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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*

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Abstract: Escapin is an L-amino acid oxidase in the ink of a marine snail, the sea hare *Aplysia californica*, which oxidizes L-lysine (**1**) to produce a mixture of chemicals which is antipredatory and antimicrobial. The goal of our study was to determine the identity and relative abundance of the constituents of this mixture, using molecules generated enzymatically with escapin and also using products of organic syntheses. We examined this mixture under the natural range of pH values for ink—from ≈5 at full strength to ≈8 when fully diluted in sea water. The enzymatic reaction likely forms an equilibrium mixture containing the linear form α-keto-ε-aminocaproic acid (**2**), the cyclic imine Δ^1-piperidine-2-carboxylic acid (**3**), the cyclic enamine Δ^2-piperidine-2-carboxylic acid (**4**), possibly the linear enol 6-amino-2-hydroxy-hex-2-enoic acid (**7**), the α-dihydroxy acid 6-amino-2,2-dihydroxy-hexanoic acid (**8**), and the cyclic aminol 2-hydroxy-piperidine-2-carboxylic acid (**9**). Using NMR and mass spectroscopy, we show that **3** is the major component of this enzymatic product at any pH, but at more basic conditions, the equilibrium shifts to produce relatively more **4**, and at acidic conditions, the equilibrium shifts to produce relatively more **2**, **7**, and/or **9**. Studies of escapin's enzyme kinetics demonstrate that because of the high concentrations of escapin and L-lysine in the ink secretion, millimolar concentrations of **3**, H_2O_2, and ammonia are produced, and also lower concentrations of **2**, **4**, **7**, and **9** as a result. We also show that reactions of this mixture with H_2O_2 produce δ-aminovaleric acid (**5**) and δ-valerolactam (**6**), with **6** being the dominant component under the naturally acidic conditions of ink. Thus, the product of escapin's action on L-lysine contains an equilibrium mixture that is more complex than previously known for any L-amino acid oxidase.

Keywords: amino acids · enols · imino compounds · oxidation

Introduction

L-Amino acid oxidases (LAAOs; E.C. 1.4.3.2) are enzymes that oxidatively deaminate L-amino acids.[1] LAAOs differ in their substrate specificities. LAAOs can function as toxins in defensive or offensive systems. Examples include the venomous enzymes of snakes in which LAAOs function in prey capture and in defense from predators,[2] secreted enzymes for allelopathy between bacterial species,[3–5] enzymatic chemical defenses in marine algae against parasites[6] and in mucus of fish against microbes,[7,8] and enzymatic antimicrobial and antipredator defenses in secretions of land and marine snails.[9–13] One type of marine snail—the sea hare—has FAD-containing LAAOs in its ink.[11,12] "Escapin" is the LAAO in the ink of the sea hare *Aplysia californica*.[10] When attacked by predators, *A. californica* co-releases escapin in ink and escapin's substrate L-lysine in opaline. This mixture contains escapin's products and acts as a chemical

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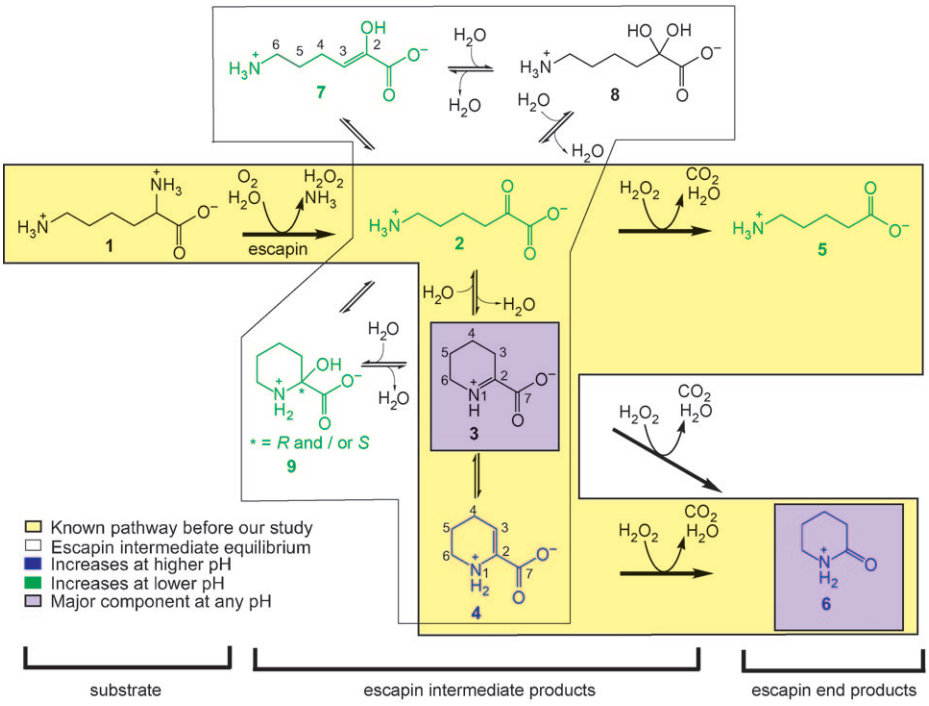
defense against predators such as California spiny lobsters and wrasses.[13,14] The preferred substrates of escapin in vitro are L-lysine and L-arginine, similar to that of LAAOs in many other species of sea hares.[10,12,15] But since L-lysine is ≈300 times more concentrated than L-arginine in *A. californica*'s opaline (≈145 vs ≈0.5 mM), L-lysine is escapin's primary substrate in situ.[16] Sea hares also have LAAOs in their egg masses, which may protect the eggs from microbes.[10–12]

A goal of our work is to identify bioactive compounds produced by the escapin pathway, and by extension, those of other LAAOs. Previous work on LAAOs suggested and in some cases demonstrated the identity of molecules in this pathway.[17–26] This scheme is summarized in the portion of Scheme 1 highlighted in yellow. This shows that LAAOs oxidize L-lysine (**1**) to form α-keto-ε-aminocaproic acid (**2**), and in the process produce H_2O_2 and NH_3. Acid **2** can cyclize to yield an equilibrium mixture also containing the imine, Δ^1-piperidine-2-carboxylic acid (**3**), and the enamine, Δ^2-piperidine-2-carboxylic acid (**4**). H_2O_2 may react with **2** to form δ-aminovaleric acid (**5**) and with **3** or **4** to form δ-valerolactam (**6**). However, the ratio of **5** and **6** is highly variable, possibly due to different conditions of the LAAO reactions. Our preliminary work on escapin suggested that the chemistry of this LAAO, and thus perhaps other LAAOs, may be more complicated than suggested by published studies and as depicted in Scheme 1, and that the identity of the molecular species in the equilibrium mixture and which dominate under natural conditions for these enzymes are unknown. One factor that may influence the reactions and equilibrium in the escapin/L-lysine pathway in vivo is pH, since the pH of sea hare ink is ≈5 when secreted and ≈8 when diluted in sea water,[27] and since pH is known to affect some reactions in the general LAAO pathway in vitro.[24,28] This issue of identifying molecular species in an equilibrium mixture, quantifying their relative abundances, and determining their bioactivity has analogs in other fields, such as in food sciences, where sugars exist as an equilibrium mixture and identifying the molecular species responsible for sweetness is important in product development.[29]

The specific aims of this study were threefold. First, we wanted to identify all of escapin's enzymatic reaction products with L-lysine, which we term "escapin intermediate product", as well as the subsequent non-enzymatic reaction products resulting from interactions among these enzymatic products, which we call "escapin end product". Second, we wanted to identify their relative abundance under the natural pH conditions of ink. Third, we wanted to evaluate escapin's enzymatic activity at different pH values to quantify its production toward predicting the concentrations of the products of LAAO activity under natural conditions.

Results and Discussion

Identification of the dominant molecular species in escapin's intermediate products at neutral pH: Escapin intermediate product of lysine (EIP) is an equilibrium mixture; its major component was identified as **3** by NMR and mass spectroscopy. EIP was prepared by incubating escapin, L-lysine·HCl, and catalase (to prevent the reaction of H_2O_2 with EIP). The presence of anion peaks at *m/z* 126 and 144 in ESI-MS experiments using neutral carrier solvents indicated that EIP contains at least two major species in the EIP equilibrium mixture (Scheme 2b). Molecular formulae for molecules corresponding to these ion peaks were determined by HR-ESI-TOF-MS as $C_6H_9NO_2$ (*m/z* 126.0555 $[M-H]^-$, Δ+0.5 mmu; *m/z* 128.07165 $[M+H]^+$, Δ+0.4 mmu) and $C_6H_{11}NO_3$ (*m/z* 144.0660 $[M-H]^-$, Δ−0.1 mmu). On the



Scheme 1. Summary of the compounds of the escapin/L-lysine pathway in the ink and opaline secretion of sea hares. The portion of the pathway highlighted in yellow summarizes the state of knowledge of the generic L-amino acid oxidase/L-lysine pathway from the literature before our study. L-lysine (**1**), α-keto-ε-aminocaproic acid (**2**), Δ^1-piperidine-2-carboxylic acid (**3**), Δ^2-piperidine-2-carboxylic acid (**4**), δ-aminovaleric acid (**5**), δ-valerolactam (**6**), 6-amino-2-hydroxy-hex-2-enoic acid (**7**), 6-amino-2,2-dihydroxy-hexanoic acid (**8**), and 2-hydroxy-piperidine-2-carboxylic acid (**9**).

other hand, ^{1}H NMR spectra of this mixture reveal that one of the equilibrium mixture's molecular species dominates this aqueous solution (Scheme 2b, second spectrum). Since NMR spectroscopy observes molecules in aqueous conditions, it gives realistic ratios of the molecular species. In D_2O, the intensity of the mixture's CH_2 signal at 2.98 ppm became smaller and totally disappeared after 24 h, decoupling the CH_2 signals at 1.88 and 3.76 ppm, indicating that the CH_2 protons at 2.98 ppm are exchangeable with deuterium in D_2O (Scheme 2b, bottom spectrum). This indicates the existence of equilibrium between **3** and **4**, **2** and **7**. To avoid this exchange and to stabilize this compound, further NMR experiments were performed in 90% H_2O/10% D_2O under neutral pH conditions. An absence of olefinic protons in ^{1}H NMR spectra (Scheme 2b, second spectrum) indicated that this major compound in EIP is not **4** or the linear enol, 6-amino-2-hydroxy-hex-2-enoic acid (**7**) (Scheme 2c). 2D NMR experiments established the connectivity of all proton and carbon atoms in this compound. The C-H connectivity was established by HMQC experiments to be the following: CH_2-3 (δ_H=2.98 ppm; δ_C=29.4 ppm), CH_2-4 (δ_H=1.88 ppm; δ_C=19.0 ppm), CH_2-5 (δ_H=1.91 ppm; δ_C=21.5 ppm), CH_2-6 (δ_H=3.76 ppm; δ_C=47.5 ppm) (Table 1). The ring system was established starting from C3 on the basis of COSY correlation from H3 to H4, H4 to H5, and H5 to H6. Long-range coupling between H3 and H6 ($J_{3,6}$=2.7 Hz) is consistent with a structure that has an imine group in the six-membered ring structure **3**. This spin system is extended on the basis of HMBC correlations from H3 and H6 to imine carbon C2 (δ_C=184.5 ppm) and carboxylic carbon C7 (δ_C=164.9 ppm) (Table 1), thus establishing that **3** is the major component in EIP. Assignment of the imine carbon was confirmed based on a down field shift of the imine carbon with protonation on the imine nitrogen atom (174 ppm at pH 10 to 184 ppm at pH 7 and pH 1)[30,31] (Table 1). A small pH effect on the carboxyl carbon might occur because of the formation of an intramolecular salt bridge to inhibit protonation on the carboxyl group under acidic conditions.

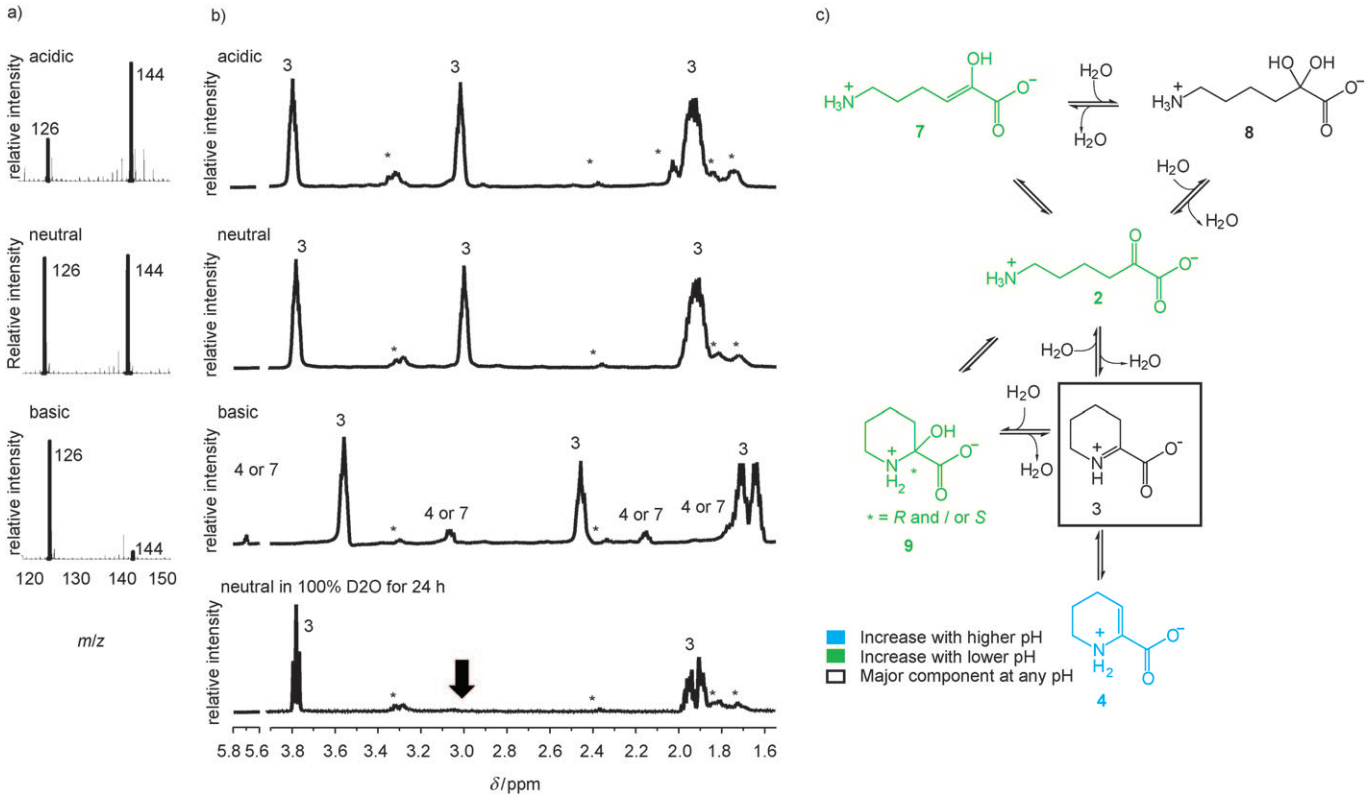
Effect of pH on the equilibrium of escapin's intermediate products: The effect of pH on the equilibrium mixture of EIP is summarized in Scheme 2c. Compound **3** is the dominant species in the equilibrium at any pH. Basic pH shifts the equilibrium towards **4**. Acidic pH shifts the equilibrium towards molecular species with an anion peak at *m*/*z* 144, which include **2**, **7**, and **9**. These results were obtained by NMR and mass spectroscopy at different pH values. 2D-NMR analysis showed that under all pH conditions, **3** was the major component, although the chemical shifts of the dominant proton signals in EIP at pH 9 were different from those at pH 1 and 7 (Scheme 2b). An olefinic proton at δ 5.70 ppm was observed as minor components at pH 9 (Scheme 2b, third spectrum), indicating that **4** or **7** was produced in greater relative amounts under higher pH conditions. HMQC and COSY experiments assigned partial structure for **4** or **7** (Table 2). However, the likelihood for this being **7** is low because the cyclic forms dominate in this mixture based on mass spectral data (Scheme 2a, c). An α-keto carbon at 190–204 ppm,[32] an expected key signal for **2**, the direct product of escapin, was not observed in ^{13}C and HMBC experiments at any pH. Small proton signals from 1.7–1.9 ppm at pH 1 and pH 7, 2.0–2.1 ppm at pH 9, 3.2–3.4 ppm at pH 1, 7, and 9 were observed (Scheme 2b, Table 3). However, these protons could not be assigned because their signals were low intensity and overlapping. MS analysis was performed using three different carrier solvents at different pH values. As pH decreased, the intensity of the ion peak at 144, corresponding to **2**, **7**, or the cyclic aminol, 2-hydroxy-piperidine-2-carboxylic acid (**9**), increased relative to the intensity of the peak at 126, corresponding to **3** or **4** (Scheme 2a, c). Signals ≈3.5 ppm might be methylene protons next to nitrogen atoms of **2**, **7**, **9**, and/or the α-dihydroxy acid, 6-amino-2,2-dihydroxy-hexanoic acid (**8**). Signals at 2.3 ppm and 1.65–1.85 ppm might be the other methylene protons of **2**, **7**, **8**, and/or **9**. However, full assignment was not possible because of overlapping signals and lack of sufficient HMBC cross peaks due to low concentrations of these molecular species. Although it is not possible to give clear assignment for these molecular species, **7** might not be present under the acidic and neutral conditions because the olefinic proton was not observed in ^{1}H NMR spectra under acidic pH conditions when mass spectra show ions corresponding to **7**. Ion signals corresponding to **8** were not observed at any pH in mass spectra, indicating that **8** might not readily ionize under our mass spectrometric conditions; however, it might exist in aqueous solutions.

Synthesis of putative escapin intermediate products: We synthesized the putative compounds in EIP to verify our

Table 1. NMR data of imine (**3**) at three different pH conditions in 90% H_2O/10% D_2O.[a]

Position	pH 1				pH 7				pH 10			
	δ_H	δ_C	HMBC (H to C)	COSY	δ_H (J in Hz)	δ_C	HMBC (H to C)	COSY	δ_H	δ_C	HMBC (H to C)	COSY
2		184.4				184.5				174.0		
3	2.96	29.5	2,4,5	4,6	2.98	29.4	2,4,5,7	4,6	2.47	29.4	2,4,5	4,6
4	1.87	19.1	2,3,5,6	3,5	1.88 (2.7)	19.0	2,3,5,6	3,5	1.71	20.8	3,5	3,5
5	1.89	21.6	3,4,6	4,6	1.91	21.5	3,4,6	4,6	1.66	23.4	4	4,6
6	3.75	47.6	2,4,5	3,5	3.76 (2.7, 5.8)	47.5	2,3,4,5,7	3,5	3.56	50.3	2, 4	3,5
7		164.9				164.9				163.7		

[a] All proton signals are multiplets. J value in pH 7 was analyzed by decoupling caused by deuterium exchange (Scheme 2b, bottom spectrum).



Scheme 2. Identification of the molecular species in the equilibrium mixture of escapin intermediate products, and the effect of pH on the equilibrium mixture. a) Changes in ESI-TOF mass spectrum in negative mode for escapin intermediate products. Aqueous carrier solvents with different pH values were used. Top: acidic conditions, using 50% MeOH+0.1% HCOOH. Middle: neutral conditions, using 50% MeOH. Bottom: Basic conditions, using 50% MeOH+0.5% NH_3. This shows that basic conditions favor the 126-Dalton form and acidic conditions favor the 144-Dalton form. b) Effect of pH and D_2O on ^1H NMR spectrum of escapin intermediate products generated by dissolving 5 mg of freeze-dried EIP in 0.5 mL of a 90% H_2O/10% D_2O mixture (except bottom spectrum). Top: acidic conditions with 0.4% TFA (pH 1); second: neutral conditions with double distilled H_2O (pH 7); third: basic conditions with 100 mM Na_2CO_3 (pH 9–10), **3** is the major molecular species at any pH, and **4** (or **7**) is observed only at pH 9. * denotes signals that could not be assigned; these might be signals for **2** or **8**–**9**. We have no experimental evidence for the existence of **8**, but it is theoretically possible. Bottom: neutral condition with 100% D_2O. After this mixture was held for 24 h in D_2O at room temperature, the CH_2 signal at 2.98 ppm indicated by solid arrow disappeared, causing decoupling of the CH_2 signals at 1.88 and 3.76 ppm and thus indicating that the CH_2 protons at 2.98 ppm are exchangeable with deuterium in D_2O. Imine–enamine conversion between **3** and **4** and/or keto–enol conversion between **2** and **7** makes this proton of **3** exchangeable with deuterium in the NMR solvent. c) Summary of the effects of pH on the equilibrium of EIP. **2**, **7**, and **9** correspond to 144 (*m*/*z*) and **3** and **4** correspond to 126 (*m*/*z*) in mass spectra (see Scheme 2a). **8** corresponds to 162 (*m*/*z*), but it was not observed in mass spectra under these experimental conditions.

Table 2. NMR data of a spin system reasonable for enamine (**4**) or enol (**7**) in 90% H_2O/10% D_2O at pH 10.[a]

Position	δ_H mult (*J* in Hz)	δ_C	COSY
2		N/A	
3	5.70 t (4.1)	112.7	4
4	2.16 td (4.1, 6.5)	24.8	3,5
5	1.75 m (N/A)	24.0	4,6
6	3.06 t (5.55)	44.5	5
7		N/A	

[a] N/A: Cross peaks were not observed because of low sensitivity or overlap of signals in HMQC experiment. HMBC data were not available because of low sensitivity and signal overlap as well.

identifications of their presence in the escapin pathway. We used a non-enzymatic synthetic pathway starting with pipecolinic acid ethyl ester, as described by Lu and Lewin.[24] ^1H NMR spectra and mass spectra show that **3** is the major product of this synthetic EIP and **4** is the minor one, as expected. Comparison of ^1H NMR spectra of natural EIP and synthetic EIP revealed identical signals corresponding to **3** (Figure S1, Supporting Information).

Determination of escapin end products: To determine the molecules in the escapin end products for L-lysine (EEP), a solution of EEP prepared at pH 7 was compared with candidate compounds using ^1H NMR spectroscopy.[18,21] ^1H NMR spectra of EEP (Figure 1a, top) showed two sets of spin systems, corresponding to **5** and **6** (Figure 1). These two compounds were purified by HPLC from EEP mixture and molecular formulae were confirmed by HR-ESI-TOF-MS as $C_5H_{11}NO_2$ (*m*/*z* 116.0710 $[M-H]^-$, $\Delta-0.2$ mmu) for **5** and C_5H_9NO (*m*/*z* 100.0767 $[M+H]^+$, $\Delta+0.5$ mmu) for **6**. Experiments in which EEP was spiked with standards confirmed the identity of these compounds (Figure 1a, second

Table 3. List of candidate NMR signals for **2**, **8**, and **9** at three different pH conditions in 90% H_2O/10% D_2O.[a]

Serial number of the signals	pH 1 δ_H	pH 1 δ_C	pH 1 COSY	pH 7 δ_H	pH 7 δ_C	pH 7 COSY	pH 10 δ_H	pH 10 δ_C	pH 10 COSY
1	1.7 m	N/A	2, 3, 5	1.69 m	N/A	3	1.76 m	28.50 or 24.13	3
2	1.8 m	19.0	1, 3, 5	1.78 m	21.1	3	1.86 m	24.0	4
3	2 m	34.5	1, 2	2.32 t	N/A	2	2.32 t	21.8	1
4	2.44 t	N/A	3	3.26 m	40.7	1,2	3.29 t	44.4	2
5	3.27 m	43.5	1, 2						

[a] These spin systems are candidates for structures **2**, **8**, and **9** but could not be assigned in this equilibrium mixture. N/A: Cross peaks were not observed because of low sensitivity or overlap of signals in HMQC experiment. HMBC data were not available because of low sensitivity and signal overlap as well.

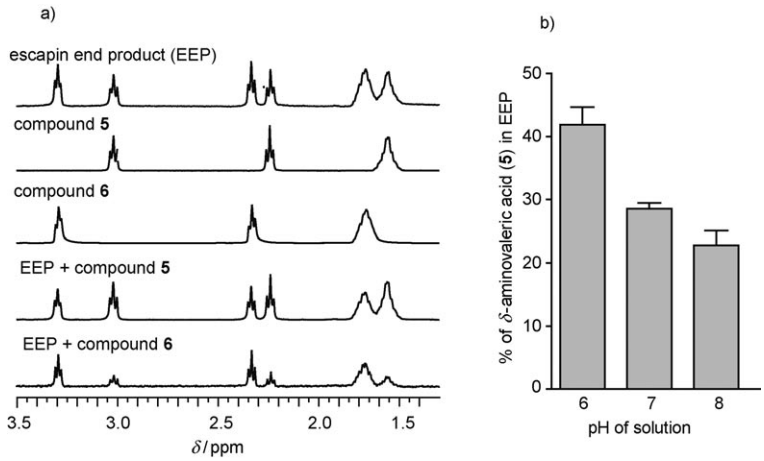


Figure 1. Identification of escapin end products as δ-aminovaleric acid (**5**) and δ-valerolactam (**6**), and the effect of pH on the ratio of two forms. EEP was generated by adding H_2O_2 (50 μmol) to EIP generated from lysine·HCl (13.7 μmol). D_2O was added to make a final concentration of 10%. a) ^{1}H NMR spectra. Top spectrum: Escapin end product (EEP); Second spectrum: **5**; Third spectrum: **6**; Fourth spectrum: mixture of EEP+**5**; Bottom spectrum: mixture of EEP+. b) Change in ratio of integral value of ^{1}HNMR signal of **5** to (**5**+**6**) in EEP as pH changes from 6 to 8. Values are means ±S.E.M., $N=5$. **5** and **6** were purchased from Sigma-Aldrich.

to fifth). Thus, the conversion of the components of EIP to their respective components of EEP involves a reaction of **2** with H_2O_2 to form **5** and a reaction of **4** with H_2O_2 to form **6**, respectively. Additionally, we believe that **3** can be converted directly to **6** by a mechanism similar to the conversion of **2** to **5**.

An equilibrium between **5** and **6** is theoretically possible. We tested this by incubating of either **5** (1.5 mg) or **6** (1.5 mg) in H_2O (50 μL) at pH 2, 7, 9, or 11, for 4 h at room temperature. We did not find any conversion between the two forms in ^{1}H NMR spectra. We conclude that there is no conversion between **5** and **6** within a biological context.

In previous work on LAAOs, **5** is described as the only or major product in some reactions and **6** the only or major product in others.[18–21] Which product occurs or dominates may depend on experimental conditions, such as purity of the enzymes, pH, concentrations of enzyme and substrate, temperature, and other factors that may influence the equilibrium. In the next sections, we explore some of these factors, such as pH and chemical background, for escapin.

Effect of pH on conversion of escapin intermediates to escapin end products: A pH-dependent change in the position of the equilibrium in the EIP mixture was observed using ESI-MS and NMR (Scheme 2). Since the 145-Dalton component increased at acidic condition, **2** and/or **9** were expected to have stronger signals in the NMR spectra under acidic conditions. However, we could not identify the molecular species as **2** or **9** in the NMR spectra of EIP because of overlapping of signals. So, we employed an indirect method—comparing the ratio of EEP at different pH values. This solution was prepared by mixing EIP and H_2O_2 in phosphate buffered solutions of pH 6, 7, or 8. The relative amount of **5** in this mixture increased at lower pH values and decreased at higher pH values (Figure 1). These changes may reflect an increased relative amount of **2** at lower pH. Scheme 1 provides a final summary of escapin's reaction with L-lysine.

Effect of pH on escapin's kinetics: We examined the effect of pH on the kinetics of the reaction of escapin with L-lysine as the substrate (Table 1). Initial rates of reaction were determined in air-saturated buffer by monitoring the rate of oxygen consumption in the pH range from 4.5 to 8.5. The values of $^{app}V_{max}$ and $^{app}(V_{max}/K_m)$ increased with increasing pH and reached limiting values above pH 8, indicating that an unprotonated group in the active site of the enzyme is required for the reaction with L-lysine as substrate catalyzed by escapin. The pK_a value in the $^{app}V_{max}$ pH-profile was lower than the corresponding pK_a value in the $^{app}(V_{max}/K_m)$ pH-profile, consistent with decreases of the $^{app}K_m$ values to limiting values of 0.03 to 0.05 mM with increasing pH (Table 1). Thus, while the $^{app}V_{max}$ values increased with increasing pH, the $^{app}K_m$ values decreased.

With L-lysine as substrate, the $^{app}K_m$ value at pH 5 is ≈0.8 mM. Assuming that 1 mg of escapin that is released in ≈1 mL (i.e., the amount released during a bout of inking) is combined with 145 mM L-lysine (i.e., its concentration in opaline), then escapin is more than 99% saturated with the substrate. Under these conditions, enzymatic turnover of escapin with L-lysine would take as little as ≈5 s to produce 1 mM H_2O_2 and ≈30 s to produce ≈5 mM H_2O_2. At higher pH values, the reactions are even faster. For example, at pH 8, it would take less than 1 s to produce 1 mM H_2O_2 and ≈1 min to produce ≈80 mM H_2O_2. Thus, escapin remains active at the naturally low pH of ink, and owing to the high concentrations of escapin and lysine in the defensive secretion, prodigious amounts of its reaction products are produced.

Conclusions

The mixture resulting from the escapin/lysine reaction in the ink secretion of sea hares is complex. It is generated by an initial enzymatic step that generates an equilibrium mixture of components, and followed by non-enzymatic reactions between these components and H_2O_2. The balance within the equilibrium mixture is dependent on the mixture's pH, which ranges from ≈5 in full strength ink to ~8 when diluted in sea water. The enzymatic step of the escapin pathway generates Δ^1-piperidine-2-carboxylic acid (**3**) and H_2O_2 as the major components, regardless of the pH value. Minor forms include α-keto-ε-aminocaproic acid (**2**), 6-amino-2-hydroxy-hex-2-enoic acid (**7**), and aminol 2-hydroxy-piperidine-2-carboxylic acid (**9**), which increase in abundance at lower pH, and Δ^2-piperidine-2-carboxylic acid (**4**), which increases in abundance at higher pH. The non-enzymatic reaction of H_2O_2 with these intermediate forms results in the end products δ-aminovaleric acid (**5**) and δ-valerolactam (**6**), with **6** dominating under all pH conditions but the relative abundance of the latter increasing under acidic conditions. The complexity of the mixture generated by escapin in aqueous solution is greater than previously realized for any L-amino acid oxidase. The pH effect on the equilibrium in EIP and on the production of end products shows that pH affects the activity of EIP and EEP. Our results with escapin may indicate equally complex mixtures are generated by other L-lysine oxidases. The products of the escapin pathway can now be tested in biological assays to determine which are antipredatory chemical defenses or antimicrobial agents.

Experimental Section

Animals: Sea hares *Aplysia californica* Cooper 1863 were collected in California by Marinus Scientific (Garden Grove, CA, USA). They were dissected on the day of arrival in our laboratory. Experiments were performed within the university regulations and national guidelines.

Collection of ink and isolation of escapin: The ink glands were dissected from anesthetized animals and frozen at −80°C until used. Purple ink was collected by gently squeezing dissected ink glands in a Petri dish with the blunt end of a scalpel handle. Escapin was isolated and purified using an ÄKTA 100 Automated FPLC. A two-step purification process involving gel filtration followed by purification using cation exchange was performed according to Yang et al.[10]

Preparation of the intermediate products of escapin's oxidation of L-lysine: The method of Meister (1952) was modified to make "escapin intermediate product for L-lysine" (EIP). L-Lysine monohydrochloride (10 mg) was incubated with escapin (3 μg) and catalase (130 μg) in double distilled H_2O (1 mL) at 30°C on a shaker for up to 20–24 h until L-lysine was completely consumed, as determined by thin layer chromatography. This solution was then filtered using an Amicon Ultra-4 Centrifugal Filter Device (Millipore Corp., Billerica, MA, USA) to remove escapin and catalase, and then stored at −80°C until used later. To make "escapin end product for L-lysine" (EEP), the same procedure was performed except that catalase was not included in the incubation and concentration of escapin (0.6 μg) in H_2O mL. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Spectroscopy: NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer using conventional pulse sequence, and FID data were processed using MestRe Nova software (Mestrelab Research) on Windows XP. 10% D_2O/90% H_2O was used as solvent for all experiments except that in Scheme 2b, bottom spectrum. Chemical shift was referenced for internal TSP. Mass spectra (ESI) were obtained using a Waters Q-TOF micro mass spectrometer and Applied Biosystems QSTAR XL.

Enzyme kinetics and pH profiles: Enzyme activity was measured in air-saturated 50 mM potassium phosphate, 150 mM NaCl, in the pH range from 4.5 to 8.5, at 25°C, by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen monitoring system (Hansatech Instrument, Ltd.). The reactions were started by the addition of escapin to a 1 mL reaction mixture, with the final concentration of enzyme in the 5 to 20 μg mL^{-1}. At any given pH, the final concentrations of L-lysine spanned from 0.2- to 5-times the $^{app}K_m$ values, which corresponded to 0.01 to 7 mM depending upon the pH. Data were fit with KaleidaGraph software (Synergy Software, Reading, PA). The apparent kinetic parameters $^{app}V_{max}$, $^{app}(V_{max}/K_m)$, and $^{app}K_m$ for L-lysine as substrate for escapin in air-saturated buffers were determined by fitting initial rates of reaction at different concentrations of substrate to the Michaelis–Menten equation for one substrate. The pH dependence of the $^{app}V_{max}$ and $^{app}(V_{max}/K_m)$ values were determined by fitting initial rate data to equation 1, which describes a curve with a slope of +1 and a plateau region at high pH. Y is the pH-independent value of the kinetic parameter of interest, and K_a is the dissociation constant for the ionization of groups which are relevant for catalysis.

$$\log Y = \log\left(\frac{Y}{1+\left(\frac{10^{-pH}}{10^{-pKa}}\right)}\right) \quad (1)$$

Conversion of EIP to EEP at different pH values: Potassium phosphate buffer was used as the incubation solution for the conversion of EIP to EEP, since the type of buffer affected the results. Freeze-dried EIP (2.5 mg, 13.7 μmol lysine equivalent) and H_2O_2 (50 μmol in 5.6 μL of 30% solution) were mixed with 100 mM potassium phosphate buffer and adjusted pH to 6, 7, and 8 to a final volume of 0.5 mL. Reactions were performed for 1.5 h at 25°C (Thermomixer, Eppendorf), and products were analyzed using NMR. For high resolution mass spectroscopy, compound **5** and **6** were purified by Beckman HPLC System GOLD equipped with Nomura Chemical Develosil RPAQUEOUS column. Water - methanol gradient containing 0.1% trifluoroacetic acid was used for elution of the compounds.

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