Review

The Molecules and Mechanisms Underlying the Antimicrobial Activity of Escapin, an L–Amino Acid Oxidase from the Ink of Sea Hares

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Abstract: Many marine animals use chemicals to defend themselves and their eggs from predators. Beyond their ecologically relevant functions, these chemicals may also have properties that make them beneficial for humans, including with biomedical and industrial applications. For example, some chemical defenses are also powerful antimicrobial or anti-tumor agents with relevance to human health and disease. One such chemical defense, Escapin, an L–amino acid oxidase in the defensive ink of the sea hare *Aplysia californica*, and related proteins have been investigated for their biomedical properties. This review details our current understanding of Escapin's antimicrobial activity, including the array of chemicals generated by Escapin's oxidation of its major substrates, L–lysine and L–arginine, and mechanisms underlying these molecules' bactericidal and bacteriostatic effects on planktonic cells and the prevention of formation and removal of bacterial biofilms. Models of Escapin's effects are presented, and future directions are proposed.

Keywords: antimicrobial; *Aplysia*; biofilm; chemical defense; Escapin; L–amino acid oxidase

1. Introduction

This review is about Escapin, an L–amino acid oxidase in the ink of a gastropod mollusk, the sea hare *Aplysia californica*. Escapin and related proteins have been studied in different contexts. First, they have been studied in the context of natural history and chemical ecology, to examine their use as chemical defenses to protect sea hares from predators and their eggs from fouling organisms including bacteria. Second, they have been used in studies of natural products chemistry and drug discovery, in the search for molecules with applications for human health and disease. This review provides a short description of their role ecological roles, then a more extensive treatment of their antimicrobial properties related to human applications. This includes identification of molecules produced by Escapin's oxidation of its substrates L–lysine and L–arginine, and the cellular and molecular mechanisms whereby these molecules act as antimicrobial agents against planktonic and biofilm forms of bacteria.

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2. Escapin in the Context of Chemical Ecology

Gastropod molluscs are renowned for their use of chemical defenses as protection against predators, especially those gastropods with reduced or absent shells such as the Euopisthobranchia and Nudipleura [1]. There are many reviews on the subject of chemical ecology and identification of deterrent compounds in these species. Many of these deterrents are diet-derived rather than synthesized *de novo*, and many are terpenoids [2-7}. Some chemical deterrents are constitutively present, including in the skin and mucus. Other chemical deterrents are released only upon attack by a predator, an example being the ink of sea hares (Figure 1). This ink is actually a mixture of secretions of two glands – the ink gland and the opaline gland – which are simultaneously released into the mantle cavity and then expelled through a siphon toward the site of predatory attack [8]. The ink gland secretes a deep purple ink, and the opaline gland releases a whitish opaline that polymerizes and becomes viscous on contact with water. The feeding deterrent properties of sea hare ink have been reviewed elsewhere [9-12].

Figure 1. An inking sea hare, *Aplysia californica*. Escapin is found in the ink gland secretion, and its substrate, L–lysine, is found in the opaline gland secretion, both of which are mixed and released to form the ink shown emanating from this sea hare. Reproduced with permission Genevieve Anderson.

3. The Chemistry of Escapin

3.1. Escapin is an LAAOs, One of Several Types of AAOs

L–Amino acid oxidases such as Escapin are one type of amino acid oxidase (AAO). The AAOs are enzymes that oxidize amino acids and in the process produce hydrogen peroxide (H_2O_2) and ammonium. The AAOs are found broadly in animals and microbes and differ in substrate specificity and function. Phylogenetic analysis of AAOs based on gene sequences identify several clusters or types [13], as shown in Figure 2. One group is L–amino acid oxidases, which are flavin-dependent enzymes that oxidize L–amino acids. They are diverse and contain several groups including two groups from animals (one for vertebrates, one for gastropods), one group from fungi, as a substrate, and various bacterial LAAOs that do not always form distinct clusters (Figure 2). A second group is the D–amino acid oxidases (DAAOs), which are FAD-containing proteins and use D-amino acids as substrates. A third group is the L–lysine ε-oxidases and related proteins,

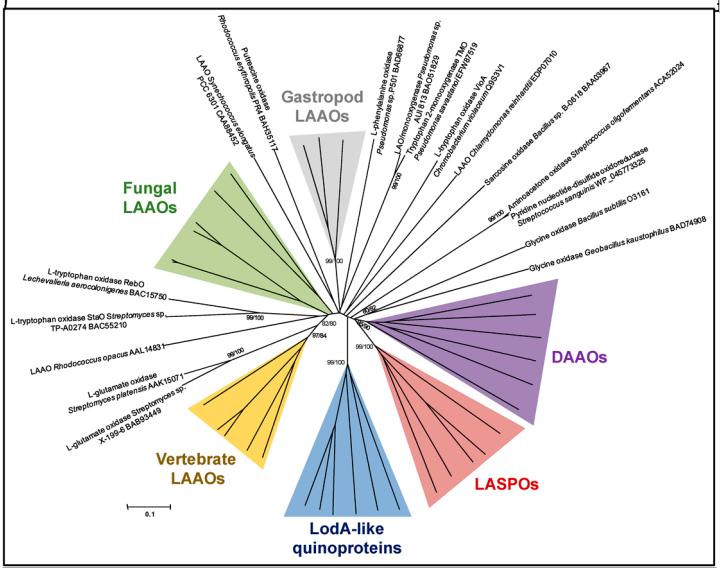

Figure 2. Phylogenetic relationships of enzymes with amino acids oxidase activity. LAAOs, L–amino acid oxidases; DAAOs, D-amino acid oxidases; LASPOs, L–aspartate oxidases; LodA-like quinoproteins, L–lysine ε-oxidases. Reproduced with permission from Campillo-Bocal et al., *Marine Drugs*; published by MDPI. See this reference for a description of methods used to generate this phylogeny.

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which contain a quinone cofactor, an example being LodA, an enzyme synthesized by melanogenic marine bacterium *Marinomonas mediterranea*. A fourth group is the L–aspartate oxidases, which are flavoproteins that use L–aspartate as a substrate.

A phylogenetic analysis of a selection of LAAOs was performed by Kamiya et al. [5] and is shown in Figure 3. This analysis shows that LAAOs in gastropods including Escapin and its homologues (Aplysianin A, Cyplasin L, APITI) and related compounds (Achacin) form one cluster, LAAOs from vertebrates including snake venoms and fish mucus molecules (AIP) form another cluster, and both clusters are distinct from a bacterial (cyanobacterium) LAAO. An Escapin homologue has also been found in another gastropod, *Biomphalaria glabrata* [14].

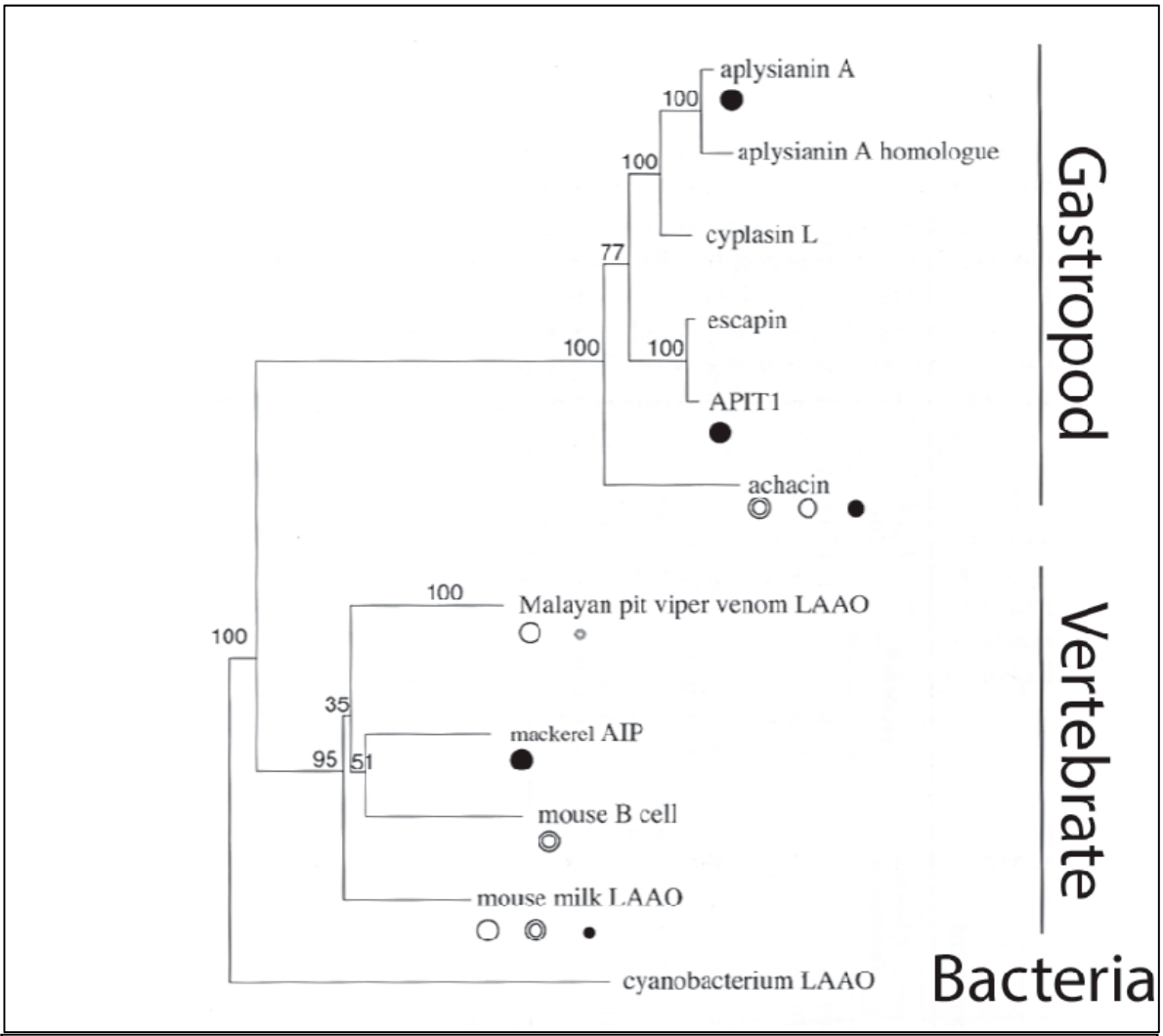

Figure 3. A phylogenetic tree and substrate specificity of L -amino acid oxidases, based on gene sequence, from gastropods, vertebrates, and bacteria. Substrate specificity is indicated by the symbols, where closed circles represent basic amino acids, open circles represent aromatic amino acids, and double circles represent aliphatic amino acids. Size of circles represents relative activity. Aplysianin A (*Aplysia kurodai* [15]); Aplysianin A homologue (*Aplysia californica* [16]); Cyplasin L (*Aplysia punctata* [17]); Escapin (*Aplysia californica* [18]); APIT1 (*Aplysia punctata* [19, 20]); Achacin (*Achatina fulica* [21, 22]); pit viper venom (*Calloselasma rhodostoma* [23]); AIP (*Scomber japonicus* [24]); mouse B cell (*Mus musculus* [25]); mouse milk LAAO (*Mus musculus* [26]). Modified from Fig. 10.4 of Kamiya et al. [5].

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3.2. Homologues of Escapin

Each sea hare species expresses several LAAOs with an organ-specific expression pattern, and an organ can express more than one type of LAAO (*A. punctata* [17, 19, 20, 27-29]). Escapin has several homologues; including some expressed in the ink gland (Dactylomelin P in *Aplysia dactylomela* [30] and APIT, Cyplasin L, and Cyplasin S in *Aplysia punctata* [17, 19, 20]) and some expressed in other tissues (Aplysianin A precursors expressed in the albumen gland of *A. californica*: [15]).

3.3 Escapin's Substrates and Products

Escapin and its homologues prefer as substrates basic amino acids: L–lysine and L–arginine [15, 18-20]. The principal natural substrate for Escapin in the ink of sea hares is L–lysine, since it is in much higher concentration in ink than is L–arginine [31]. Sea hares store Escapin and its substrates in separate reserve pools: Escapin in the ink gland, and L–lysine in the opaline gland [29]. Escapin and its substrate are only mixed when the defensive secretion is deployed and released. In fact, Escapin is kept in specific (amber) vesicles in the ink gland, separate from other (purple) vesicles in the ink gland that contain Aplysioviolin, another chemical defense [12, 29, 32, 33].

Studies of the chemistry of Escapin show that Escapin's oxidation of L–lysine produces a mixture of ingredients that changes quickly over time [34], as shown in Figure 4. The first step is Escapin's oxidative deamination of L–lysine (**compound 1**), which produces an equilibrium mixture of compounds called "escapin intermediate products of lysine" (EIP-K, or just EIP). EIP includes α-keto-

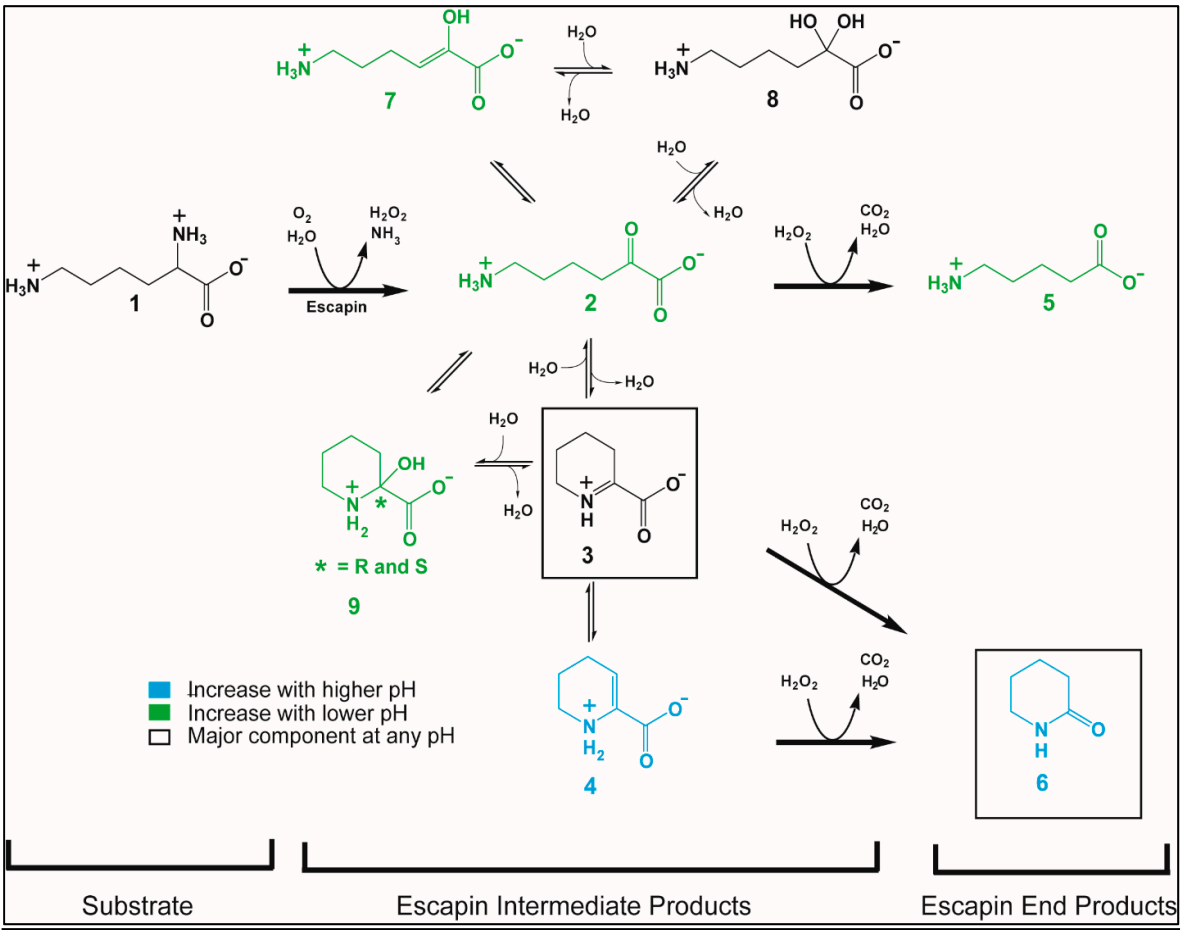

Figure 4. Summary of the chemistry of the reaction of escapin with L–lysine, including the effects of pH on the relative composition of the molecular species in the equilibrium mixture. This series of chemical reactions produces products that interact with each other to form a diverse group of molecules at low millimolar concentrations within a few seconds of the release of the secretion. Modified from Kamio et al. [34].

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ε-aminocaproic acid (**compound 2**), Δ^1-piperideine-2-carboxylic acid (**compound 3**), Δ^2-piperideine-2-carboxylic acid (**compound 4**), 6-amino-2-hydroxy-hex-2-enoic acid (**compound 7**), possibly 6-amino-2,2-dihydroxy-hexanoic acid (**compound 8**), 2-hydroxy-piperidine-2-carboxylic acid (**compound 9**), H_2O_2, and ammonium. Three of these compounds, **compounds 2**, **3**, and **4**, then react non-enzymatically with H_2O_2 to yield a mixture of δ-aminovaleric acid (**compound 5**) and δ-valerolactam (**compound 6**), called "escapin end products of L–lysine" (EEP-K, or EEP). The pH of *A. californica* ink is *ca.* 5.0 at full strength, in contrast to a pH of *ca.* 8.0 for seawater. This is significant because pH affects the equilibrium among Escapin's reaction products: the naturally low pH of the secretion favors the linear forms of EIP (**compound 2** and **compound 5**), though the cyclic forms (**compound 3** and **compound 6**) dominate at any pH. A kinetic analysis of Escapin showed that incubation of Escapin and L–lysine at natural concentrations produces millimolar concentrations of hydrogen peroxide, ammonia, and other reaction products within seconds [34].

4. Bioactivity of Escapin's Products

4.1. Anti-Predatory Agents.

The efficacy of Escapin's products as a feeding deterrent depends on the identity of the molecules and predators. Hydrogen peroxide is a deterrent against fish and crustaceans [35-37], and EIP is also deterrent against fish [36, 37]. So far, there is no evidence of synergy between H_2O_2 and EIP in antipredatory effects.

4.2. Antimicrobial Agents.

Escapin's products inhibits the growth of several types of microbes, including Gram-negative and Gram-positive bacteria, fungi, yeast, and mold, with minimum inhibitory concentrations of between.25 and 65 μg/ml [18]. Homologues of Escapin have been shown to have antitumor properties [5, 15, 19, 38], but Escapin has not been tested for such properties. Escapin is an effective antimicrobial agent against both planktonic bacteria and biofilms, and, as described in this section, some of its mechanisms of action are known.

4.2.1. Planktonic Bacteria. Escapin has both bacteriostatic and bactericidal effects on planktonic cells. The bacteriostatic effect, in which growth is inhibited, is mediated by H_2O_2 alone, without a contribution from EIP or EEP. This is shown through equal bacteriostatic effects of H_2O_2 and of Escapin products when Escapin's substrate is either L–lysine or L–arginine (both of which are equally effective substrates in the production of H_2O_2) (Fig. 5A) [18]. Homologues of Escapin also appear to be bacteriostatic, at least in large part because of the effects of H_2O_2 [5,15,19,22,39]. Escapin's bactericidal effects, however, are not due only to H_2O_2, a surprising

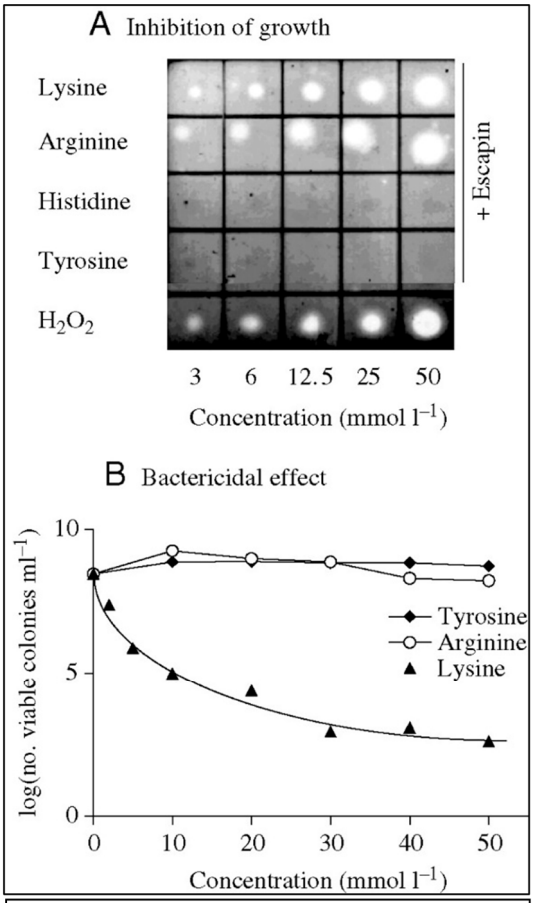

Figure 5. Antimicrobial effects of Escapin on planktonic bacteria, *E. coli*. (A) Bacteriostatic effect: plate assay of growth inhibition. *E. coli* cells were grown in the presence of Escapin and the indicated amino acid, or in H_2O_2 alone. (B) Bactericidal effect: *E. coli* cells were grown in media, and then incubated with Escapin and the indicated amino acid. Reproduced with permission from Yang et al., 2005, *Antimicrobial Agents and Chemotherapy*; published by the American Society for Microbiology.

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result. This can be seen in Fig. 5B, in which L–lysine but not L–arginine is an effective substrate in Escapin's bactericidal effects [18].

Another important observation in understanding Escapin's mechanisms of bactericidal action was a synergistic effect of H_2O_2 and EIP when L–lysine, but not L–arginine, was used as the substrate [40]. Figure 6 shows that a mixture of H_2O_2 and EIP using L–lysine as the substrate is by far the most effective bactericidal agent of those tested, more than either alone, more than H_2O_2 and EEP, and more than H_2O_2 and EIP when L–arginine was the substrate [40]. The effect can be powerful, reducing the number of cells by more than seven log units in some cases (Fig. 6). This demonstrates that the bactericidal effect is due to molecules other than H_2O_2 alone. Furthermore, Figure 7 shows the concentration dependence of this potent synergistic bactericidal effect for *E. coli*, with a maximum effect of *ca.* 13 mM EIP-K and 2.5 mM H_2O_2. Such concentration-response relationships where both higher and lower concentrations are less effective than concentration in between is known as the Eagle effect, and it has been reported for a variety of microbes and antimicrobial agents [41-43].

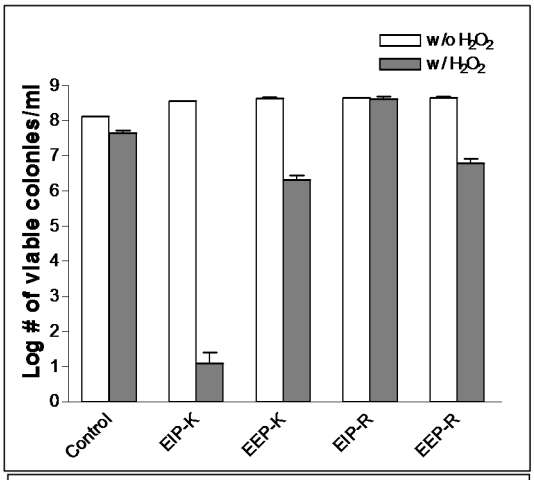
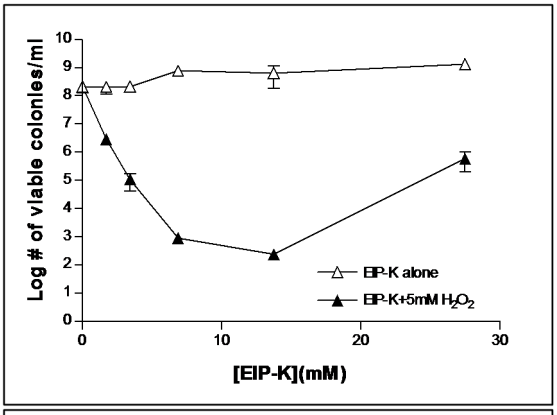


Figure 6. Bactericidal effects on *E. coli* of Escapin products at 45 mM in the absence or presence of 10 mM H_2O_2. EIP is Escapin Intermediate Products; EEP is Escapin End Products; K indicates that L–lysine was the substrate; R indicates that L–arginine was the substrate. The compositions of EIP-K and EEP-K are shown in Figure 4. From Ko et al. 2008. Reproduced with permission from Ko et al. 2008, Antimicrobial Agents and Chemotherapy; published by the American Society of Microbiology.



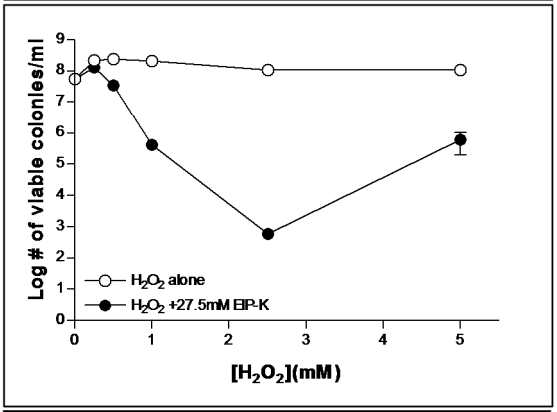


Figure 7. Concentration dependence of synergy between EIP and H_2O_2 on *E. coli*. Same legend as in Figure 6. Reproduced with permission from Ko et al. 2008, Antimicrobial Agents and Chemotherapy; published by the American Society of Microbiology.

The synergistic action of co-presented H_2O_2 and EIP might result from the generation of novel, strongly bactericidal compounds from the chemical reaction between H_2O_2 and components in EIP. This hypothesis was tested by presenting H_2O_2 and EIP either simultaneously or sequentially, and then determining if synergy occurred. A short (10 min) co-treatment with H_2O_2 and EIP was sufficient to generate long-lasting bactericidal effects [40,44], but a 10-min presentation with either H_2O_2 or EIP only followed by brief rinsing and 10-min treatment of the other showed no synergy [40]. This supports the idea that the synergy of H_2O_2 and EIP is due to novel compounds generated by their chemical interactions, assuming that the effect of either H_2O_2 alone or EIP alone is long lasting (at least longer than 10 min).

One identified effect of H_2O_2 and EIP is a rapid and long-lasting DNA condensation [44]. A two-min treatment with H_2O_2 and EIP causes significant DNA condensation(Fig. 8) and killing, and 10-min treatment causes a maximal effect that lasts at least 70 h. Consistent with an effect on DNA is that H_2O_2 and EIP act preferentially on fast-growing cells (*i.e.* cells in their log-growth phase) *vs.* cells in their stationary phase [18]. Additionally, Escapin's bactericidal effects do not require protein synthesis [18,40].

DNA condensation might occur because of alterations in the oxidation process. Bacteria with a single missense mutation in the oxidation regulatory gene, *oxyR*, are resistant to EIP and H_2O_2. This

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effect is a specific response to oxidative stress (i.e. H_2O_2), since temperature stress combined with EIP (but not H_2O_2) does not produce the bactericidal effect [44]. Experiments using mutants for several single DNA-binding proteins suggest that EIP and H_2O_2 function through a combination of DNA-binding proteins [44]. Experiments with chelators and scavengers suggest that hydroxyl radicals may mediate these effects [44].

The results to date indicate that the powerful, rapid, and long-lasting bactericidal effect of Escapin's oxidation of L–lysine is that this reaction generates a rich array of highly reactive molecules that affect DNA of fast-growing planktonic bacterial cells. Hydroxyl radicals and possibly other oxidative agents generated by Escapin may interact in a specific way with the oxidation regulatory gene, *oxyR*. In turn, *oxyR* interacts with several DNA binding proteins, including Dps and H-NS, causing irreversible DNA condensation and inhibition of DNA unwinding mechanisms, thus arresting the initiation of DNA replication and initiating the degradation of DNA [44]. The role of molecules in EIP in this process is less clear. EIP can move across bacterial cell membranes (Mihika T. Kozma, P. C. Tai, and C. D. Derby, unpublished data), so its effects could be effected either by binding to receptor proteins on the bacterial cell membrane or by interacting with intracellular targets. EIP might play a role in stabilizing the oxidative response from H_2O_2 and thus in inducing the irreversible DNA condensation and degradation. Future studies must investigate not only the independent effects of EIP and H_2O_2 but also the interactive effects of the combination of the two, because of the synergistic effects of the two components.

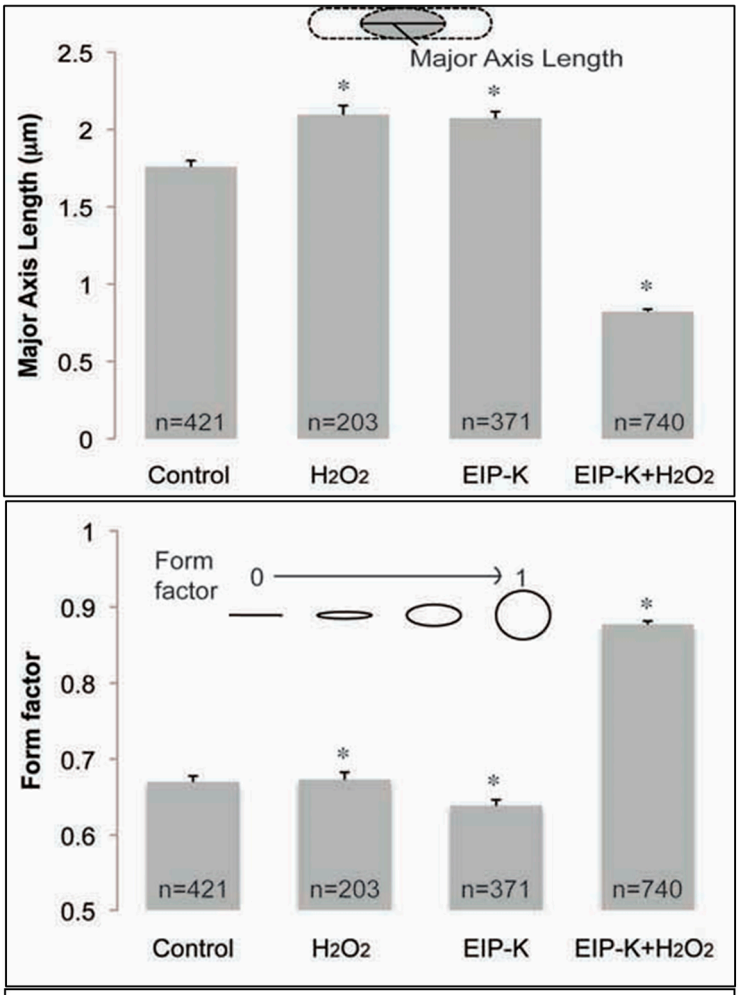

Figure 8. DNA condensation following treatment with H_2O_2, EIP, or the combination of the two. DNA condensation was quantified using length of the major axis of the nucleoid (upper figure) and form factor, where 1.0 is a perfect circle and 0.0 is a straight line (lower figure). Reproduced with permission from Ko et al. 2008, Antimicrobial Agents and Chemotherapy; published by the American Society of Microbiology.

4.2.2. Bacterial Biofilms.

In nature, most microbes exist as biofilms rather than as planktonic cells. Biofilms are communities of cells attached to surfaces and encased in a self-produced extracellular matrix called extracellular polymeric substances (EPSs) [45,46]. The life cycle of biofilms includes attachment of planktonic cells to a surface, growth of the biofilm, and dispersal (Fig. 9). Biofilms are highly dynamic, with environmental conditions supporting either growth or emigration of bacterial cells from the biofilm. The structure and composition of EPSs in biofilms is variable but typically includes an abundance of polysaccharides in addition to proteins, nucleic acids, and lipids, held together through physicochemical interactions [47]. The EPSs provide biofilms with resistance to environmental

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perturbations, including to antimicrobials, which makes them a challenge in medical and industrial settings [48-50]. Consequently, development of antibiofilm strategies is an active field.

Escapin's products have been tested as antimicrobial agents against biofilms of the pathogen *Pseudomonas aeruginosa*. This included examining inhibition of biofilm formation and disruption of established biofilms, and if EIP and H_2O_2 acted synergistically [51]. In these experiments, chemically synthesized EIP was used as it has similar activities as the products of Escapin's action on L-lysine [40,44]. Significant effects were found with very low concentrations, in the micromolar range. For example, in 5 hr assays of inhibition of biofilm formation in microtiter plates, biofilms exposed to 96 μM H_2O_2 were 30% smaller than controls, biofilms exposed to 3 μM EIP were 25% smaller than controls, and biofilms exposed to the combination of the two (96 μM H_2O_2 + 3 μM EIP) were 65% smaller than the control. Assays of dispersal of established biofilms by chemical agents involved growing biofilms in flow cells for 20 h, then exposing them to agents for 30 min, and then quantifying biomass using microscopy. These assays show that a combination of EIP at 50 μM + H_2O_2 at between 0.03 and 3 μM caused 40% clearance of biofilms, while each alone caused very little clearance; thus, over this range of concentrations, EIP + H_2O_2 had a synergistic effect in disrupting biofilms (Fig. 10). Together, these results show that micromolar, and in some cases even nanomolar, concentrations of EIP and H_2O_2 can affect biofilm formation or disruption. These concentrations are significantly lower than those causing bactericidal effects on planktonic bacteria.

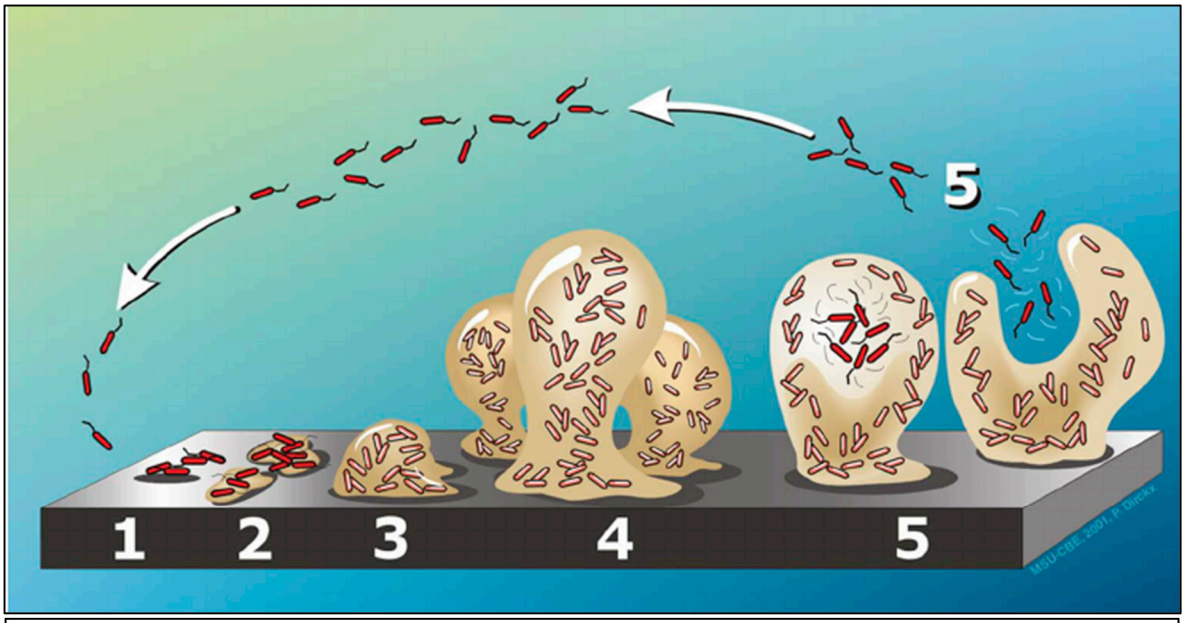

Figure 9. Structure and dynamics of bacterial biofilms. 1, reversible attachment; 2, irreversible attachment; 3, maturation 1; 4, maturation 2; 5, dispersion. From P. Dirckx, Center for Biofilm Engineering, Montana State University, Bozeman.

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The biology of Escapin's action against biofilms is still largely unexplored, with the most fundamental questions surrounding its mechanisms of activity. The work carried out to date generated several clues that are likely to be part of the story. First, sub-micromolar concentrations of H_2O_2 in combination with EIP resulted in significant biofilm disruption (Fig. 10) and reduced biofilm formation. Second, EIP increased swimming motility (Fig. 11) and yet did not affect swarming or other types of motility. Several hypotheses that incorporate these observations are plausible, potentially involving either intracellular or extracellular factors that affect biofilms. An essential component of a microbial biofilm is the extracellular matrix, which physically anchors the community to the substratum. The matrix is comprised of diverse biopolymers and ions, which contribute to its structural integrity. A matrix building block used by diverse biofilm-forming microorganisms is extracellular DNA (eDNA) [52]. EIP promotes DNA condensation and it could potentially influence eDNA concentrations and three-dimensional structure in the biofilm matrix, resulting in biofilm disruption. Alternatively, surfactants can contribute to biofilm detachment [53,54]. EIP, with its mixture of cationic and amphipathic structures, could act as a surfactant to promote biofilm detachment. Additionally, the negatively charged functional groups of several EIP components (Fig. 1) could work as chelators, interacting with calcium ions in the biofilm matrix and reducing its stability [55,56]. Another potential mode of action for Escapin on biofilms is to interact with cellular signaling networks that influence biofilm detachment. One of the surprising findings of our work was the low concentrations of H_2O_2 that affected biofilm disruption. While no work to date has focused on sub-micromolar concentrations of hydrogen peroxide, nitric oxide concentrations in the same range can promote biofilm dispersal [57]. Nitric oxide targets cyclic di-GMP signaling in *P. aeruginosa* [58,59] and hydrogen peroxide may work similarly and be potentiated by EIP. EIP and H_2O_2 enhanced swimming motility while decreasing biofilm formation. These traits have been inversely linked via cyclic di-GMP signaling pathways and related

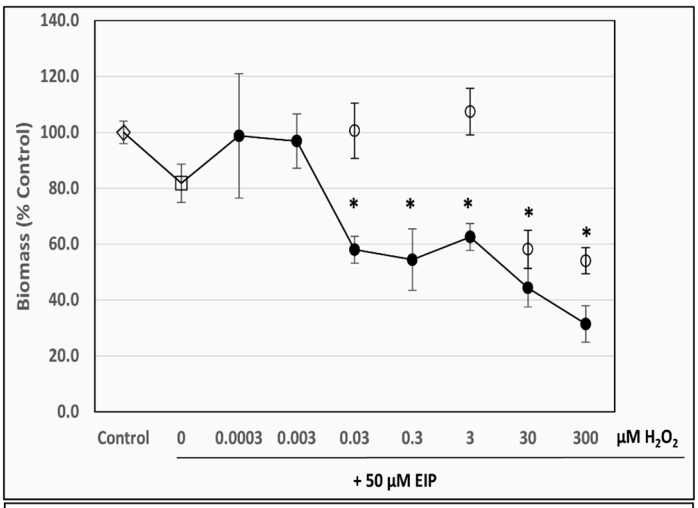

Figure 10. Dispersal ability of EIP + H_2O_2 against *P. aeruginosa* biofilms (i.e., biofilm disruption). Flow cell-cultivated *P. aeruginosa* biofilms (20-h) were analyzed post-treatment by confocal microscopy. Open diamond, untreated control; open square, 50 μM EIP alone; open circle, H_2O_2 alone; filled circle, EIP + H_2O_2. Values are means ± SEM for three replicates for each experimental condition. Reproduced with permission from Santiago et al. 2016, *Antimicrobial Agents* and Chemotherapy; published by the American Society for Microbiology.

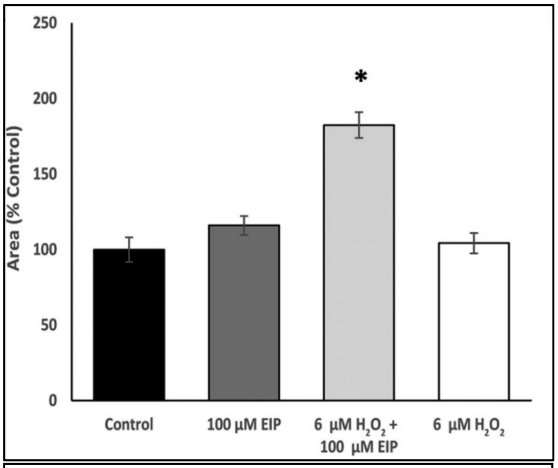

Figure 11. Effects of EIP + H_2O_2 on motility of *P. aeruginosa* after treatment for 2 h at 37°C. Swimming motility was quantified as the area of the motility zone for each treatment. Values are means ± SEM for two replicates for each treatment. Values for treatments were normalized to levels in the untreated controls after the mean of the values for each of the control replicates was determined. Reproduced with permission from Santiago et al. 2016, *Antimicrobial Agents and Chemotherapy*; published by the American Society for Microbiology.

regulatory elements in *P. aeruginosa* and suggest that EIP may act by interacting with this network [60].

5. Conclusions

In response to the global spread of antibiotic resistance, there is a demand for compounds that have the ability to attenuate microbial pathogenicity yet are not biocidal [61]. The logic for using compounds with anti-virulence capabilities is to control infections while reducing the strong selection for resistant phenotypes caused by standard antibiotics. This is particularly important for Gram-negative bacteria such as *P. aeruginosa* for which the presence of outer membranes renders many antibiotics impermeable to the cells. To date, there are a limited number of compounds that have both anti-virulence and anti-biofilm characteristics. A recent review on therapeutics for biofilm eradication argued that a multi-pronged approach is required to effectively manage biofilm infections, due to their complexity at many levels [62]. From this standpoint, Escapin has potential as a therapeutic agent and warrants further attention. Progress in evaluating its use as a therapeutic agent requires a greater knowledge of its mechanisms of action. As described in this this review, some of the independent and synergistic actions of EIP and H_2O_2 are known, but much more detailed information is necessary. A challenge working with EIP is that there is variation in the activity of the mixture, since EIP and H_2O_2 are reactive and the ratios of the components in the equilibrium mixture may be different with each reaction and over time. To facilitate research into the mechanism of EIP, a consistent mixture of EIP should be available. The development of a synthetic approach to making EIP solved part of this requirement [34]. To move beyond the associated uncertainty, a synthetic EIP made from known concentrations of the chemical components needs to be developed.

Acknowledgments: The experimental work on the antimicrobial aspects of Escapin was supported by The Georgia Research Alliance and Georgia State University, and work on chemical ecology and antipredatory effects of Escapin was supported by grants from the National Science Foundation. We thank our students and collaborators for their substantial contributions to the experimental work and ideas presented in this review

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Jörger, K.M.; Stöger, I.; Kano, Y.; Fukuda, H.; Knebelsberger, T.; Schrödl, M. On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia. *BMC Evol. Biol.* **2010,** *10*, 323. DOI: 10.1186/1471-2148-10-323

[2] Avila, C. Natural products of opisthobranch molluscs: a biological review. *Oceanogr. Mar. Biol. Annu. Rev.* **1995**, *33*, 487–559.

[3] Cimino, G.; Fontana, A.; Gavagnin, M. Marine opisthobranch molluscs: chemistry and ecology in sacoglossans and dorids. *Curr. Organic Chem*. **1999**, *3*, 327–372.

[4] Paul, V.J., Arthur, K.E.; Ritson-Williams, R.; Cliff, R.; Sharp, K. Chemical defenses: from compounds to communities. *Biol. Bull.* **2007**, *213*, 226–251. DOI: 10.2307/25066642

[5] Kamiya, H.; Sakai, R.; Jimbo, M. Bioactive molecules from sea hares. In *Molluscs: From Chemo-ecological Study to Biotechnological Application. Progress in Molecular and Subcellular Biology. Marine Molecular Biotechnology*. Cimino, G.; Gavagnin, M., Eds.; Springer-Verlag: Berlin-Heidelberg, Germany, **2006**; pp. 215–239, ISBN-978-3-540-30879-9

[6] Benkendorff, K. Molluscan biological and chemical diversity: secondary metabolites and medicinal resources produced by marine molluscs. *Biol. Rev. Camb. Philos. Soc.* **2010,** *85*, 757–775. DOI: 10.1111/j.1469-185X.2010.00124.x

[7] Bornancin, L.; Bonnard, I.; Mills, S.C.; Banaigs, B. 2017. Chemical mediation as a structuring element in marine gastropod predator-prey interactions. *Nat. Prod. Rep.* **2017,** *34*, 644. DOI: 10.1039/c6np00097e

[8] Walters, E.T.; Erickson, M.T. Directional control and the functional organization of defensive responses in *Aplysia*. *J. Comp. Physiol. A* **1986,** *159*, 339–351. DOI: 10.1007/BF00603980

[9] Carefoot, T.H. *Aplysia*: its biology and ecology. *Oceanogr. Mar. Biol. Annu. Rev.* **1987**, *25*, 167–284.

[10] Johnson, P.M.; Willows, A.O.D. Defense in sea hares (Gastropoda, Opisthobranchia, Anaspidea): multiple layers of protection from egg to adult. *Mar. Freshw. Behav. Physiol.* **1999,** *32*, 147–180. DOI: 10.1080/10236249909379045

[11] Derby, C.D. Escape by inking and secreting: marine molluscs avoid predators through a rich array of chemicals and mechanisms. *Biol. Bull.*, **2007**, *213*, 274–289. DOI: 10.2307/25066645

[12] Derby, C.D.; Aggio, J.F. Neuroecology of chemical defenses. *Integr. Comp. Biol.* **2011**, *51*, 771–780. DOI: 10.1093/icb/icr063

[13] Campillo-Brocal, J.C.; Lucas-Elío, P.; Sanchez-Amat, A. Distribution in different organisms of amino acid oxidases with FAD or a quinone as cofactor and their role as antimicrobial proteins in marine bacteria. *Mar. Drugs* **2015**, *13*, 7403–7418. DOI: 10.3390/md13127073

[14] Hathaway, J.J.; Adema, CM; Stout, B.A.; Mobarak, C.D.; Loker, E.S. Identification of protein components of egg masses indicates parental investment in immunoprotection of offspring by *Biomphalaria glabrata* (Gastropoda, Mollusca). *Dev. Comp. Immunol.* **2010**, *34*, 425–435. DOI: 10.1016/j.dci.2009.12.001

[15] Jimbo, M.; Nakanishi, F.; Sakai, R.; Muramoto, K.; Kamiya, H. Characterization of L-amino acid oxidase and antimicrobial activity of aplysianin A, a sea hare-derived antitumor-antimicrobial protein. *Fish. Sci.* **2003**, *69*, 1240–1246. DOI: 10.1111/j.0919-9268.2003.00751.x

[16] Cummins, S.F.; Nichols, A.E.; Amare, A.; Hummon, A.B.; Sweedler, J.V.; Nagle, G.T. Characterization of *Aplysia* enticin and temptin, two novel water-borne protein pheromones that act in concert with attractin to stimulate mate attraction. *J. Biol. Chem.* **2004,** *279*, 25614–25622. DOI: 10.1074/jbc.M313585200

[17] Petzelt, C.; Joswig, G.; Stammer, H.; Werner, D. Cytotoxic cyplasin of the sea hare, *Aplysia punctata*, cDNA cloning, and expression of bioactive recombinants in insect cells. *Neoplasia* **2002,** *4*, 49–59. DOI: 10.1038/sj.neo.7900202

[18] Yang, H.; Johnson, P.M.; Ko, K.-C.; Kamio, M.; Germann, M.W.; Derby, C.D.; Tai, P.C. Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*. *J. Exp. Biol.* **2005,** *208*, 3609–3622. DOI: 10.1242/jeb.01795

[19] Butzke, D.; Machuy, N.; Thiede, B.; Hurwitz, R.; Goedert, S.; Rudel, T. Hydrogen peroxide produced by *Aplysia* ink toxin kills tumor cells independent of apoptosis via peroxiredoxin I sensitive pathways. *Cell Death Differ*. **2004,** *11*, 608–617. DOI: 10.1038/sj.cdd.4401385

[20] Butzke, D.; Hurwitz, R.; Thiede, B.; Goedert, S.; Rudel, T. Cloning and biochemical characterization of APIT, a new L-amino acid oxidase from *Aplysia punctata*. *Toxicon* **2005**, *46*, 479–489. DOI: 10.1016/j.toxicon.2005.06.005

[21] Obara, K.; Otsuka-Fuchino, H.; Sattayasai, N.; Nonomura, Y.; Tsuchiya, T.; Tamiya, T. Molecular cloning of the antibacterial protein of the giant African snail, *Achatina fulica* Férussac. *Eur. J. Biochem.* **1992,** *209*, 1–6. DOI: 10.1111/j.1432-1033.1992.tb17254.x

[22] Ehara, T.; Kitajima, S.; Kanzawa, N.; Tamiya, T.; Tsuchiya, T. Antimicrobial action of achacin is mediated by L-amino acid activity. *FEBS Lett.* **2002,** *531*, 509–512. DOI: 10.1016/S0014-5793(02)03608-6

[23] Ponnudurai, G.; Chung, M.C.; Tan, N.H. Purification and properties of the L-amino acid oxidase from Malayan pit viper (*Calloselasma rhodostoma*) venom. *Arch. Biochem. Biophys.* **1994,** *313*, 373–378. DOI: 10.1006/abbi.1994.1401

[24] Jung, S.-K.; Mai, A.; Iwamoto, M.; Arizono, N.; Fujimoto, D.; Sakamaki, K.; Yonehara, S. Purification and cloning of an apoptosis-inducing protein derived from fish infected with *Anisakis simplex*, a causative nematode of human anisakiasis. *J. Immunol.* **2000,** *165,* 1491–1497. DOI: 10.4049/jimmunol.165.3.1491

[25] Mason, J.M.; Naidu, M.D.; Barcia, M.; Porti, D.; Chavan, S.S.; Chu, C.C. IL-4-inducing gene-1 is a leukocyte L-amino acid oxidase with an unusual acidic pH preference and lysosomal localization. *J. Immunol*. **2004,** *173*, 4561–4567. DOI: 10.4049/jimmunol.173.7.4561

[26] Sun, Y.; Nonobe, E.; Kobayashi, Y.; Kuraishi, T.; Aoki, F.; Yamamoto, K.; Sakai, S. Characterization and expression of L-amino acid oxidase of mouse milk. *J. Biol. Chem*. **2002,** *277*, 19080–19086. DOI: 10.1074/jbc.M200936200

[27] Iijima, R.; Kisugi, J.; Yamazaki, M. A novel antimicrobial peptide from the sea hare *Dolabella auricularia*. *Dev. Comp. Immunol*. **2003,** *27*, 305–311. DOI: 10.1016/S0145-305X(02)00105-2

[28] Iijima, R.; Kisugi, J.; Yamazaki, M. L-Amino acid oxidase activity of an antineoplastic factor of a marine mollusk and its relationship to cytotoxicity. *Dev. Comp. Immunol.* **2003,** *27*, 505–512. DOI: 10.1016/S0145-305X(02)00140-4

[29] Johnson, P.M.; Kicklighter, C.E.; Schmidt, M.; Kamio, M.; Yang, H.; Elkin, D.; Michel, W.C.; Tai, P.C.; Derby, C.D. Packaging of chemicals in the defensive secretory glands of the sea hare *Aplysia californica*. *J. Exp. Biol.* **2006,** *209*, 78–88. DOI: 10.1242/jeb.01972

[30] Melo, V.M.M.; Duarte, A.B.G.; Carvalho, A.F.F.U.; Siebra, E.A.; Vasconcelos, I.M. Purification of a novel antibacterial and haemagglutinating protein from the purple gland of the sea hare, *Aplysia dactylomela* Rang, 1828. *Toxicon* **2000,** *38*, 1415–1427. DOI: 10.1016/S0041-0101(99)00234-2

[31] Kicklighter, C.E.; Shabani, S.; Johnson, P.M.; Derby, C.D. Sea hares use novel antipredatory chemical defenses. *Curr. Biol.* **2005,** *15*, 549–554. DOI: 10.1016/j.cub.2005.01.057

[32] Kamio, M.; Grimes, T.V.; Hutchins, M.H.; van Dam, R.; Derby, C.D. The purple pigment aplysioviolin in sea hare ink deters predatory blue crabs through their chemical senses. *Anim. Behav.* **2010,** *80*, 89–100. DOI: 10.1016/j.anbehav.2010.04.003

[33] Kamio, M.; Nguyen, L.; Yaldiz, S.; Derby, C.D. 2010. How to produce a chemical defense: structural elucidation and anatomical distribution of aplysioviolin and phycoerythrobilin in the sea hare *Aplysia californica*. *Chem. Biodivers.* **2010,** 7, 1183–1197. DOI: 10.1002/cbdv.201000006

[34] Kamio, M.; Ko, K.-C.; Zheng, S.; Wang, B.; Collins, S.L.; Gadda, G.; Tai, P.C.; Derby, C.D. The chemistry of escapin: identification and quantification of the components in the complex mixture generated by an L-amino acid oxidase in the defensive secretion of the sea snail *Aplysia californica*. *Chem. Eur. J.* **2009,** *15*, 1597–1604. DOI: 10.1002/chem.200801696

[35] Aggio, J.F.; Derby, C.D. Hydrogen peroxide and other components in the ink of sea hares are chemical defenses against predatory spiny lobsters acting through non-antennular chemoreceptors. *J. Exp. Mar. Biol. Ecol.* **2008,** *363*, 28–34. DOI: 10.1016/j.jembe.2008.06.008

[36] Nusnbaum, M.; Derby, C.D. Effects of sea hare ink secretion and its escapin-generated components on a variety of predatory fishes. *Biol. Bull.* **2010,** *218*, 282–292. DOI: 10.1086/BBLv218n3p282

[37] Nusnbaum, M.; Derby, C.D. Ink secretion protects sea hares by acting on the olfactory and non-olfactory chemical senses of a predatory fish. *Anim. Behav.* **2010,** 79: 1067–1076. DOI: 10.1016/j.anbehav.2010.01.022

[38] Yamazaki, M. Antitumor and antimicrobial glycoproteins from sea hares. *Comp. Biochem. Physiol. C* **1993,** *105*, 141–146. DOI: 10.1016/0742-8413(93)90185-N

[39] Kanzawa N, Shintani S, Ohta K, Kitajima S, Ehara T, Kobayashi H, Kizaki H, Tsuchiya T. Achacin induces cell death in HeLa cells through two different mechanisms. *Arch. Biochem. Biophys.* **2004,** *422*,103–109. DOI: 10.1016/j.abb.2003.12.007

[40] Ko, K.-C.; Wang, B.; Tai, P.C.; Derby, C.D. Identification of potent bactericidal compounds produced by escapin, an L-amino acid oxidase in the ink of the sea hare *Aplysia californica*. *Antimicrob. Agents Chemother.* **2008,** *52*, 4455–4462. DOI: 10.1128/AAC.01103-08

[41] Eagle, H.; Musselman, A.D. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. *J. Exp. Med.* **1948**, *88*, 99–131. **DOI:** 10.1084/jem.88.1.99

[42] Stevens, D.L.; Gibbons, A.E.; Bergstrom, R.; Winn, V. The Eagle effect revisited: efficacy of clindamycin, erythromycin, and penicillin in the treatment of streptococcal myositis. *J. Infect. Dis.* **1988,** *158*, 23–28. DOI: 10.1093/infdis/158.1.23

[43] Fleischhacker, M.; Radecke, C.; Schultz, B.; Ruhnke, M. Paradoxical growth effects of the echinocandins caspofungin and micafungin, but not of anidulafungin, on clinical isolates of *Candida albicans* and *C. dubliniensis*. *Eur. J. Clin. Microbiol. Infect. Dis.* **2008**, *27*, 127–131. DOI: 10.1007/s10096-007-0411-4

[44] Ko, K.-C.; Tai, P.C.; Derby, C.D. Escapin, a bactericidal agent in ink secretion of the sea hare *Aplysia californica*, acts through irreversible DNA condensation in *E. coli*. *Antimicrob. Agents Chemother.* **2012,** *56*, 1725–1734. DOI: 10.1128/AAC.05874-11

[45] Costerton, J.W.; Stewart, P.S.; Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science* **1999**, *284*, 318–1322. DOI: 10.1126/science.284.5418.1318

[46] Houry, A.; Gohar, M.; Deschamps, J.; Tischenko, E.; Aymerich, S.; Gruss, A, Briandet R. Bacterial swimmers that infiltrate and take over the biofilm matrix. *Proc. Natl. Acad. Sci. U.S.A.* **2012,** *109*, 13088–13093. DOI:10.1073/pnas.1200791109

[47] Flemming, H.C.; Wingender, J. The biofilm matrix. *Nat. Rev. Microbiol.* **2010**, *8*, 623–633. DOI: 10.1038/nrmicro2415

[48] Donlan, R.M. Biofilms on central venous catheters: is eradication possible? *Curr. Top. Microbiol. Immunol.* **2008,** *322*, 133–161. PMID: 18453275

[49] Hatt, J.K.; Rather; P.N. Role of bacterial biofilms in urinary tract infections. *Curr. Top. Microbiol. Immunol.* **2008,** *322*, 163–192. PMID: 18453276

[50] Simões, M.; Simões, L.C.; Vieira, M.J. A review of current and emergent biofilm control strategies. *LWT–Food Sci. Technol.* **2010,** *43*, 573–583. DOI: 10.1016/j.lwt.2009.12.008

[51] Santiago, A.J.; Ahmed, M.N.A.; Wang, S.-L.; Damera, K.; Wang, B.; Tai, P.C.; Gilbert, E.S.; Derby, C.D. Inhibition and dispersal of *Pseudomonas aeruginosa* biofilms by combination treatment of escapin intermediate products and hydrogen peroxide. *Antimicrob. Agents Chemother.* **2016,** *60*, 5554–5562. DOI:10.1128/AAC.02984-15

[52] Ibáñez de Aldecoa, A.L.; Zafra, O.; González-Pastor, J.E. Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. *Front. Microbiol.* **2017**, *8*, 1390. DOI: 10.3389/fmicb.2017.01390

[53] Díiaz De Rienzo, M.A.; Stevenson, P.S.; Marchant, R.; Banat, I.M. *Pseudomonas aeruginosa* biofilm disruption using microbial surfactants. *J. Appl. Microbiol.* **2016**, *120*, 868–876. DOI: 10.1111/jam.13049

[54] e Silva, S.S.; Carvalho, J.W.P.; Aires, C.P.; Nitschke, M. Disruption of *Staphylococcus aureus* biofilms using rhamnolipid biosurfactants. *J. Dairy Sci.* **2017**, *100*, 7864–7873. DOI: http://dx.doi.org/10.3168/jds.2017-13012

[55] Flemming, H.–C. EPS – then and now. *Microorganisms* **2016**, *4*, 41. DOI: 10.3390/microorganisms4040041

[56] van der Waal, S.V.; van der Sluis, L.W. Potential of calcium to scaffold an endodontic biofilm, thus protecting the micro-organisms from disinfection. *Med. Hypotheses* **2012,** *79*, 1–4. DOI: 10.1016/j.mehy.2012.03.012

[57] Barraud, N.; Kelso, M.J.; Rice, S.A.; Kjelleberg, S. Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases. *Curr. Pharm. Design* **2015**, *21*, 31–42. DOI : 10.2174/1381612820666140905112822

[58] Barraud, N.; Schleheck, D.; Klebensberger, J.; Webb, J.S.; Hassett, D.J.; Rice, S.A.; Kjelleberg,S. Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J. Bacteriol.* **2009**, *191*, 7333–7342. DOI: 10.1128/JB.00975-09

[59] Kim, S.K.; Lee, J.H. Biofilm dispersion in *Pseudomonas aeruginosa. J. Microbiol.* **2016**, *54*, 71–85. DOI: 10.1007/s12275-016-5528-7

[60] Li, K.; Yang, G.; Debru, A.B., Li, P.; Zong, L; Li, P.; Xu, T.; Wu, W.; Jin, S. Bao, Q. *SuhB* regulates the motile-sessile switch in *Pseudomonas aeruginosa* through the Gac/Rsm pathway and c-di-GMP signaling. *Front. Microbiol.* **2017**, *8*, 1045. DOI 10.3389/fmicb.2017.01045

[61] Vale, P.F.; McNally, L.; Doeschl-Wilson, A.; King, K.C.; Popat, R., Domingo-Sananes, M.R.; Allen, J.E.; Soares, M.P.; Kümmerli, R. Beyond killing: can we find new ways to manage infection? *Evol. Med. Public Health* **2016**, 2016, 148–157. DOI: 10.1093/emph/eow012

[62] Koo, H.; Allan, R.N.; Howlin, R.P.; Stoodley, P.; Hall-Stoodle, L. Targeting microbial biofilms: current and prospective therapeutic strategies. *Nature Rev. Microbio.* **2017**, *15*, 740–755. DOI: 10.1038/nrmicro.2017.99