Reports

Characterization of Compounds That Induce Symbiosis Between Sea Anemone and Anemone Fish

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The species-specific partnership between the sea anemone and anemone fish in many parts of the Indo-Pacific region is a well-known phenomenon. Chemicals secreted by the sea anemone to elicit symbiotic behavior of the fish have been studied for two host-guest pairs, *Radianthus kuekenthali* (sea anemone)–*Amphiprion perideraion* (anemone fish) and *Stoichactis kenti*–*A. ocellaris*. A new pyridinium compound, amphikuemin, which induces characteristic attracted swimming (toward the chemical stimulus) in *A. perideraion*, has been isolated from *R. kuekenthali* and characterized. Several other chemicals that elicit characteristic symbiotic movements have also been identified.

THE SYMBIOSIS BETWEEN ANEMONE fishes and giant sea anemones has long interested biologists (*1*–*3*). Since Davenport and Norris (*4*) did their experimental analysis, it has been believed that an acclimating process is needed to establish a symbiotic association between anemone fishes and sea anemones and that protection against fatal sea anemone sting is acquired by the fish during this process (*5*, *6*). However, Miyagawa demonstrated (*7*) that naïve juveniles of four species of anemone fish, including *Amphiprion perideraion* and *A. ocellaris* described below, are innately protected from anemone species that are symbiotic with *Amphiprion*. She also found that the fish recognize their specific partner anemone on the basis of chemicals secreted by the anemone, thus leading to species-specific symbiosis; visual cues do not play an important role in recognition of the host at the first encounter. After the planktonic period, which lasts for 7 to 10 days after hatching, juveniles enter the benthic life, search for their partner anemone, and begin the symbiosis. However, the chemical agents as well as the mechanism leading to this species-specific symbiosis remain open to question.

We report here the characterization of chemicals that induce symbiosis or synomones (*8*) of marine origin. Two host-guest pairs were used in these studies, *Radianthus kuekenthali* Kwietniewski [=*Heteractis crispa* (*9*)] sea anemone–*Amphiprion perideraion* Bleeker (fish) and *Stoichactis kenti* Haddon & Shackleton [=*Stichodactyla gigantea* (*9*)]–*A. ocellaris* Cuvier. Because of the difficulty of breeding *A. perideraion* in an aquarium, young fish collected in Okinawa waters were used for the assay (Fig. 1).

Figure 1 depicts the assay vessel and the responses of a fish attracted to stimuli extracted from an anemone: *A. perideraion* after administration of the crude mucous extract of *R. kuekenthali* (Fig. 1A) or pure "amphikuemin" (Fig. 1B). Note the similarity in the occurrence of repeated movements toward the sample ("attracted swimming") of the fish induced by the two different samples. Active upstream and downstream movement (from section 1 to 8) by the fish is because of its sensitivity to the chemical stimulus. When control seawater or an inactive fraction was administered, the fish stayed throughout at the starting point (Fig. 1C). In contrast, when the crude mucous extract of its symbiotic anemone (*S. kenti*) had reached the fish *A. ocellaris* (after 6 to 7 minutes) the movements of the fish were mostly confined to the area (from section 5 to 8) of sample administration (Fig. 1D).

The sea anemones were collected off Sesoko Island near the Okinawa Expo Memorial Park Aquarium. They were squeezed at 1-week intervals to collect the mucus. Alternatively, the animals were killed and homogenized (Fig. 2). The mucus or homogenate was extracted with ethyl acetate after *p*H of the medium had been adjusted to 10.5; the organic layer induced much weaker attracted swimming activity response than that in-

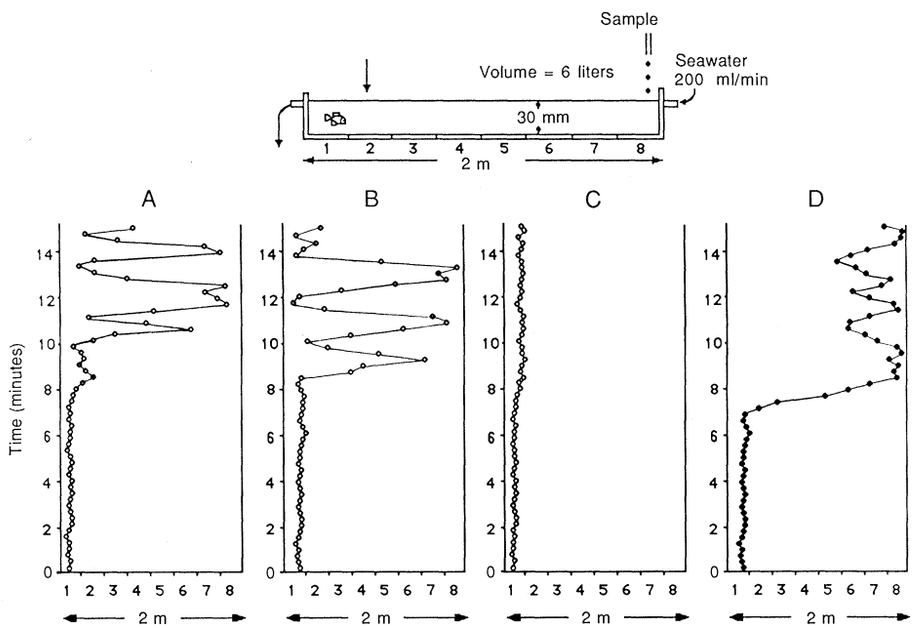

Fig. 1. Bioassay of the synomones between two symbiotic pairs of sea anemones and anemone fishes. The acrylic assay vessel, 200 cm (length) by 5 cm (height) by 8 cm (width), was filled to a height of 3 cm and continuously supplied with seawater from one end at a flow rate of 200 ml/min. The vessel was marked every 25 cm into eight sections to monitor the behavioral response of a fish. Fish *A. perideraion*, 2 to 4 cm in length, were collected in Okinawa waters and were kept in a tank with *R. kuekenthali* for a period of up to 3 months; a fish was separated from the sea anemone 30 minutes prior to the assay. One fish was confined to the downstream end for 15 minutes by a transparent plastic mesh introduced at the point indicated by the arrow; only the fish that did not swim upstream for 5 minutes after removal of the mesh were used (about 50% of the test fish). Individual samples of the test chemicals were dissolved in seawater and added dropwise, 3 ml/min, from the upstream end. Behavior and movement of the fish were observed for 15 minutes, while its locations were recorded by an observer every 15 seconds using the marked sections as the guide. Assays were repeated twice for each sample with a different fish. The graph illustrates the movement of an experimental fish exposed to a stream of crude mucous extract (A), amphikuemin **1**, tank concentration $\sim 10^{-10} M$ (B), and control seawater (C). The same vessel was used in the assays with *A. ocellaris* and *S. kenti* (D); however, two naïve test fish were used for each assay, because the more synchronous movement of this species led to less variation in assay results when the fish were paired. The fish were attracted to the chemical stimulus and stayed around sections 5 to 8 (D).

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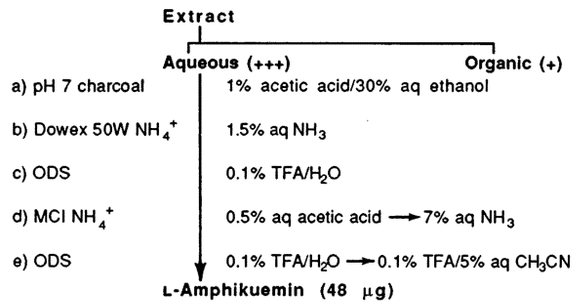

Fig. 2. Isolation of amphikuemin **1**. Ten *Radianthus kuekenthali* (15 kg) were homogenized, then extracted three times with acetic acid: aqueous methanol (1%:20%); the resulting extract was adjusted to *p*H 10.5 and extracted with ethyl acetate. The organic layer contained the aplysinopsins and dihydroaplysinopsins (see text), but the bulk of activity was retained in the aqueous phase. After removal of ethyl acetate from the aqueous phase, the latter was passed through a column of active charcoal (Nakarai, 500 ml), and the column was eluted with acidic aqueous ethanol. The eluate was successively fractionated by chromatography with Dowex 50W NH_4^+ (Nakarai), octadecylsilane (ODS) (YMC gel, Yamamura Kagaku), MCI gel CK10V NH_4^+ (Mitsubishi), and ODS (Radial Pak, Waters) to yield 48 μg of amphikuemin. TFA, trifluoroacetic acid.

duced by the aqueous layer. Fractionation of the aqueous layer, according to Fig. 2, yielded 48 μg of a cationic compound from *R. kuekenthali* as the sole active factor as monitored by the attracted swimming assay. Its strong activity was reproducible, and the induced behavioral response was comparable to that seen from assays with the crude extract. The synomone from the host that is recognized by the young *A. perideraion* has been named amphikuemin. The structure **1** of amphikuemin (Fig. 3) was determined by (i) hydrolysis, which yielded L-lysine; (ii) spectroscopic data: mass-to-charge ratio (*m/z*) 308 (M^+, by secondary ionization mass spectrometry), ultraviolet absorption maximum (in H_2O) 220 nm and molar extinction coefficient (ε, 8300) and 260 nm (ε, 4500), proton nuclear magnetic resonance (NMR) spectroscopy (*10*); and (iii) synthesis. The synthetic L-specimen was identical with natural amphikuemin in all respects including biological activity.

The organic layer with weak activity (Fig. 2) was extensively fractionated by silica gel 60 (Merck), Develosil 60-5 (Nomura), and Radial Pak SI-5 (Waters) high-performance liquid chromatography with chloroform: methanol:aqueous ammonia and benzene: methanol:aqueous ammonia as solvents to give a total of six dihydroaplysinopsins (a few milligrams of each) exemplified by **2** and ten aplysinopsins (a few milligrams of each) exemplified by **3** (Fig. 3). Aplysinopsins have frequently been isolated from sponges (*11, 12*); dihydroaplysinopsins have also been recently reported (*13*). The structures of **2** and analogs (difference in tautomeric form, site, and number of *N*-methylation in ring C, also X = bromine or hydrogen) were determined from NMR spectroscopy data. The dihydro compounds elicited attracted swimming, but the effective dose of $10^{-6}M$ is much weaker than that of amphikuemin ($10^{-10}M$). The structures of aplysinopsin **3** and analogs were derived by comparison with published data (*11, 12*) and synthesis (*14*). Variations in their structures again reside in ring C, the stereochemistry of the double bond between positions 8 and 1′, and the presence or absence of bromine. The fish responded to these chemicals; the aplysinopsins induced the fish to perform a head up and down "seesaw" movement (effective dose $10^{-6}M$), a characteristic behavior observed in nature.

There was wide individual variation in the response of fish to dihydroaplysinopsins. Furthermore, we found that unidentified species of dinoflagellates, symbiotic with the *R. kuekenthali* used in these studies, produce several of the aplysinopsins. Therefore, these compounds probably do not play substantial roles in attracting fish. In addition to *A. perideraion, R. kuekenthali* also live symbiotically with *A. clarkii* (Bennett) in the sea; however, neither the amphikuemin nor the aplysinopsins and dihydroaplysinopsins exhibited synomonal activity toward *A. clarkii*. It is thus clear that two different species of symbiotic fishes recognize their common host through different chemicals.

Similar extraction of the sea anemone *Stoichactis kenti*, collected off Kuroshima Island in Okinawa and assayed against its symbiotic fish *A. ocellaris*, led to the identification of tyramine **4** and tryptamine **5**, which induced attracted swimming with tail wagging and active searching behavior, respectively, both at a dose of $10^{-6}M$. When tryptamine was added to a fraction that induced a weak swimming response, the potency of this fraction was increased 100-fold. However, the activity of this fraction, even after addition of tryptamine, was far less than that of the crude secretion, and, moreover, further purification led to reduction in the activity. We conclude that the synomonal activity of the secretion is caused by tyramine and by the synergistic effect of tryptamine together with multiple unidentified chemicals. Tyramine was also found in the mucus of *R. kuekenthali*, but had no

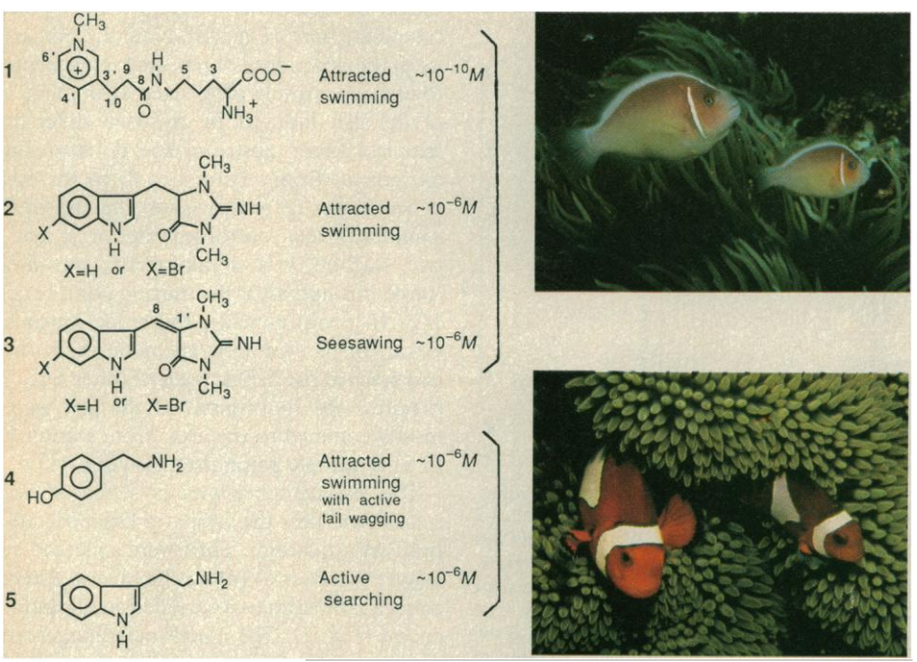

Fig. 3. Synomones involved in the symbiosis between *Radianthus kuekenthali–Amphiprion perideraion* (top) and *Stoichactis kenti–A. ocellaris* (bottom). Attracted swimming: active, oriented movement paralleling that observed with administration of crude mucous extract (Fig. 1A). Seesawing: head up and down movement observed in *A. perideraion* in symbiotic association with *R. kuekenthali*. Active tail wagging: wagging of tail paralleling that observed in naïve *A. ocellaris* in response to chemical factors released from *S. kenti*. Active searching behavior: searching behavior without definite direction away from the region of sample administration. Activity is expressed in orders of magnitude of the sample concentration in the tank that induces positive response in over 80% of test fish. [Photographs by Masayuki Abe (top) and Hideaki Usuki (bottom)].

effect on its two symbiotic fishes *A. perideraion* and *A. clarkii*.

One of the chemicals responsible for the *A. perideraion–R. kuekenthali* symbiosis, amphikuemin, is particularly active and elicits a swimming pattern mimicking that caused by a crude mucous extract from the anemone. The heterocyclic nitrogen of amphikuemin is made quaternary, which presumably increases affinity to the negatively charged lipid surface of receptor membranes; *N*-demethyl-L-amphikuemin (a synthetic intermediate) was totally devoid of activity. The activity of the synthetic quaternary salt 4′-demethyl-L-amphikuemin was greatly reduced to $2.0 \times 10^{-8} M$, thus indicating the very specific structural requirement. However, since the experiments were carried out with young fish in an assay vessel, it is not known whether the same set of compounds is responsible for leading the naïve juvenile fish to their host at the initial encounter in the sea. In contrast to the *A. perideraion–R. kuekenthali* pair, it appears that induction of the swimming movement in *A. ocellaris–S. kenti* depends on the synergistic action of multiple chemicals not yet identified. We have also shown that different species of anemone fish recognize the same anemone species by different chemicals. Thus a variety of chemicals differing in structural type, activity level, and function are involved in maintaining the species-specific association between the sea anemone and anemone fish.

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Radar Glory from Buried Craters on Icy Moons

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Three ice-covered moons of Jupiter, in comparison with rocky planets and Earth's moon, produce radar echoes of astounding strengths and bizarre polarizations. Scattering from buried craters can explain these and other anomalous properties of the echoes. The role of such craters is analogous to that of the water droplets that create the apparition known as "the glory," the optically bright region surrounding an observer's shadow on a cloud. Both situations involve the electromagnetic phenomenon of total internal reflection at a dielectric interface, operating in a geometry that strongly favors exact backscattering. Dim surface craters are transformed into bright glory holes by being buried under somewhat denser material, thereby increasing the intensity of their echoes by factors of hundreds. The dielectric interface thus formed at the crater walls nicely accounts for the unusual polarizations of the echoes.

SINCE THE FIRST OBSERVATIONS more than a decade ago, a major puzzle in planetary science has been the unexpected results of radar studies of Europa, Ganymede, and Callisto, large ice-clad moons of Jupiter. These radar echoes can exceed the strengths that would be obtained if the moons were perfectly reflecting spheres. This, plus the odd polarizations and weak angular dependence of the echoes, characterizes their profound differences from the relatively well-understood radar signatures of terrestrial planets and Earth's moon (*1*). A new model is developed here in which the anomalous characteristics of the icy-moon echoes can be attributed to inherent scattering properties of buried craters.

Four previous attempts at explaining the observations have been less satisfactory. Ostro and Pettengill (*2*) proposed scattering by surface craters, but this mechanism cannot easily explain the measured echo strengths. Goldstein and Green (*3*) invoked random subsurface scattering events to model the observations, but this also has difficulty in accounting for the strengths of the echoes. Hagfors *et al.* (*4*) suggested scattering from smooth-gradient centers of refraction; this can explain the strengths but does not produce a good match to the observed polarizations. Eshleman (*5*) demonstrated that a decoupling of two characteristic modes of propagation may be fundamental in producing the odd polarizations and suggested several ways that the refracting centers of Hagfors *et al.* could be modified to produce the decoupling. Although the echo properties (*1*) can all be matched in this way, there is no other compelling reason to believe that such centers exist.

One of the suggested modifications to the refraction model involved sharp radial gradients in refractivity. These discontinuities could cause trapping of the two characteristic propagation modes, analogous to the trapping that occurs in dielectric waveguides and optical fibers, thereby differentially shifting their phases and decoupling them as required. Moreover, Eshleman (*5*) noted that such a trap might provide the necessary backward focusing even in the absence of the smooth refraction proposed by Hagfors *et al.* (*4*). A particularly simple model of this type is introduced here. It consists of a single negative discontinuity of refractive index in a hemispheroidal geometry, or more descriptively, a buried crater. This model is appealing, since it is known that both cratering and resurfacing have occurred on the icy moons.

Perfect geometrical shapes cannot explain the observations (*5*) and are not expected to occur. Thus the craters are considered to be hemispheroidal with their imperfections causing a breakup of the coherent annulus of the glory circle (the locus of intersection of the backscattered rays and a plane normal to the incident rays at the crater) into discrete patches or glints. From an extension of a quasi-wave-optical analysis of spheroidal

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