Isolation and Structural Elucidation of Novel Mycosporine-Like Amino Acids as Alarm Cues in the Defensive Ink Secretion of the Sea Hare *Aplysia californica*

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Three new mycosporine-like amino acids (MAAs), aplysiapalythines A, B, and C (**1**, **2**, and **3**, resp.) were isolated from opaline, a glandular component of the defensive ink secretion of sea hares (*Aplysia californica*) collected from waters off southern California. Here, we report the structure of these MAAs determined by mass spectrometry and NMR data. These new MAAs are structurally related to two known MAAs that are also present in sea hare opaline, *i.e.*, asterina 330 (**4**) and palythine (**5**), and for which we also provide detailed data here for comparison. The fact that three of the five MAAs that we identified from sea hare opaline are novel molecules is interesting given that this represents a relatively large addition to the current list of known MAAs. This is likely because most researchers have identified MAAs through HPLC with UV detection, which is imprecise given similarities in UV spectra for different MAAs. Our findings suggest that there is much greater diversity of MAAs than is currently known. Results published elsewhere show that **1**, **2**, and **4** are alarm cues for conspecific sea hares at natural concentrations, but **3** and **5** are not.

Introduction. – Mycosporine-like amino acids (MAAs) are widely distributed in marine organisms including bacteria, plants, invertebrates, and vertebrates [1]. Animals are generally thought to be incapable of synthesizing MAAs, but rather they are thought to sequester MAAs from their diet or from symbionts; however, this view has been questioned [2]. MAAs, of which *ca.* 30 are known, are traditionally considered to be screens against solar radiation, but other functions have also been proposed, including anti-oxidants, osmotic regulation, providing a source of amino acids in embryonic development, and induction of spawning [3–10].

A new function for MAAs was recently identified from our work on chemical defenses of sea hares (*Aplysia californica*) collected from the waters of southern California [11]. We found that MAAs are present in high concentrations in opaline, which is one of the glandular products of the sea hares' ink secretion. Of the five major MAAs in opaline of these sea hares, three are novel, and two of these novel MAAs, together with nucleic acids and nucleosides in the ink component of the sea hares' ink secretion, function as intraspecific alarm cues [11]. In this article, we describe the isolation and the structure elucidation of these novel MAAs.

Results and Discussion. – Opaline from specimens of *Aplysia californica* collected from waters of southern California was fractionated using liquid/liquid partitioning, gel filtration, and reversed-phase HPLC guided by assays using alarm behavior of small juveniles. This yielded compounds with UV absorbance around 330 nm and, from MS and NMR, they were identified as MAAs. Of the five major MAAs in opaline, three, **1**–**3**, were new structures, and two were previously identified MAAs, asterina 330 (**4**) and palythine (**5**) (*Fig. 1*). Compounds **1**–**3** are structurally related to **5**, an imino-MAA, and **1** is the decarboxylated analog of porphyra-334, which has been known for three decades [10]. Compound **1** is *N*-isopropanolpalythine (=aplysiapalythine A (APA)), **2** is *N*-ethylpalythine (=aplysiapalythine B (APB)), and **3** is *N*-methylpalythine (=aplysiapalythine C (APC)).

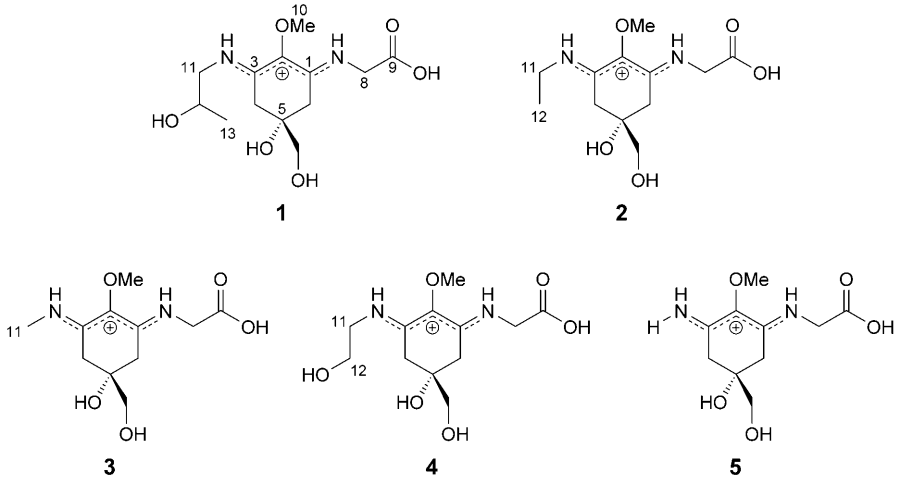

Fig. 1. *Molecular structures of five mycosporine-like amino acids,* **1**–**5**, *in opaline of wild-caught sea hares* Aplysia californica

Aplysiapalythine A (**1**) showed a strong maximum UV absorption at 332 nm (*Fig. 2*), which is characteristic for MAAs, and a $[M+H]^+$ ion peak at *m*/*z* 303.1614 in ESI-MS, indicating the molecular formula $C_{13}H_{22}N_2O_6$. The structure of **1** was determined by ^1H,^{13}C, COSY, HMQC, and HMBC NMR experiments in D_2O. All H-atoms were assigned to the corresponding C-atoms by HMQC data except exchangeable H-atoms of the OH and NH groups. HMQC and HMBC experiments revealed the following (see the *Table*): one C=O group (δ(C) 177.0); one olefinic O-bearing C-atom (δ(C) 127.8); two imine C-atoms (δ(C) 162.0 and 162.8); two CH_2 groups bonded to N-atom (δ(C) 49.0 and 52.42); two CH_2 groups (δ(C) 35.51 and 35.36); one quaternary C-atom (δ(C) 73.6); and one CH_2 group bonded to an O-atom. The one C=O and two C=N bonds accounted for all but one degree of unsaturation required by the molecular formula, indicating a cyclic structure for **1**. Further analysis of HMBC experiments revealed an imino-mycosporine ring. ^1H-NMR Resonances of CH_2(7) (δ 3.56), and CH_2(4) (δ 2.87) and CH_2(6) (δ 2.71, 2.78) showed HMBC correlation to the quaternary C(5)-atom (δ 73.6). ^1H-NMR Resonance of CH_2(4) (δ 2.87) showed HMBC

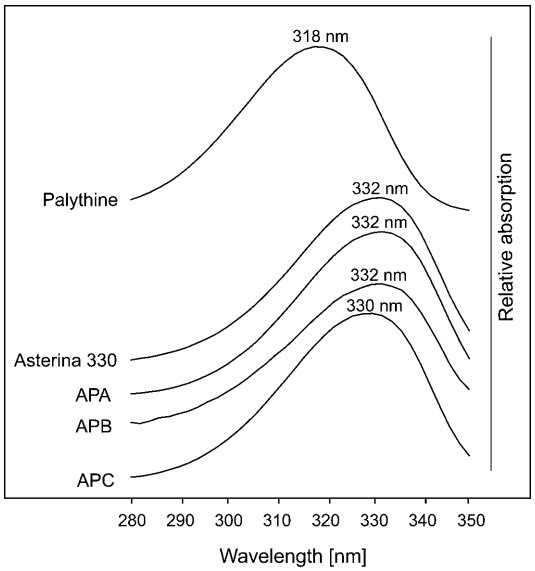

Fig. 2. *UV Absorption spectra of mycosporine-like amino acids isolated from opaline of wild-caught sea hares* Aplysia californica. Wavelength for maximum UV absorption is shown for palythine (**5**), asterina 330 (**4**), aplysiapalythine A (APA; **1**), aplysiapalythine B (APB; **2**), and aplysiapalythine C (APC; **3**). Data were collected from HPLC peaks with a photodiode array.

correlation to C(3) (δ 162.8) and C(2) (δ 127.8), while CH_2(6) showed correlation to C(1) (δ 61.5) and C(2) (δ 127.8). Finally, HMBC correlation from carbinol Me(10) to C(2) established an imino-mycosporine ring structure. HMBC Correlation from CH_2(8) (δ 4.00) to C(9) (δ 177.0) and C(1) (δ 162.0) substructure corresponds to palythine (**5**). The 2-hydroxyethyl group was identified by 2D-NMR experiments. HMBC Correlation from CH_2(11) (δ 3.40) to C(3) located the 2-hydroxyethyl group at the iminic N-atom connected to C(3) to establish planar structure of **1**. The configuration of the cyclohexane ring at C(5) was reported in [10]. The configuration at C(12) was not determined.

Aplysiapalythine B (**2**) had a characteristic potent maximum UV absorption at 332 nm (*Fig. 2*) and gave a $[M+H]^+$ ion peak at *m*/*z* 273.1288 in the ESI-MS, leading to the molecular formula $C_{12}H_{20}N_2O_5$. 1D- and 2D-NMR Analysis showed that **2** also had the palythine substructure and an Et group. HMBC Correlation from a CH_2 group (δ(H) 3.46) of the Et moiety to C(2) (δ(C) 162.3) and C(3) (δ(C) 127.6) located the Et group at the imine N-atom connected to C(3) (*Table*), thus establishing the structure of **2**.

Aplysiapalythine C (**3**) had a characteristic potent maximum UV absorption at 330 nm (*Fig. 2*) and gave a $[M-H]^-$ ion peak at *m*/*z* 257.1137 in ESI-MS, implying the molecular formula $C_{11}H_{18}N_2O_5$. Compound **3** also has the palythine substructure and a Me group. HMBC Correlation from Me(11) (δ 3.07) to C(3) (δ(C) 161.4) located the Me group at imine N-atom connected to C(3), establishing the structure of **3** (*Table*).

Table. *NMR Data of Aplysiapalythines A, B, and C* (**1**–**3**, resp.)*, Purified from Opaline in Sea Hares* Aplysia californica *Collected in Waters off the Coast of Southern California.* δ in ppm, J in Hz. Arbitrary numbering.

Position	Aplysiapalythine A (**1**)			Aplysiapalythine B (**2**)			Aplysiapalythine C (**3**)		
	δ(H)	δ(C)	HMBC	δ(H)	δ(C)	HMBC	δ(H)	δ(C)	HMBC
1		162.0 (*s*)			161.2 (*s*)			159.0 (*s*)	
2		127.8 (*s*)			127.6 (*s*)			125.6 (*s*)	
3		162.8 (*s*)			162.3 (*s*)			161.4 (*s*)	
4	2.87 (*s*)	35.5 (*t*)	3, 5, 6, 7	2.83 (*s*)	35.2 (*t*)	2, 3, 5, 6	2.87 (*s*)	33.3 (*t*)	2, 3, 5, 6, 7
5		73.6 (*s*)			73.6 (*s*)			73.6 (*s*)	1, 2, 4, 6, 7
6	2.71 (*d*, J = 17.5), 2.78 (*d*, J = 17.5)	35.4 (*t*)	1, 2, 4, 5	2.71 (*d*, J = 17.5), 2.77 (*d*, J = 17.5)	34.9 (*t*)	1, 2, 5	2.71 (*d*, J = 17.7), 2.60 (*d*, J = 17.7)	33.2 (*t*)	1, 2, 4, 5, 7
7	3.56 (*s*)	69.9 (*t*)	5, 6	3.57 (*s*)	70.0 (*t*)	5, 6	3.59 (*s*)	68.1 (*t*)	5
8	4.00 (*s*)	49.0 (*t*)	1, 9	3.98 (*s*)	49.0 (*t*)	1, 9	3.99 (*s*)	46.9 (*t*)	1, 9
9		177.0 (*s*)			178.0 (*s*)			175.8 (*s*)	
10	3.60 (*s*)	61.5 (*q*)	2	3.59 (*s*)	61.2 (*q*)	2	3.60 (*s*)	59.5 (*q*)	2
11	3.40 (*dd*, J = 7.6, 14.5), 3.47 (*dd*, J = 4.16, 14.5)	52.4 (*t*)	3, 12	3.46 (*q*, J = 7.3)	41.2 (*t*)	2, 3, 12	3.07 (*s*)	30.2 (*q*)	3
12	3.98 (*m*)	69.2 (*d*)		1.22 (*t*, J = 7.3)	17.0 (*q*)	11			
13	1.21 (*d*, J = 6.4)	21.9 (*q*)	11, 12						

The structures of **4** as asterina 330 and of **5** as palythine were confirmed by ESI-MS, and NMR and UV spectra. ESI-MS showed that **4** had a $[M+H]^+$ ion peak at *m/z* 289.1426, which is appropriate for the molecular formula $C_{12}H_{20}N_2O_6$. Compound **5** had a $[M-H]^-$ ion peak at *m/z* 243.0994, which implies the molecular formula $C_{10}H_{16}N_2O_5$. NMR Analysis revealed that the ^1H and ^{13}C chemical-shift data were consistent with the literature values for **4** and **5** [12–14]. UV Maxima for **4** and **5** were 332 and 318 nm, respectively. The slight difference in UV maximum for **4** is likely due to experimental conditions such as pH.

Conclusions. – We identified three novel MAAs in the defensive secretion of sea hares collected from waters of southern California, two of which function as intraspecific alarm cues in sea hares. All three MAA alarm cues are structurally related to the imino-MAA palythine **5**. They are *N*-isopropanolpalythine (=aplysiapalythine A (APA); **1**), *N*-ethylpalythine (=aplysiapalythine B (APB); **2**), and *N*-methylpalythine (=aplysiapalythine C (APC); **3**; *Fig. 1*). Two other known MAAs were also found in sea hare ink: asterina 330 (**4**) and palythine (**5**).

Our identification of three new MAAs out of the five that we identified in opaline is noteworthy in light of the fact that less than 30 MAAs are currently known [1][9][15]. This finding is probably due to our experimental approach of identifying MAAs using ESI-MS and NMR rather than relying on the more typically used HPLC with UV detection, which can be relatively imprecise [16][17]. Thus, we propose that there exists a much greater diversity of MAAs than is currently known.

MAAs function as sunscreen in many organisms [7], and this is probably also true of sea hares [4][5]. MAAs may also have other functions in organisms, including scavenging oxygen radicals, osmoregulation, light capture for photosynthesis, and as a nitrogen source [3][8][9]. However, our demonstration that **1**, **2**, and **4** act as intraspecific alarm cues at natural concentrations [11] is the first demonstration in any organism of MAAs acting as semiochemicals, *i.e.*, a chemical message between individuals of the same species [18]. Thus, the MAAs in opaline act together with a pyrimidine (uracil) and two nucleosides (uridine, cytidine) in ink, the other glandular component of ink secretion of sea hares [19], to constitute a potent alarm cue. These seemingly redundant signals in the co-released opaline and ink may be used, because one set, the MAAs, probably cannot be synthesized and are acquired solely from their diet of red algae, whereas the cues in ink can be synthesized. Furthermore, it is speculated that the MAAs in opaline may have evolved their semiochemical function secondary to their original function as sun screens [11].

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Experimental Part

General. HPLC: *Beckman* system equipped with a *168* photodiode array set at 200–600 nm with a *Develosil* pre-aqueous column (0.46 × 250 mm; *Nomura Kagaku Co.*, Japan). UV Spectra: *Beckman 168* photodiode array detector. NMR Spectra: 500-MHz *Bruker Avance* spectrometer (D-Rheinstetten) equipped with a triple resonance cryoprobe. ESI-MS: *Waters Q-TOF micro*.

Collection of Animals and Opaline. Sea hares *Aplysia californica* Cooper 1863 of 200–300 g of body weight were collected in waters off the coast of southern California by *Marinus Scientific* (Newport Beach, CA, USA). These animals were shipped to our laboratory in Atlanta, and opaline glands were dissected on the day the animals arrived. Opaline glands were frozen at $-80°$ until used. Opaline was collected by spinning opaline glands at 30,000*g* to separate the fluid from the gland tissue.

Extraction and Isolation. To isolate the alarm cues in opaline, 39 ml of opaline was first partitioned *via* a modified *Kupchan*'s scheme [20]. Because nearly all of the opaline components partitioned into the H_2O fraction, we combined the hexanes, $CHCl_3$, AcOEt, and BuOH partitions for a behavioral bioassay of alarm response, as described in [11][19]. We tested this combined fraction and the H_2O partition at natural concentrations, using seawater and opaline as negative and positive controls, resp. Only the H_2O-soluble partition elicited a frequency of alarm responses different from seawater. This fraction was further purified by passing it through lipophilic *Sephadex LH-20* with 100% H_2O as the mobile phase at a flow rate of 0.3 ml min^{-1}. This produced four fractions (based on characteristics on TLC), one of which produced an alarm response. Thus, this fraction was combined and further purified by passing through *Sephadex* (*Superdex Peptide* column, *GE Healthcare*) with 100% H_2O 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. Three fractions produced a trend toward a significant difference from seawater ($P = 0.063$), so we combined them for further purification by prep. thin layer reversed chromatography (*Partisil KC-18* silica gel, 60 Å, 200-μm thick, *Whatman*) developed with MeOH/H_2O 60:40 as solvent. Based on UV absorbance, two bands were collected separately from MeOH/H_2O 70:30. Each band was dried and tested separately in the bioassay. Only the top band elicited an alarm response. This fraction was further purified *via* HPLC using a *Phenomenex Luna* reversed-phase (RP) *C18* silica-gel 5-μ column (250 mm × 10 mm) with a mobile phase of MeOH/H_2O 3:97 at 0.8 ml min^{-1}. Three peaks elicited an alarm response and were further purified separately *via* another round of HPLC using a *Develosil RP Aqueous* reversed-phase *C30* silica-gel 5-μ column (250 mm × 4.6 mm) with a mobile phase of 100% H_2O at 0.8 ml min^{-1} to yield compounds **1**, **2**, and **4**. Compounds **3** and **5** were purified in the same way, from 20 ml of opaline. UV Spectra were obtained from this chromatography with a photodiode array.

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