent peak of *m/z* 197, suggested that D_2 is dihydroxyphenylalanine. The positions of the two hydroxy groups of D_2 were assigned to the 3- and 4-positions by CH-COSY of the ^{13}C NMR spectrum at 145.03, 143.72, 128.42, 116.76, and 119.45 ppm, according to Nelson et al. (1972). The COLOC data also confirmed this assignment. Furthermore, D_2 was identified with an authentic sample of 3,4-dihydroxyphenylalanine (DOPA) by TLC and HPLC.

By subtraction of the molecular formula and ^{13}C NMR signals for the three constituents determined so far (histidine, glycine, and DOPA in halocyamine A and histidine, threonine, and DOPA in B) from those for the whole molecules of halocyamines, the common residual moiety (BDT) was estimated to be $C_{10}H_8N_2Br$. UV spectra suggested that this moiety contained an indole chromophore. The NMR spectral data of BDT in halocyamine A (Table II, part 1) indicated that a cis-disubstituted double bond (J = 9.5 Hz) was attached to the indole ring and that one of the double-bond protons was coupled with the acid amide proton at 9.04 ppm (d, J = 10.5 Hz). From the results of ^1H–^1H COSY, the six-membered ring moiety of the indole ring was trisubstituted. As shown in Table II (part 1), nuclear Overhauser effects (NOESY) were observed between the BDT-8 proton (5.92 ppm) and the BDT-4 proton (7.55 ppm) and between the BDT-9-NH proton (9.04 ppm) and the BDT-2 proton (7.73 ppm). From these findings the binding positions of the bromine atom and the enamine moiety were determined to be BDT-6 and BDT-3, respectively. Thus, BDT consists of a 6-bromo-8,9-didehydrotryptamine chromophore (Figure 3). In halocyamine B, the results of the NMR data (Table II, part 2) gave the same conclusion with the structure of BDT (Figure 3).

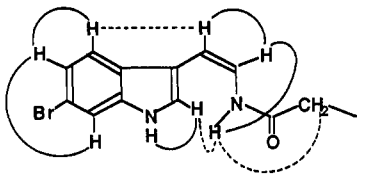

FIGURE 3: Assignment of BDT by COSY (—) and NOESY (--).

The sequence of these constituents of the respective halocyamine was determined by NMR analysis and Edman degradation analysis. Nuclear Overhauser effect was observed in halocyamine A (Table II, part 1) between the acid amide proton of glycine (9.16 ppm) and the 2-methine proton of DOPA (4.43 ppm), in addition to the effect between the BDT-9-NH proton and the methylene protons of glycine. By analysis of halocyamine A on a protein sequencer, histidine was detected as the N-terminal amino acid residue (13.5% recovery), and glycine was detected at the third position from the N-terminus (58.4%) (Table IV). The configurations of histidine and DOPA were assigned to the L configuration by HPLC (Weinstein et al., 1982). The structure of halocyamine A was thus determined to be L-histidyl-L-6,7-dihydroxyphenylalanylglycyl-6-bromo-8,9-didehydrotryptamine as shown in Figure 1. The structure of halocyamine B was similarly determined. Nuclear Overhauser effects were observed in halocyamine B (Table II, part 2) between the acid amide proton of histidine and the 2-methine proton of DOPA, between the acid amide proton of DOPA and the 2-methine proton of threonine, and between the BDT-9-NH proton and the 2-methine proton of histidine. Threonine was detected as the N-terminal amino acid residue (116% recovery), and histidine was detected at the third position from the N-terminus (26.9%) by protein sequencer analysis (Table IV). Histidine, threonine, and DOPA were again assigned to the L form. Thus, the structure of B was determined to be L-threonyl-L-6,7-dihydroxyphenylalanyl-L-histidyl-6-bromo-8,9-didehydrotryptamine as shown in Figure 1.

Antimicrobial Activity. Halocyamine A showed inhibitory effects on the growth of the Gram-positive bacteria *B. subtilis* (MIC 50 μg/mL), *B. megaterium* (MIC 50 μg/mL), and *B. cereus* (MIC 100 μg/mL) and the yeast *Cryptococus neoformans* (MIC 100 μg/mL) (Table V). Halocyamine B showed an antimicrobial spectrum similar to that of A (data not shown).

Cytotoxic Activity. The neuroblastoma N-18 cells cultured in the presence of halocyamine A (160 μM) for 24 h became round, detached from the dish, and died (Figure 4). All of the neurites were shrunk. In the case of neuronal cells cultured from rat fetal brain, addition of halocyamine A (100 μM) resulted in degeneration of the neurite and, furthermore, of the soma (Figure 5). We also demonstrated that almost all of the Hep-G2 cells died and floated in the medium after treatment with 200 μM halocyamine A for 24 h, while cytochalasin B and colchicine, inhibitors for the action of cytoskeletal proteins, did not exert such an effect on cell growth at the same concentrations as that of halocyamine A (data not shown).

Distribution among Hemocytes. Hemocytes were separated into the three fractions, U-1, U-2, and P fractions. Under

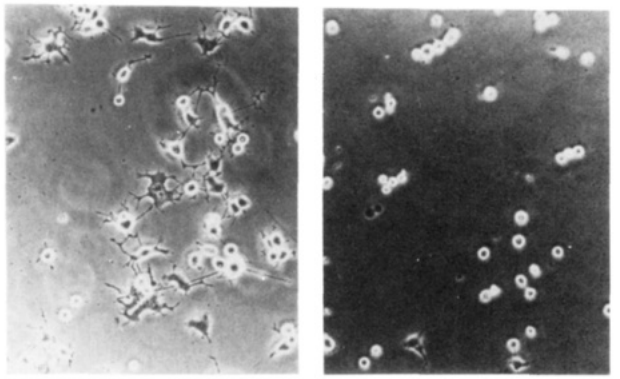

FIGURE 4: Cytotoxic effect of halocyamine A against neuroblastoma N-18 cells. Cells were cultured in the presence and absence of halocyamine A at a concentration of 160 μM for 24 h. DMSO at a concentration of 0.5% was used as a solvent control.

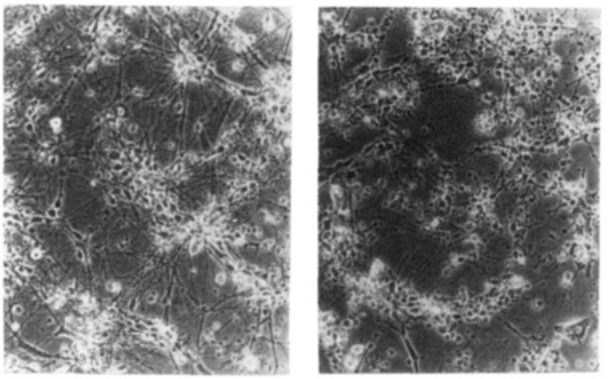

FIGURE 5: Cytotoxic effect of halocyamine A against neuronal cells cultured from rat fetal brain. Cells were cultured in the presence and absence of halocyamine A at a concentration of 100 μM for 24 h. DMSO at a concentration of 0.5% was used as a solvent control.

microscopic observations, it was found that the U-1 and U-2 fractions contained several cell types of hemocytes but the P fraction contained only the "morula"-like cells, which are the most abundant (more than 50%) among the *H. roretzi* hemocytes. Halocyamine A and B were detected in the P fraction (i.e., morula-like cells) but scarcely found in the U-1 or U-2 fraction (Figure 6). Thus, the intracellular total concentration of halocyamine A and B in the morula-like cells may reach a level higher than about 10 mg/mL.

Discussion

This paper describes the isolation, structure determination, characterization, and distribution of novel antimicrobial substances designated as halocyamine A and B from hemocytes of the solitary ascidian *H. roretzi*. Prior to this paper, several antimicrobial substances have been isolated from colonial ascidians (Rinehart et al., 1981, 1984; Ireland et al., 1982; Kobayashi et al., 1984, 1988; Ishibashi et al., 1987), but all of them have been extracted from whole bodies of animals. Thus, halocyamine A and B were the first antimicrobial substances that have been isolated from a defined tissue, the hemocyte, of the ascidians, although the antimicrobial activities of the two halocyamines were lower than those of the antimicrobial substances of colonial ascidians described above by approximately 2 orders of magnitude.

The structures of halocyamine A and B were determined to be His-DOPA-Gly-BDT and Thr-DOPA-His-BDT, re-

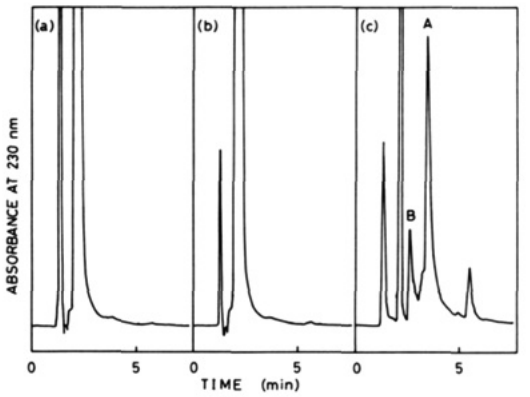

FIGURE 6: Analytical HPLC on a column (0.6 × 15 cm) of YMC pack A312 of acetone extracts of (a) U-1 (20 μL), (b) U-2 (20 μL), and (c) P (1 μL) fractions obtained by Percoll gradient centrifugation of *H. roretzi* hemocytes. The elution conditions were the same as those described in Figure 2.

spectively (Figure 1). Thus, they have interesting structural features. One of the features to be noticed in these structures is the presence of DOPA as a constituent. Two kinds of substances that contain DOPA and/or DOPA derivatives have been isolated from hemocytes of ascidians. One of them is a group of "tunichromes", reducing blood pigments found in the ascidians *Ascidia nigra* and *Molgula manhattensis* (Bruening et al., 1985; Oltz et al., 1988). Tunichromes contain DOPA and/or DOPA derivatives and pyrogallol groups. Another one is "ferreascidin" from the ascidian *Pyura stolonifera* (Hawkins et al., 1986; Dorselt et al., 1987). This compound is a DOPA-containing protein and has been proposed to function as an iron-binding protein. Both tunichrome and ferreascidin have not been reported so far to show antimicrobial or cytotoxic activity. The second structural feature of halocyamines is that one of their constituents is a tryptamine derivative containing a bromine atom. Almost all "eudistomines", antimicrobial and antiviral substances isolated from the ascidian *Eudistoma olivaceum*, have been reported to contain one atom of bromine (Reinhart et al., 1984; Kobayashi et al., 1984). Bromoeudistomin D, a chemically synthesized derivative containing two atoms of bromine, is known to induce contraction of skeletal muscle by releasing calcium from sarcoplasmic reticulum (Nakamura et al., 1986). Eudistomidin A, a novel calmodulin antagonist isolated from the ascidian *Eudistoma glandes*, also contains one bromine atom (Kobayashi et al., 1986).

H. roretzi has several cell types of hemocytes (Fuke, 1979). By analytical HPLC, halocyamines A and B were only found in the morula-like cells (Wright, 1981). With respect to the biosynthesis of halocyamines, two possibilities could be considered. One is that halocyamines could be byproducts or metabolites derived from foreign substances as in the case of tetrodotoxin in fishes. Another is that halocyamines could be synthesized inside morula cells. Our findings that several DOPA-containing peptides were isolated from whole hemocytes of *H. roretzi* (Azumi, preliminary result) may indicate the latter possibility. The determination of amino acid sequences of these peptides, now in progress, may clarify whether they are the precursors of halocyamines or not.

Although the biological role of halocyamines in relation to defense mechanisms of *H. roretzi* is still unknown, intracellular and extracellular functions of halocyamines could be considered. Since almost all of the hemocytes of *H. roretzi* showed phagocytosis (Fuke, 1979), halocyamines highly accumulated in morula-like cells (the total concentration may be higher than 10 mg/mL) would function intracellularly as antibacterial

Table II: NMR Spectra Data of Halocyamines A and B [δ (ppm), in Dimethyl-d_6 Sulfoxide]

		carbon (75 MHz)		proton (300 MHz)				
	position[a]	ppm	mult[b]	ppm	mult	J (Hz)	^{1}H–^{1}H COSY	NOESY
				(Part 1) Halocyamine A				
BDT	1			11.85	s			
	2	124.72	CH	7.73	s			
	3	109.59	Q					
	3a	125.59	Q					
	4	119.90	CH	7.55	d	8.5		
	5	121.73	CH	7.15	dd	8.5, 2.0		
	6	114.24	Q					
	7	114.12	CH	7.61	d	2.0		
	7a	136.43	Q					
	8	102.10	CH	5.92	d	9.5		
	9	118.39	CH	6.69	t	9.5		
	9-NH			9.04	d	10.5		
Gly	1	167.39	Q					
	2	42.72	CH_2	3.97	dABq	5.5		
	2-NH			9.16	br t	6.5		
DOPA	1	171.96	Q					
	2	54.94	CH	4.43	m			
	3	36.46	CH_2	2.65	dd	14.0, 10.5		
				2.98[c]				
	4	128.42	Q					
	5	116.76	CH	6.81	d	1.8		
	6	145.03	Q					
	7	143.72	Q					
	8	115.24	CH	6.59	d	8.0		
	9	119.47	CH	6.47	dd	8.0, 1.8		
	2-NH			8.84	br d	6.8		
His	1	170.27	Q					
	2	53.10	CH	3.84	t	5.0		
	3	29.62	CH_2	2.93	dd	15.5, 7.5		
				3.03	dd	15.5, 5.0		
	4	132.88	Q					
	6	135.13	CH	7.65	s			
	8	116.37	CH	6.90	s			
				(Part 2) Halocyamine B				
BDT	1			11.60	s			
	2	124.79	CH	7.68	d	1.5		
	3	109.56	Q					
	3a	125.56	Q					
	4	119.99	CH	7.49	d	8.0		
	5	121.83	CH	7.09	dd	8.0, 2.0		
	6	114.29	Q					
	7	114.03	CH	7.52	d	2.0		
	7a	136.42	Q					
	8	102.79	CH	5.88	d	9.5		
	9	18.47	CH	6.57	t	10.0		
	9-NH			9.22	d	10.0		
His	1	168.52	Q					
	2	52.24	CH	4.78	dd	9.0, 5.5		
	3	29.50	CH_2	2.93	dd	15.0, 9.0		
				3.08	dd	15.0, 5.0		
	4	130.29	Q					
	6	133.67	CH	8.47	br s			
	8	116.94	CH	7.16	br s			
	2-NH			8.64	d	7.5		
DOPA	1	171.34	Q					
	2	54.82	CH	4.42	m			
	3	36.47	CH_2	2.56	dd	14.0, 10.0		
				2.78	dd	14.0, 4.0		
	4	128.01	Q					
	5	116.57	CH	6.65	d	1.5		
	6	144.84	Q					
	7	143.76	Q					
	8	115.32	CH	6.53	d	8.0		
	9	119.88	CH	6.40	dd	8.0, 1.5		
	2-NH			8.68	d	8.0		
Thr	1	166.85	Q					
	2	57.71	CH	3.55	d	6.0		
	3	65.79	CH	3.87	q	6.0		
	4	19.20	CH_3	1.10	d	6.0		

[a] The signals were assigned by ^{1}H–^{1}H and ^{13}C–^{1}H COSY methods and confirmed by a COLOC method. [b] The multiplicities were determined by a DEPT method. [c] 3.00 ppm, dd, J = 14 and 5.6 Hz, in CH_3OD.

Table III: Amino Acid Compositions of Halocyamine A and B

amino acid	relative amount A	B
His	1.00[a]	1.00[a]
Thr		1.22
Gly	0.94	
DOPA[b]	0.68[c]	0.82[c]

[a] Amount of histidine residue in the respective halocyamine was defined as 1.00. [b] Standard amino acid, DOPA, was eluted at the same position as that of leucine. This amino acid was determined as DOPA on the basis of the NMR data of isolated fragment D_2 (see Results). [c] Determined from the color yield of standard DOPA.

Table IV: Amino Acid Sequences of Halocyamine A and B

cycle no.	recoveries of PTH-amino acids (%)[a] A	B
1	His (14)	Thr (116)
2	DOPA[b] (5)	DOPA[b] (11)
3	Gly (58)	His (27)
4	nd[c]	nd[c]

[a] Determined on the basis of the initial amount of the respective halocyamine. [b] Standard PTH-DOPA was eluted at the same position as that of PTH-His. This PTH-amino acid was determined as PTH-DOPA on the basis of NMR spectra (Table II) and amino acid composition analysis (Table III) of the respective halocyamine (see Results). [c] Not detected.

Table V: Antimicrobial Spectrum of Halocyamine A

test organisms	MIC (μg/mL) PA[a]	TSA[a]	SDA[a]
Staphylococcus aureus FDA 209p	>100	100	
Bacillus subtilis NIHJ PCI219	50	50	
Bacillus cereus FDA 5	100		
Bacillus megaterium IFO 12108	50		
Escherichia coli NIH JC-2	>100	>100	
Escherichia coli PG 8s	25		
Proteus vulgaris IFO 3988	>100		
Candida albicans IFO 0583		>100	>100
Cryptococcus neoformans IFO 0410		>100	100
Aspergillus niger IFO 4066		>100	>100
Penicillium chrysogenum IFO 4626		>100	>100

[a] Medium: PA, Penassay agar at 37 °C for 10 h; TSA, Trypticase soy agar at 28 °C for 2 days; SDA, Sabouraud dextrose agar at 28 °C for 3 days.

agents against bacteria that had invaded the hemolymph and had been phagocyted into the cells. On the other hand, a unique cellular response of hemocytes designated as the "contact reaction" has been reported in *H. roretzi* (Fuke, 1980). This reaction is reciprocal lyses of hemocytes possibly triggered by the self- and non-self-recognition mechanism between allogenic hemocytes, when they are allowed to contact with each other. If this recognition mechanism could work also when hemocytes contact foreign materials such as bacteria and the lysis of hemocytes could occur, halocyamines released from the cell would function extracellularly as antimicrobial agents against bacteria in the hemolymph even when they are diluted in the hemolymph. Subcellular localization of halocyamines and elucidation of their release upon the contact reaction would clarify their roles in defense mechanisms of *H. roretzi*.

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Registry No. Halocyamine A, 122548-03-2; halocyamine B, 122548-04-3.

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