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Note

Separation of mycosporine-like amino acids in marine organisms using reversed-phase high-performance liquid chromatography

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Mycosporine-like amino acids are water-soluble nitrogenous substances with strong absorption maxima in the range 310–360 nm[1]. As shown in Fig. 1, nine mycosporine-like amino acids, mycosporine-Gly (3) (λ_{max}310 nm)[2], palythine (4) (λ_{max} 320 nm)[3–5], shinorine (5) (λ_{max}334 nm)[6,7], porphyra-334 (6) (λ_{max}334 nm)[7,8], asterina-330 (7) (λ_{max}330 nm)[9], palythinol (8) (λ_{max}332 nm)[10], palythenic acid (9 and 10) (λ_{max} 337 nm)[11] and palythene (11) (λ_{max}360 nm)[10,12], and two related compounds (1[13] and 2[14]) have been isolated from several marine animals (starfish, zoanthid, mussel and cod eggs) and plants (red algae). However, their rôles and biogenesis *in vivo* remain unknown. From their structural similarity and the variety of origins, the series of compounds are supposed to be related to one another, probably originating from shikimic acid, and to be distributed widely among numerous marine organisms. Preliminary results of our survey on the distribution of mycosporine-like amino acids in

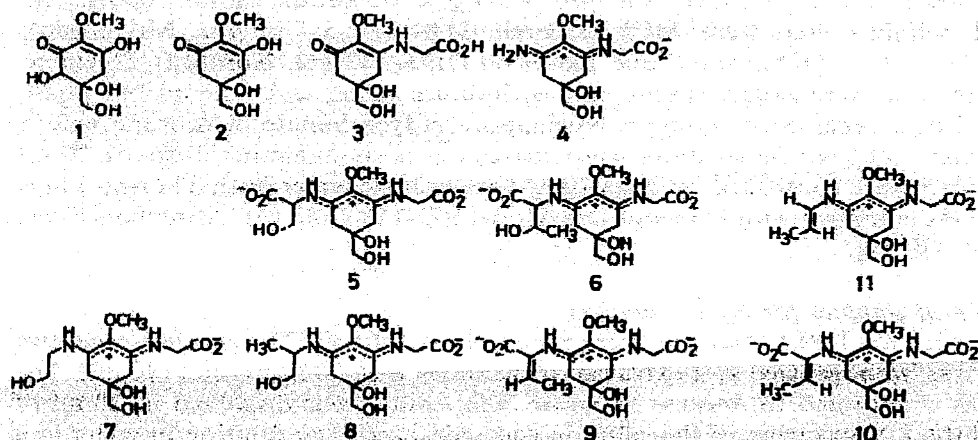

Fig. 1. Structures of mycosporine-like amino acids 3–11 and the related substances 1 and 2 isolated from marine organisms.

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marine organisms have shown that they are almost ubiquitous among algae and invertebrates, suggesting that they have important rôles in biological systems. Our interest in mycosporine-like amino acids is focused on their compositions in marine organisms and the structures of their biogenetically related metabolites. In the present work, we describe a method for separation of mycosporine-like amino acids 3–10 and for rapid determination of their compositions in marine organisms by using reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals

MCI gel CHP-20 (porous styrene polymer, 75–150 μm; Mitsubishi), charcoal (activated, chromatographic grade; Wako), acetic acid and ethanol (Wako) and glass-distilled water were used.

Samples

Mycosporine-like amino acids 3–6, 9 and 10 were isolated from the ascidian *Halocynthia roretzi*, and 7 was isolated from the starfish *Asterina pectinifera* as previously reported[9,11]. Substance 8 was kindly donated by Dr. Takano[10].

The seaweeds *Geldium amansii* and *Codium fragile* were collected at Misaki in Kanagawa Prefecture in April, the seaweed *Padina crassa* and the sea sponge *Halychondira japonica* at Okinawa Islands in May, the zoanthid *Palythoa tuberculosa* at Ishigaki Island in May, and the mussel *Mythilus edulis*, the starfish *Asterina pectinifera* and the ascidian *Halocynthia roretzi* at Asamushi in Aomori Prefecture in November. The antarctic krill *Euphasia sperba* was obtained as frozen material. Marine organisms were stored at −20°C until they were used.

Chromatographic apparatus and conditions

The HPLC apparatus consisted of an ALTEX pump Model 100A, a Rheodyne injector Model 7125 equipped with a 20-μl loop, a JASCO spectrophotometer Model UVIDEC-100III and a System Instruments Intelligent Integrator Model 7000A. The prepacked columns used were MCI Hypersil ODS HY-5U (5 μm, Mitsubishi), ALTEX Ultrasphere ODS (5 μm) and Develosil ODS-3 (3 μm, Nomura), 25 cm × 4.6 mm I.D., and were eluted isocratically with dilute acetic acid. The mobile phase was filtered and degassed by using a Nucleopore polycarbonate membrane with a pore diameter of 0.2 μm. Separations were carried out at room temperature (*ca.* 20°C) or at a temperature controlled by a constant-temperature water-bath Thermo Elites Model BH-41 equipped with Neocool Dip Model BD-11 (Yamato). Absorbance was detected at 330 nm.

Preparation of samples for HPLC analysis

Samples for HPLC analysis were prepared as follows. The marine organism (*ca.* 20 g) was homogenized and extracted three times with 70% ethanol (40 ml). The extract was evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml of water and the UV spectrum of the solution was measured after dilution in water to a convenient concentration. An aliquot (0.5–10 ml) of the solution, whose optical density was *ca.* 1 at around 330 nm when it was diluted in water to 40 ml, was applied on

TABLE I

RETENTION TIMES OF MYCOSPORINE-LIKE AMINO ACIDS ON THREE ODS COLUMNS AT ROOM TEMPERATURE

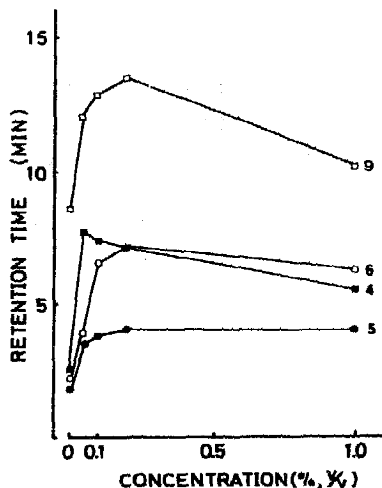
Eluent: 0.1 % acetic acid in water; flow-rate 1.0 ml/min.

Column	*Retention time (min)*			
	5	*6*	*4*	*9*
Hypersil	3.81	6.51	7.44	13.00
Ultrasphere	3.85	8.62	8.84	20.19
Develosil	4.39	9.04	2.39	10.26

a column of CHP-20 (5 cm × 7 mm I.D.), which was then eluted with water (15 ml). The eluate was applied on a column of charcoal (3.5 × 1.5 cm I.D.), which was eluted with water (20 ml) and then with 50 % ethanol, monitoring the UV absorption. The 50 % ethanol fractions showing UV absorption in the range 310–340 nm were collected (50–100 ml) and evaporated to dryness *in vacuo*. The residue was dissolved in 500 μl of water and aliquots of the solution were used for HPLC analysis.

RESULTS AND DISCUSSION

Mycosporine-like amino acids 3–10 were eluted from a charcoal column by addition of 50 % ethanol to water, whereas substances 1, 2 and 11 could not be recovered from the column even by use of higher contents of ethanol. On the other hand, only substance 11 was absorbed on a column of CHP-20 and was eluted from the column by addition of 10 % ethanol to water. For the HPLC analysis of mycosporine-like amino acids, crude extracts of marine organisms were purified by a charcoal



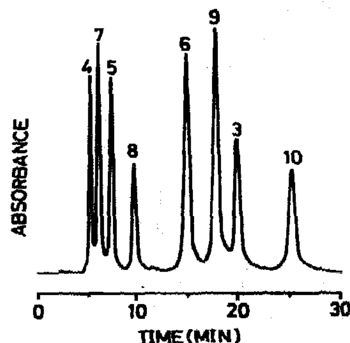
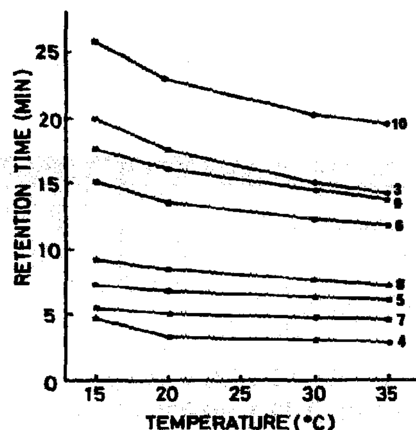


Fig. 2. Effect of the concentration of acetic acid in the mobile phase on retention times of mycosporine-like amino acids 4–6 and 9. Column: Hypersil ODS (25 cm × 4.6 mm I.D.). Flow-rate: 1 ml/min. Room temperature.

Fig. 3. Separation of mycosporine-like amino acids 3–10 on Develosil ODS column (25 cm × 4.6 mm I.D.). Mobile phase: 0.02 % acetic acid in water; flow-rate, 1.0 ml/min. Temperature: 15°C.



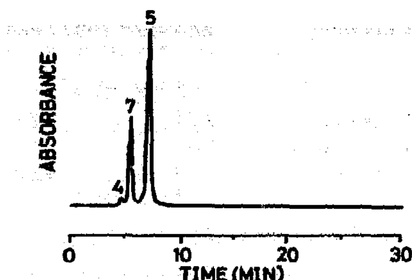


Fig. 4. Effect of temperature on retention times of mycosporine-like amino acids 3–10. Other conditions as in Fig. 3.

Fig. 5. Chromatogram of mycosporine-like amino acids of the red alga *Geldium amansii*. Peak numbers and chromatographic conditions as in Fig. 3.

column following a CHP-20 column. A mixture of components purified from each extract was dissolved in water and aliquots (1–20 μl) of the solution were injected for HPLC analysis.

The separations of mycosporine-like amino acids were carried out on reversed-phase columns using isocratic elution with dilute acetic acid. The retention times of amino acids 4–6 and 9 were obtained on three different columns under identical solvent and flow conditions at room temperature (Table I). The retention time of 4 on Develosil ODS was much shorter than those on the other two columns. The difference may result from different amounts of free silanol groups. However, the substances 5, 6 and 9 were eluted in the same order from the three columns, according to their hydrophobic properties.

On Hypersil ODS, maximum retentions of 5, 6 and 9 were obtained at 0.2% acetic acid, whereas that of 4 was achieved at 0.05% acetic acid (Fig. 2). The difference is due to the presence of two carboxyl groups in substances 5, 6 and 9 but only one in 4.

Complete separation of substances 3–10 was achieved with Develosil ODS

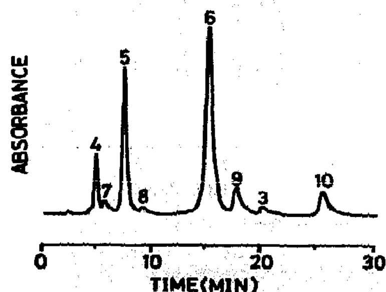


Fig. 6 Chromatogram of mycosporine-like amino acids of the antarctic krill *Euphasia sperba*. Peak numbers an l chromatographic conditions as in Fig. 3.

TABLE II

COMPOSITIONS OF MYCOSPORINE-LIKE AMINO ACIDS IN MARINE ORGANISMS

	Relative molar ratio							
	3	*4*	*7*	*8*	*5*	*6*	*9*	*10*
Alga								
Rhodophyceae								
Geldium amansii	–	1.0	9.5	–	23	–	–	–
Chlorophyceae								
Codium fragile	–	1.0	0.1	–	0.1	1.3	–	–
Phaeophyceae								
Padina crassa	–	1.0	0.9	1.9	15	9.4	–	–
Invertebrate								
Porifera								
Halychondria japonica	–	1.0	0.1	<0.1	0.2	0.1	<0.1	–
Coelenterate								
Palythoa tuberculosa	0.07	1.0	–	0.03	–	–	–	–
Arthropoda								
Euphasia speba	–	1.0	0.1	<0.1	1.9	3.8	0.4	0.6
Mollusca								
Mythilus edulis	0.2	1.0	0.1	–	2.4	0.9	0.2	–
Echinodermata								
Asterina pectinifera	0.2	1.0	0.2	<0.1	<0.1	–	–	–
Protochordata								
Halocynthia roretzi	6.8	1.0	<0.1	<0.1	0.6	0.9	0.3	<0.1

using 0.02% acetic acid as mobile phase at 15°C (Fig. 3). As shown in Fig. 4, their retentions depended on temperature to different extents, and baseline separation of each peak was obtained at 15°C. Under these conditions, substances 3–10 were separately eluted in order of their hydrophobic properties, from 4 of the lowest hydrophobicity to 10 of the highest, within 30 min.

HPLC analyses of mycosporine-like amino acids in about 40 marine organisms were carried out under the optimum conditions described above and good separations were obtained in all cases. In Figs. 5 and 6 are displayed chromatographic profiles of the red alga *Geldium amansii* and the antarctic krill *Euphasia sperba*, respectively. Each peak was identified by comparing the retention time with that of an authentic sample and the amount of each substance was determined on the basis of the peak area obtained by an integrator. Some of the results are summarized in Table II. The data show that substance 4 is contained in all the organisms examined and occurs as the main component in several organisms.

The wide distribution of these mycosporine-like amino acids could be explained in terms of the food chain. The differences in their compositions may result from differences in the biological systems (*cf.*, metabolism) of the marine organisms.

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