Mycosporine-like amino acids are multifunctional molecules in sea hares and their marine community

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Molecules of keystone significance are relatively rare, yet mediate a variety of interactions between organisms. They influence the distribution and abundance of species, the transfer of energy across multiple trophic levels, and thus they play significant roles in structuring ecosystems. Despite their potential importance in facilitating our understanding of ecological systems, only three molecules thus far have been proposed as molecules of keystone significance: saxitoxin and dimethyl sulfide in marine communities and tetrodotoxin in riparian communities. In the course of studying the neuroecology of chemical defenses, we identified three mycosporine-like amino acids (MAAs)—*N*-ethanol palythine (= asterina-330), *N*-isopropanol palythine (= aplysiapalythine A), and *N*-ethyl palythine (= aplysiapalythine B)—as intraspecific alarm cues for sea hares (*Aplysia californica*). These alarm cues are released in the ink secretion of sea hares and cause avoidance behaviors in neighboring conspecifics. Further, we show that these three bioactive MAAs, two [aplysiapalythine A (APA) and -B (APB)] being previously unknown molecules, are present in the algal diet of sea hares and are concentrated in their defensive secretion as well as in their skin. MAAs are known to be produced by algae, fungi, and cyanobacteria and are acquired by many aquatic animals through trophic interactions. MAAs are widely used as sunscreens, among other uses, but sea hares modify their function to serve a previously undocumented role, as intraspecific chemical cues. Our findings highlight the multifunctionality of MAAs and their role in ecological connectivity, suggesting that they may function as molecules of keystone significance in marine ecosystems.

chemical signaling | pheromone | semiochemical | opisthobranch mollusk

Among the many strategies to reduce predation pressure is the use of chemical defenses. Chemicals may protect the individual releasing the molecules through a variety of mechanisms (1–5), whereas other released chemicals, called alarm cues, warn neighboring conspecifics about predation events (6–12). Despite the many examples of organisms using alarm cues, the molecular identities of the vast majority of them remain unknown (but see refs. 13–17). Elucidating the identities of the alarm cues and other chemical defenses that mediate predator–prey interactions is crucial to facilitating our understanding of the sources of these molecules (via sequestration or de novo production), the neural processing of these cues by prey species, and other roles these cues may play. An understanding of the functions of chemical cues may also lend insight into the evolution of their roles and their importance to inter- and intraspecific interactions. Many animals sequester secondary metabolites from their diet, and in many cases, these diet-derived molecules function as defenses for both the prey that produce them and for the consumers that eat and sequester them (18, 19). Some species, however, use sequestered secondary metabolites for functions different from their roles in their prey. For example, some moths and butterflies and an amphipod sequester defensive metabolites from the plants in their diets and use them unmodified or modified as sex pheromones for mate attraction (20–24). Adult California newts (*Taricha granulosa*) use tetrodotoxin as a chemical defense, whereas this molecule functions as an alarm cue for juveniles (11, 25). Dimethylsulfoniopropionate, an osmolyte in marine phytoplankton, is released and degraded to dimethyl sulfide and other metabolites, which deter zooplankton grazing (26). Dimethyl sulfide is used as a cue for foraging by birds and fish (11, 26–28). Ultimately, an understanding of the specific molecules serving as chemical defenses and the other roles they play will allow for a more complete appreciation of the role of chemicals in predator–prey interactions, linking physiological, behavioral, and ecological mechanisms influencing these exchanges.

Sea hares (*Aplysia* spp.) are excellent model species with which to investigate chemical defenses, due to their diverse suite of antipredation strategies. When attacked by predators, the sea hare *Aplysia californica* releases two defensive secretions, ink and opaline (Fig. S1). These secretions provide protection both by directly deterring attack from predators (5, 29–31) and by alarming conspecifics of nearby predators (16, 32). The release of ink and opaline occurs when sea hares are attacked, and thus they are reliable indicators of predation risk. When conspecifics detect the secretions, they move away from the point of contact and/or increase their speed of crawling (galloping), facilitating their movement to a safer location. The alarm cues in ink are uracil, uridine, and cytidine (16). Opaline also induces these alarm responses, and here we report that the bioactive molecules in opaline are mycosporine-like amino acids (MAAs), including two previously undescribed ones, derived from the sea hare's algal diet. Thus, sea hares have co-opted MAAs, which function widely as sun screens (33–38), for an alternative function as an intraspecific alarm cue. Given that MAAs are produced by few species, are relatively rare in aquatic communities, have diverse and multiple functions within and across species, and are known to cross trophic levels in aquatic communities (33–47), we suggest that they may be molecules of keystone significance (11, 17, 25, 27).

Results and Discussion

We examined behavioral responses of juvenile sea hares when exposed to opaline and its components. Opaline elicited an alarm response at a significantly higher frequency than the control stimulus, seawater (Movie S1). Consistently throughout our experiments, when opaline was used as a positive control, it evoked alarm responses in 60–90% of the sea hares, whereas seawater evoked responses from only 0–25% of the animals. To identify the components in opaline responsible for the alarm responses, we performed bioassay-guided fractionation of opaline using a series of separations based on polarity and size, as described in Fig. 1. This scheme resulted in identification of six

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peaks (T1–T6), three of which (T2, T4, and T5) each alone elicited a statistically significant alarm response when tested at its concentration in full-strength opaline (Fig. 1*E*).

These three compounds are necessary and sufficient to elicit the alarm response (Fig. 2*A*). Opaline lacking these three alarm molecules did not evoke a significant alarm response, demonstrating their necessity. A mixture of the three alarm molecules produced a statistically significant alarm response, as did opaline with alarm molecules removed and then added back, thus demonstrating their sufficiency as alarm cues in opaline. Both of these stimuli produced alarm responses similar to the level elicited by the full-strength opaline secretion. The mixture of three alarm molecules, as well as opaline, is effective over concentrations from full-strength concentration (i.e., that in opaline) to 1/500th of full-strength concentration, further supporting the sufficiency of these three molecules as the opaline alarm cues (Fig. 2*B*).

Our chemical analyses reveal that all three opaline alarm molecules are MAAs, all derivatives of the imino-MAA palythine (Fig. 3). T2 is asterina-330 (*N*-ethanol palythine), originally discovered in starfish and also present in other invertebrates (39). T4 and T5 are previously undocumented MAAs: *N*-isopropanol palythine (= aplysiapalythine A) and *N*-ethyl palythine (= aplysiapalythine B), respectively. The median concentrations (and range) of the three alarm molecules in full-strength opaline are 66 (63–90) μg mL^{-1} for asterina-330, 12 (7–14) μg mL^{-1} for aplysiapalythine A, and 10 (2–13) μg mL^{-1} for aplysiapalythine B, which equate to concentrations of 229, 40, and 37 μM, respectively. Thus, an adult sea hare, which can weigh several hundred grams, has at most a few hundred micrograms of these three MAAs in its opaline. We also identified two MAAs in opaline that our bioassay-guided fractionation indicated were not alarm cues. These molecules include one known MAA

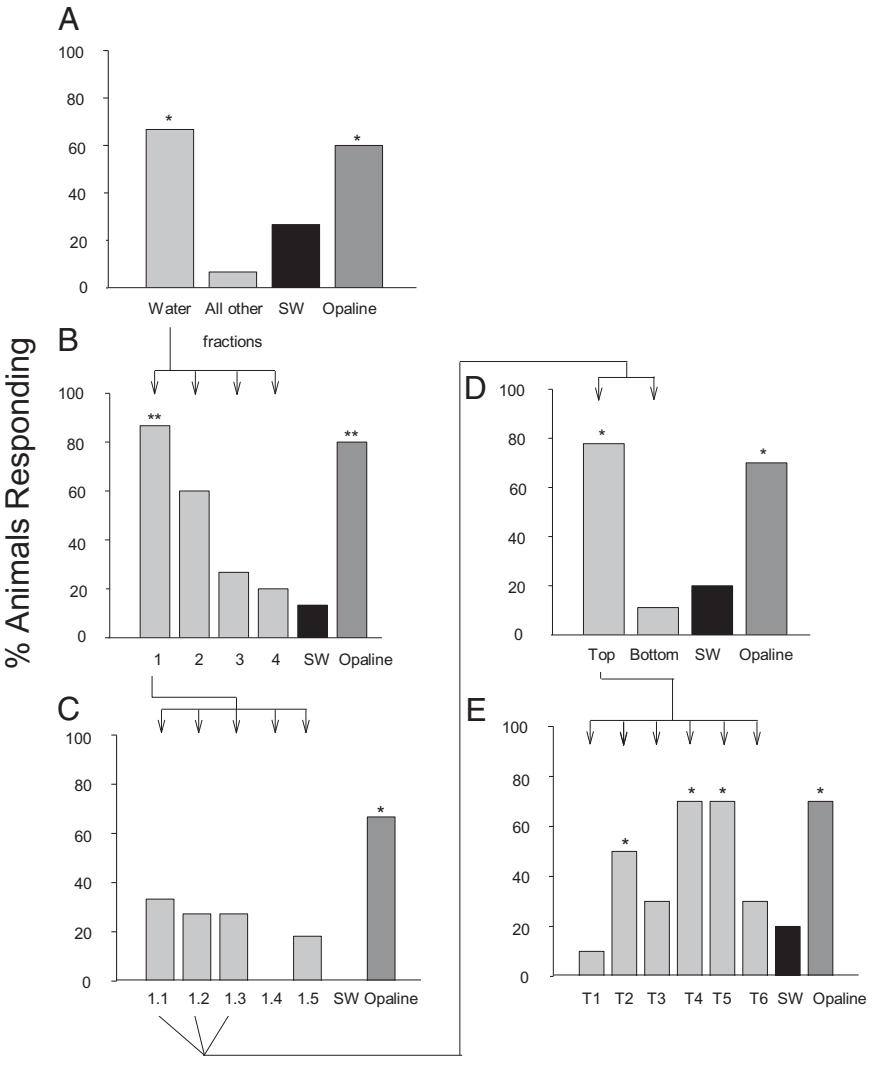

Fig. 1. Isolation of the alarm molecules in opaline via bioassay-guided fraction. Frequency of alarm response (moving away or galloping) by sea hares to chemical extracts is shown for successive fractionation steps. (*A*) Fractions resulting from solvent partitioning of opaline (all other fractions aside from the water-soluble fraction—hexanes, chloroform, ethyl acetate, and butanol fractions—were combined. (*B*) Fractionation of the water-soluble fraction in *A* using a lipophilic Sephadex LH-20 column yielded four fractions, one of which had bioactivity. (*C*) Fractionation of the bioactive fraction in *B* with a Sephadex Superdex peptide column yielded five fractions, three of which (1.1, 1.2, and 1.3) had some activity and were combined. (*D*) This combined fraction was separated by TLC, yielding 2 UV-visible bands, with the top one having significant bioactivity. (*E*) The top UV band was separated by HPLC via a C18 reversed phase column, yielding six fractions, three of which (T2, T4, and T5) had bioactivity. These active fractions were further purified with HPLC via a C30 reversed phase column and their molecular structures were identified via NMR and high resolution mass spectrometry (Fig. 3). Black bars represent seawater (negative control), clear bars represent opaline extracts, and gray bars represent opaline (positive control). n = 10–15 animals. Analysis by one-tailed McNemar test, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

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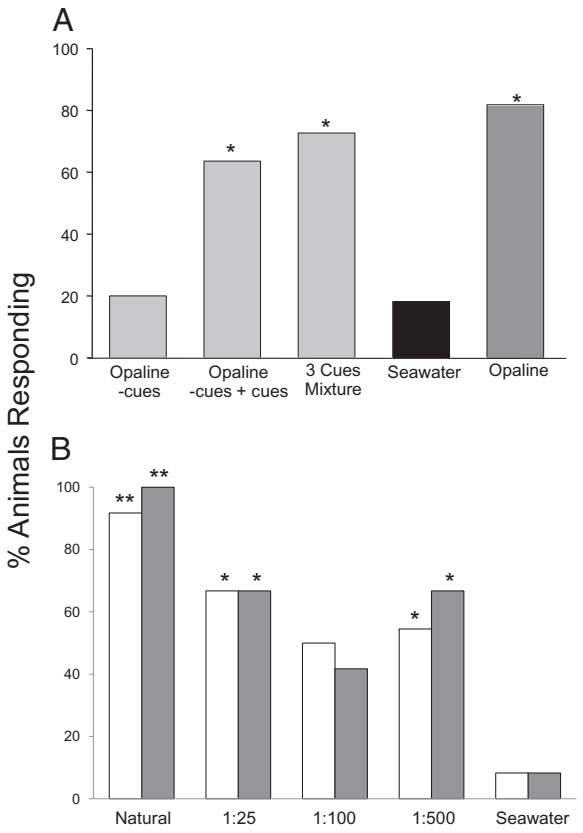

Fig. 2. Frequency of alarm response (moving away or galloping) by sea hares to opaline and the three alarm molecules. (*A*) Responses to opaline with the alarm molecules removed ("opaline – cues"), opaline with the molecules added back ("opaline – cues + cues"), and the three opaline alarm molecules ("3 cues mixture"). n = 9–11. (*B*) Responses to opaline (gray bars) and 3 cues mixture (clear bars) at concentrations in full-strength opaline and dilutions of 1:25, 1:100, and 1:500. n = 11 or 12. Analysis and symbols as in Fig. 1.

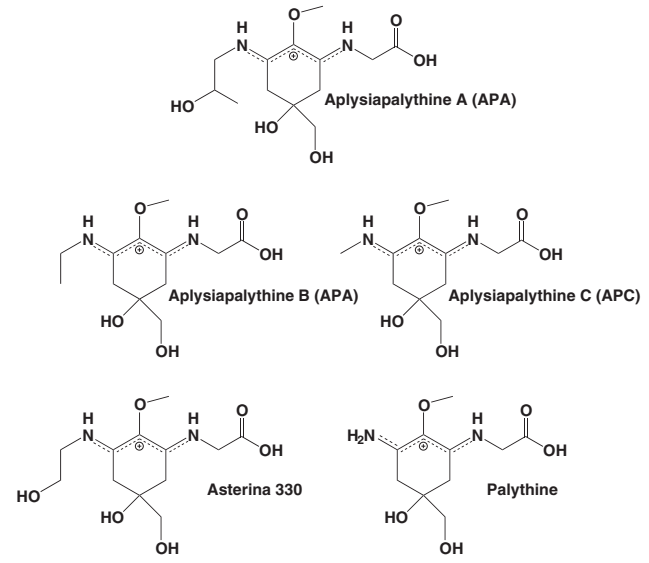

Fig. 3. Molecular structures of five mycosporine-like amino acids in opaline. Three of these—asterina-330, aplysiapalythine A, and aplysiapalythine B—are alarm cues.

(palythine) and one previously unidentified MAA (*N*-methylpalythine or aplysiapalythine C) (Fig. 3). Additional MAAs are present in opaline but at lower concentrations, and we did not characterize their molecular structure.

MAAs are present in other marine animals, including the sea hare *Aplysia dactylomela*, where they are thought to function as sun screens because of their UV absorbent properties (33–37). Marine animals, including *A. dactylomela*, are generally thought to acquire MAAs through their diet of seaweeds or from bacteria or other symbionts, rather than by de novo synthesis (33–40). We compared the levels of the five most abundant MAAs in opaline and other tissues and secretions of *A. californica* and its algal diet (Fig. 4), taking advantage of the availability of sea hares reared in the laboratory exclusively on a diet of two red algal species, *Gracilaria ferox* and *Agardhiella subulata*. Three of these five major MAAs in laboratory-raised sea hares were also major MAAs in the wild-caught sea hares (aplysiapalythine C, asterina-330, and palythine), but two were not (MAA1 and MAA2, whose structures we did not identify) (Fig. 4). Opaline had significantly higher levels of MAAs than in other tissues (Fig. 4*A*). Dorsal skin had higher levels of MAAs than ventral skin (Fig. 4*B*), probably indicative of MAAs serving as a sun screen. Both red algae contained all five of the major MAAs found in sea hare opaline, albeit at significantly lower levels (Fig. 4 *A* and *C*), whereas a brown alga (*Egregia menziesii*) that is present in the natural environment of sea hares but which is not typically eaten by them (41) does not contain detectable levels of MAAs (Fig. 4*C*). We conclude from our data and from previous work (34, 35) that sea hares acquire MAAs from their diet of red algae and preferentially concentrate some MAAs in opaline and skin, especially the skin on the dorsal surface of the animal. We found no evidence of de novo synthesis of MAAs by sea hares.

Similar to a keystone species, a molecule of keystone significance is present in a community or ecosystem across multiple trophic levels, has multiple functions, and has impacts on a community or ecosystem that are large and more profound than would be expected from its relative abundance or total biomass (11, 17, 25, 27, 48). Founding examples of molecules of keystone significance are tetrodotoxin, saxitoxin, and dimethyl sulfide (11, 17, 25, 27). MAAs have features of molecules of keystone significance. They are produced by only a small number of organisms, yet occur in a wide variety of species across multiple trophic levels, at relatively low levels, and perform a multitude of functions. MAAs are mainly produced by bacteria, phytoplankton, algal symbionts, and macroalgae, and they are transferred via trophic interactions to diverse taxonomic groups, such as sponges, cnidarians, mollusks, arthropods, echinoderms, ascidians, and fish (42, 43). MAAs are transferred up to at least several trophic levels. For example, many tropical and temperate fishes contain MAAs in their mucus (44), and the cleaner fish *Labroides dimidiatus* likely obtains its MAAs through the mucus it consumes when cleaning other fish (44), suggesting that MAAs can be transferred up to as many as three trophic levels. MAAs occur at relatively low levels, and they are multifunctional. A primary and ubiquitous function of MAAs is as sun screens through their absorption of harmful UV radiation (36, 37, 45, 46), but they also may function as antioxidants, antifoulants, osmotic regulators, spawning inducers, and inducers of the release of photosynthate from endosymbiotic algae (45–49). Our study demonstrates an alternate role of MAAs as an intraspecific cue.

Through their many functions across several trophic levels, MAAs have the potential to influence the structure and function of marine communities. For example, MAAs influence population dynamics by increasing the survivorship of phytoplankton, zooplankton, and echinoderm larvae that possess these compounds when exposed to UV radiation compared with individuals lacking MAAs (50–52). MAAs affect food web dynamics by facilitating primary productivity, protecting photosynthetic species from harmful UV radiation, allowing them to

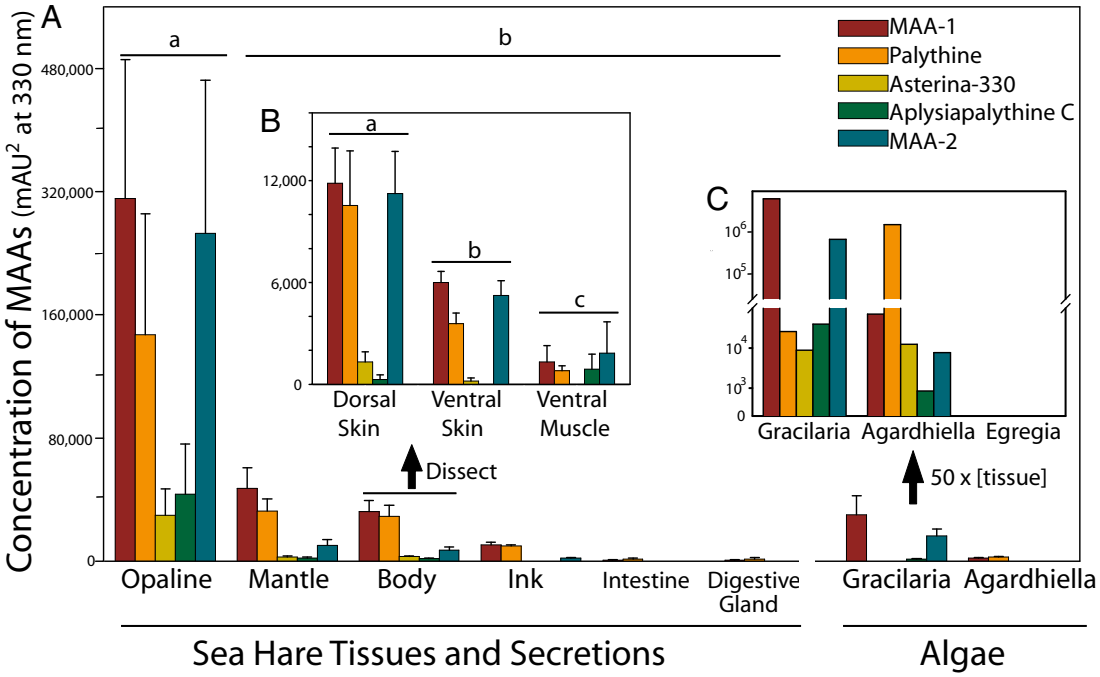

Fig. 4. Levels of mycosporine-like amino acids in sea hare tissues and secretions and in algae. Values are means ± SEM of UV absorbance for tissue at the same concentration (mAU^2 at 330 nm/g) in *A* and *B* and 50× higher concentration in *C*. (*A*) MAA concentrations are higher in opaline than in all other sea hare tissues and secretions or in two red algal species upon which these sea hares exclusively fed [ANOVA $F_{(7,95)} = 6.698$, $P = 0.000002$, $n = 3$–6 per tissue/secretion/alga; letters above bars indicate values that are significantly different in Duncan's post hoc tests, $\alpha = 0.05$]. (*B*) MAA concentrations are higher in the dorsal skin than ventral skin or ventral foot muscle [ANOVA $F_{(2,60)} = 25.681$, $P < 0.0000001$, $n = 5$ per tissue; letters above bars indicate values that are significantly different in Duncan's post hoc tests, $\alpha = 0.05$]. (*C*) All five MAAs found in sea hare opaline and other tissues are present in each of the two red algae that these sea hares fed on exclusively (*Gracilaria*, *Agardhiella*) but not in a brown alga that these sea hares did not normally eat (*Egregia*). The amount of tissue used in this quantification was 50 times that in *B*, and a single sample was analyzed for each species. The molecular structures of MAA1 and MAA2 were not determined.

survive in shallow waters where light is abundant. These molecules also impact interspecies interactions. For example, copepods that are protected from UV radiation by MAAs and pigments are less likely to migrate to deeper water (53) and likely gain a competitive advantage through their ability to feed in more shallow waters where phytoplankton are abundant. Sea hares and other herbivores likely receive a similar competitive benefit through the ability to feed openly in shallow waters and tide pools where primary producers are plentiful. Finally, MAAs may influence higher-order ecological systems. Sea stars (some of which contain MAAs) function as keystone species in some rocky intertidal systems (48). These secondary metabolites may indirectly facilitate the significant feeding impacts of sea stars on competitive dominant species by allowing them to forage in shallow waters without experiencing UV damage. As another example, sea hares consume up to 35% of their body weight in seaweeds each day (54), and as a result, areas where predation risk is high may experience changes in seaweed diversity and/or abundance due to emigration of sea hares in response to MAA alarm cues released by attacked conspecifics. A keystone role of MAAs in marine communities would transcend a diversity of organisms and trophic levels, influencing community structure and function through their influence on food web dynamics by allowing species to persist in both high predation risk and UV-stressful environments.

The role of MAAs as alarm cues is probably a derived characteristic, secondary to its role as a sun screen, osmoregulatory agent, or some other physiological function. The use of MAAs as alarm cues probably did not evolve as the result of a benefit to the sea hares releasing them in their opaline secretion. Sea hares have a planktonic larval stage, so sympatric juveniles and adults are most likely unrelated, and thus the release of alarm cues is not the result of kin selection. Rather, nearby conspecifics (receivers) probably evolved the ability to detect and respond to these cues because they are reliable indicators of predation risk.

Why have sea hares evolved the ability to identify molecules in both ink and opaline as alarm cues? One possibility is that the alarm cues in ink (uracil, uridine, and cytidine) are synthesized de novo, unlike the sequestered opaline alarm cues. Due to diet variability, there may be variation in the presence or concentration of MAAs in opaline, and possibly the ink alarm cues as well. In addition, variation in physiological condition of animals may alter synthesis of ink alarm cues. The ability to identify and respond to these different molecules may compensate for variation in the presence or abundance of these cues in sea hare secretions.

Methods

Organisms and Their Care. Juvenile (1–5 g) and adult (108–222 g) sea hares, *A. californica*, and two species of red algae, *G. ferox* and *A. subulata*, were obtained from the National Institutes of Health (NIH)/University of Miami (Miami) National Resource for *Aplysia* (Key Biscayne, FL). These sea hares were fed throughout their entire life, both in being raised at the NIH/Miami facility and when held before experiments in our laboratory, an exclusive diet of *G. ferox* and *A. subulata*. Wild-caught adult (200–300 g) sea hares, *A. californica*, and the brown alga *Egregia menziesii*, were collected off the coast of California by Marinus Scientific. Adult animals were dissected immediately upon receipt from Miami and California and thus were not fed in our laboratory. All organisms were maintained in our laboratory in aquaria with recirculating, filtered, and aerated artificial seawater (Instant Ocean; Aquarium Systems) at 20 °C. Juvenile animals, 1–5 g, were used in the behavioral analyses. Both NIH/Miami animals and wild-caught adult animals were used in the chemistry experiment.

Collection of Secretions and Tissues from Sea Hares. We obtained secretions and tissues from both NIH/Miami reared and wild-caught sea hares. Opaline and ink secretions were collected by chilling *Aplysia* in seawater for 4 h at

ECOLOGY

4 °C. Animals were then anesthetized by injection of 120–180 mL isotonic $MgCl_2$, and glands were removed by dissection. Opaline was collected by centrifuging opaline glands at 30,000 × *g* to separate the fluid from the gland tissue. Ink was collected by gently squeezing glands with a spatula. Intestine, digestive gland, body, skin from either the dorsal or ventral surface, and muscle from the foot were separately collected by dissection. Secretions and tissues were frozen at −20 °C until used.

Bioassay of Alarm Responses. To examine alarm behavior of juvenile *A. californica* in response to chemical stimuli, we used the method reported in ref. 16. Each animal was tested individually in a 39 × 26 × 5.5 cm glass arena filled with seawater to a depth of about 2.5 cm. For each trial, an animal was gently placed in the center of the arena and allowed to acclimate for 1–2 min. After this acclimation period, as the animal locomoted forward, it was presented with 75 μL of stimulus delivered about 1 cm in front of the animal at a 45° angle. After presentation of the stimulus, the sea hare was videotaped from overhead for 2 min. Animals were scored for two alarm behaviors that are elicited by opaline: moving away from the stimulus and galloping (i.e., saltatory crawling). Each stimulus was tested on each animal, with at least 1 h between presentations. In all experiments, opaline was used as a positive control and seawater was used as a negative control. The order of presentation of the stimuli to each animal was randomized, and the experimenter was unaware of the identity of the stimuli except for opaline, as we were unsuccessful at creating colored seawater to mimic this secretion. After each trial, the arena was emptied, rinsed with fresh water, and dried with a paper towel. Responses of sea hares to stimuli were statistically analyzed using a one-tailed McNemar's test ($P < 0.05$). Responses to stimuli were considered significant if they were statistically greater than the response to the paired sea water response.

Bioassay-Guided Fractionation to Separate and Identify Alarm Cues. To identify the components in opaline responsible for generating the alarm response, 39 mL of opaline was collected and processed as described above and was partitioned via a modified Kupchan partition scheme (55) (Fig. 1). This scheme yielded five partitions that differed in solubility; these were soluble in hexanes, chloroform, ethyl acetate, butanol, or water. Because nearly all of the opaline components partitioned into the water fraction, we combined the hexanes, chloroform, ethyl acetate, and butanol partitions for the bioassay. This fraction was solubilized in a 20-mL vial in a 3:1 solution of hexanes/chloroform/ethyl acetate and seawater. The vial was then placed on a rotary evaporator to remove the organic solvent, leaving the remaining extract solubilized in seawater. A volume of seawater was then added to the solubilized extract to match the volume of opaline extracted, so that the extract was tested at the concentration at which it occurs in full-strength opaline.

Only the water-soluble partition elicited a frequency of alarm response different from seawater (Fig. 1*A*). Thus, this fraction was further purified by passing it through lipophilic Sephadex LH-20 (GE Healthcare) with 100% water as the mobile phase at a flow rate of 0.3 mL min^{-1}. This produced four fractions (based on TLC characteristics), one of which produced a significant alarm response (Fig. 1*B*). This fraction was further purified by passing it through Sephadex (Superdex Peptide column; GE Healthcare) with 100% water as the mobile phase at 0.1 mL min^{-1}. This produced five fractions, none of which resulted in a response significantly different from seawater but three of which produced considerable activity (Fig. 1*C*). We combined these three fractions for further purification by preparative thin layer reversed chromatography (Partisil KC18 silica gel 60 Å, 200 μm thick; Whatman) with 60:40 methanol:water as the mobile phase. On the basis of UV absorbance, two bands were collected separately in 70:30 methanol:water. Each band was dried and tested separately in the bioassay. Only the top band elicited an alarm response (Fig. 1*D*). This fraction was further purified via HPLC [Beckman 125S solvent module coupled to a Beckman 168 photodiode array detector, using a Phenomenex Luna reversed phase C18 silica 5-μm column (250 mm × 10 mm)] with a mobile phase of 3:97 methanol:water at 0.8 mL min^{-1}. This produced six peaks (T1–T6). Three peaks (T2, T4, and T5) elicited an alarm response (Fig. 1*E*) and were further purified separately via another round of HPLC using a Develosil RP Aqueous reversed phase C30 silica 5 μm column (250 mm × 4.6 mm) (Nomura Chemical) with a mobile phase of 100% water at 0.8 mL min^{-1}.

To determine the concentrations of T2, T4, and T5 in full-strength opaline, we pooled opaline from 10 adult sea hares (~150 g each). Opaline glands were dissected as described above and stored at −80 °C until their use. Glands were thawed and centrifuged to collect opaline. One milliliter of opaline was taken from this pooled sample and passed through a C18 reversed phase column, which was flushed with 250 mL of water, and the eluate was dried in a SpeedVac concentrator. This fraction was solubilized in water and three 100-μL replicate injections were analyzed by HPLC (at 330 nm) using a Develosil RP-Aqueous C30 silica 5-μm column (250 mm × 4.6 mm) with a mobile phase of 100% water and a flow rate of 0.8 mL min^{-1}. The area under HPLC chromatogram peaks of the three molecules was calculated for each injection. To determine the concentrations of the alarm molecules, standard curves were created by analyzing the peak area of 100-μL injections of five concentrations (0.5, 0.7, 1.0, 5.0, and 25.0 μg) of each molecule. The linear regressions for the standard curves for peaks T2, T4, and T5 had R^2 values of 1.000, 1.000, and 0.999, respectively.

To examine the necessity and sufficiency of these alarm molecules to elicit an alarm response, we created opaline lacking alarm molecules and, as a control, opaline with alarm molecules removed and then added back. This was done by recombining all opaline fractions resulting from a purification process as described above for quantifying the alarm molecules in opaline. For the reversed phase C18 separation, after the column was flushed with 100% water, it was flushed with 100% methanol and the eluate was collected. The water eluate collected from the C18 separation of opaline was then passed through a C30 column via HPLC and the three alarm molecules in opaline were collected and all other compounds that eluted from the column were combined. All collected fractions (100% methanol C18 column flush and the nonalarm molecule portions eluting from the HPLC) were combined. This extract was divided into two aliquots; one had no alarm cues added (= opaline – cues), and the other had half of the total collected alarm molecules added to it (= opaline – cues + cues). These stimuli were compared with the mixture of the three alarm cues ("3 cues mixture") in behavioral assays.

Molecular Identification of the Alarm Cues. To determine the identity of the alarm cues and their derivatives, purified molecules T1–T5 were analyzed using NMR spectroscopy, mass spectrometry, and UV absorption spectroscopy. They were identified as palythine, asterina-330, aplysiapalythine C (APC), aplysiapalythine A (APA), and aplysiapalythine B (APB), respectively. Detailed data are published elsewhere (56).

Analysis of MAAs in Sea Weeds and Sea Hare Tissues. To determine whether MAAs in sea hare opaline are synthesized de novo, diet derived and used in unmodified form, or diet derived and modified by sea hares after their ingestion, we compared the composition of MAAs in the following: tissues from laboratory-reared sea hares, the two species of red algae that these sea hares were exclusively fed (*G. ferox* and *A. subulata*), and one species of brown alga (*E. menziesii*) from California that is neither in the diet of laboratory-reared animals nor in the diet of natural sea hares (41). Tissues of sea hares (opaline gland, ink gland, mantle, intestine, and digestive gland) were dissected and saved, and the rest of the animal ("body") was further dissected into three parts (dorsal skin, ventral skin, and ventral muscle). Three individual sea hares and three samples of *G. ferox* and *A. subulata* were used for this analysis. Tissue (0.03–0.06 g dry weight) was ground using mortar and pestle extracted with 10 mL of methanol. The extract was dried under vacuum, dissolved in 10 μL of methanol, and injected into Beckmann System Gold HPLC equipped with a 168 photodiode array detector and Develosil C30 column 4.6 × 250 mm (particle size 5 μm). To separate MAAs in the extract, 0.1% trifluoroacetic acid in water was used as the mobile phase at a flow rate of 1 mL/min for 15 min followed by a linear gradient to increase methanol up to 10% in 10 min. MAAs were detected at 330 nm, and the area of each MAA peak was quantified in milliabsorbance units squared (mAU^2). To compare MAA concentrations between tissues, the areas were standardized to mAU^2/g tissue (dry weight). Because the concentrations of most MAAs in *G. ferox*, *A. subulata*, and *E. menziesii* were under the detection limit, samples of these were concentrated 50 times and analyzed using the same method. The data were analyzed by a two-factor ANOVA, with tissue and type of MAA as factors, followed by Duncan's post hoc tests ($\alpha = 0.05$).

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