RESEARCH ARTICLE

Discovery of New Cytotoxic Aplaminone Derivatives from the Sea Hare *Aplysia kurodai* and Elucidation of Their Accumulation from Local Sea Algae through the Food Chain

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Abstract: The shell-less herbivorous marine mollusk (sea hare) *Aplysia kurodai*, is known to contain a variety of bioactive substances. While these compounds have been thought to originate from sea algae or their associated microbes, most of their origin and acquisition pathways are still unclear. Six new cytotoxic aplaminone derivatives, bromodopamine–terpenoid hybrid molecules, were isolated from A. kurodai. Among them, isoaplaminone had a reverse prenyl group at the C15 aliphatic chain, which is a rare structural feature from the viewpoint of terpenoid biosynthesis. Investigation for chemical components in *A. kurodai* and the sea algae collected at several different locations revealed that two major aplaminones were contained in the Laurencia complex species at specific sites. Our chemical and ecological studies provide new insights into the origin of marine alkaloid toxins and their dynamism through the food chain.

Introduction

Marine invertebrates are known to be a rich source of biologically active substances, and many isolation studies have supported this fact.[1–4] *Aplysia kurodai*, a common Japanese herbivorous mollusk that is also referred to as sea hare, has been investigated since the 1960s in an attempt to discover structurally and biologically interesting compounds.[5,6] In the 1990s, Kigoshi and co-workers isolated highly cytotoxic alkaloids aplaminone (**1**, 1.6×10^{-4}% yield based on wet wt, same as below) and neoaplaminone (**2**, 2.4×10^{-5}%), two bromodopamine analogs with an oxygenated C15 chain (Figure 1).[7,8] Interestingly, the *β*,*γ*-unsaturated ketone in **1** is chemically unstable, and autoxidized to give **2**, which has been reported to be 1.5 million times more cytotoxic than **1**. Furthermore, potent cytotoxic and actin-depolymerizing macrolides aplyronines A–H were also found in *A. kurodai* as minor constituents ($0.020–2.5 \times 10^{-5}$%).[9–11] Continuous chemical and biological studies on aplyronines have revealed that aplyronine A induces a unique protein-protein interaction between two cytoskeletal proteins: actin and tubulin.[12,13] This activity was established for aplyronine C, a minute congener of aplyronine A that lacks microtubule disassembly activity, despite exhibiting the same actin-depolymerizing activity. Therefore, investigation of minor substances, even in the case of well-studied organisms, might lead to the discovery of new compounds with significant biological activities and structural novelty, and to a better understanding of their biosynthetic and metabolic pathways.[14–17]

Over the past few years, an innovative method for comprehensive analysis of non-targeted mass spectrometry data, namely “molecular networking analysis”, has been developed.[18–20] This technique allows us to easily categorize structurally similar compounds as a “molecular family” based on MS/MS spectral similarity and to dereplicate known compounds by a search of the Global Natural Product Social Molecular Networking (GNPS) spectral library. This approach has recently been utilized for the (re)investigation of biologically important compounds such as phorbols,[21,22] depsipeptides,[23,24] and terpenoid indole alkaloids.[25,26]

It has been widely speculated that most marine natural products found in marine invertebrates are not produced by these creatures themselves, but rather by their symbiotic microbes or prey.[6,27–29] Pioneering studies on pederin and its related molecules, cytotoxic polyketides originally isolated from a terrestrial beetle[30] and marine sponges,[31–35] revealed that these polyketides were symbiont-derived toxins produced by uncultured bacteria.[36–39] However, most of the real producers or acquisition pathways of marine natural products are still unknown despite their value from a chemical ecology perspective. Especially, it is challenging to elucidate the origins of natural products derived from carnivorous and herbivorous organisms, since these compounds can sometimes diversify through bioaccumulation and food chains. To overcome this problem and elucidate the dynamism and true origins of such marine natural products, a metabolomic approach focusing on the diet and habitat of marine organisms might be effective.

Herein, we report the MS-based targeted isolation of new aplaminone derivatives from the sea hare *A. kurodai* and their acquisition pathways, origins, and proposed biosynthesis by a comprehensive LC-MS/MS analysis of the sea algae in the habitat of the sea hare.

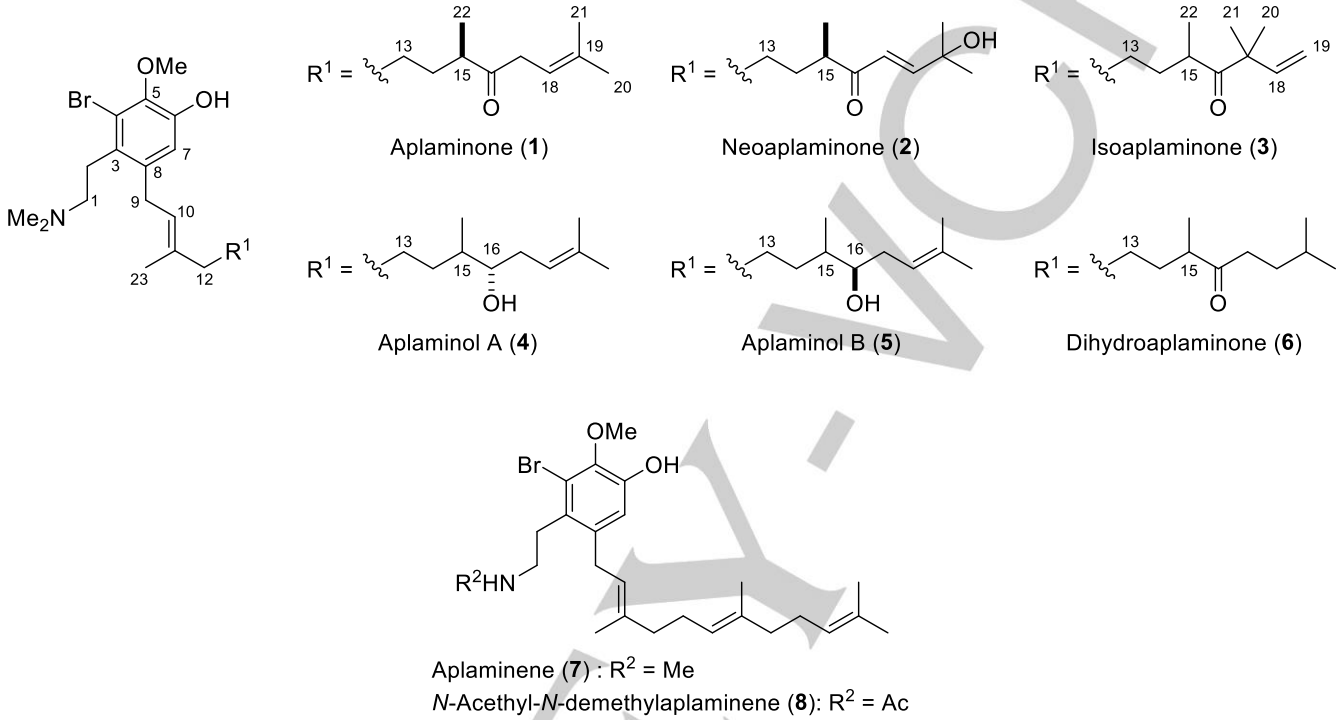


Figure 1. Structures of aplaminone (**1**), neoaplaminone (**2**), and newly isolated derivatives **3**–**8**.

Results and Discussion

Targeted Isolation of New Aplaminone Derivatives 3–8

In preliminary experiments, we found that the aqueous layers of *A. kurodai* showed cytotoxicity against the human colon cancer cell line HCT-116, while most of the cytotoxic compounds were extracted into the organic (EtOAc) layers. To enrich cytotoxic substances and remove residual salts, the aqueous layer (prepared from 21.7 kg of *A. kurodai*, wet wt) was loaded onto a TSK-G3000S polystyrene gel column and eluted with 50 and 75% aqueous EtOH to give cytotoxic fractions (IC_{50} 5.0 and 10 μg/mL, respectively). These two fractions were subjected to LC-MS/MS analysis as well as the feature-based molecular networking analysis (FBMN). Furthermore, compounds **1** and **2** were separated from the EtOAc layer of *A. kurodai* (**1**: 0.5 mg, 5.6×10^{-6}%,[40] **2**: 0.21 mg, 1.9×10^{-6}%) and used for *in-house* annotation. As expected, an aplaminone network containing 15 nodes was generated from the two fractions (Figures 2, S1, and S2, Table S1), along with major networks of phospholipids and fatty acids (Figure S1). Thus, the 50 and 75% aq. EtOH fractions were further chromatographed using a silica gel, Al_2O_3, and HPLC to afford five new minor aplaminone derivatives (Figure 1): isoaplaminone (**3**, 0.14 mg, 1.3×10^{-6}%), aplaminol A (**4**, 0.58 mg, 5.3×10^{-6}%), aplaminol B (**5**, 0.17 mg, 1.6×10^{-6}%), dihydroaplaminone (**6**, 0.044 mg, 4.1×10^{-7}%), and aplaminene (**7**, 0.13 mg, 1.2×10^{-6}%). In addition, during separation of the EtOAc layer, *N*-acetyl-*N*-demethylaplaminene (**8**, 5.9 mg, 5.9×10^{-5}%) was also isolated.

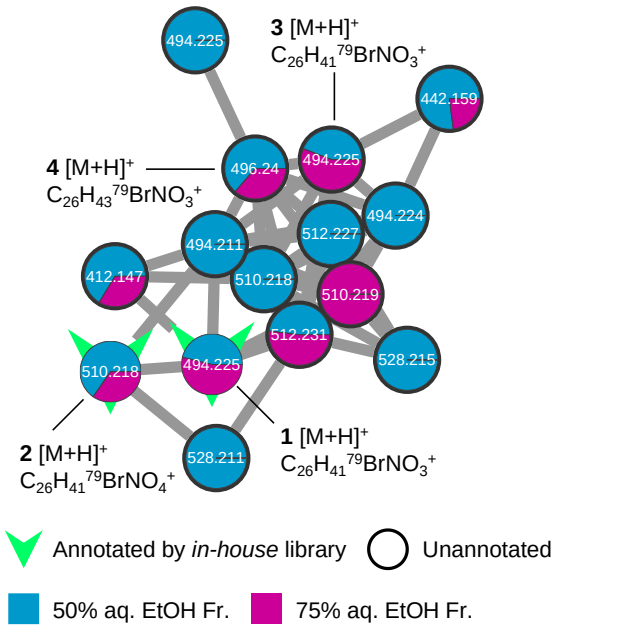


Figure 2. Aplaminone network constructed by the feature-based molecular networking analysis of the 50 and 75% aqueous EtOH fractions of *A. kurodai*.

Structure Elucidation of 3–8

Isoaplaminone (**3**) was obtained as a colorless oil with a chemical formula of $C_{26}H_{40}BrNO_3$, as confirmed by HRESIMS showing a characteristic isotope pattern of a monobrominated compound (*m/z* 494.2266 $[M+H]^+$ calcd for $C_{26}H_{41}{}^{79}BrNO_3{}^+$, 494.2264). The degree of unsaturation was seven, as with aplaminone (**1**).[7] The ^1H NMR spectrum showed an aromatic proton at δ_H 6.76 (1H, s), four olefinic protons at δ_H 5.98 (1H, dd, J = 17.4, 10.7 Hz), 5.21 (1H, m), 5.19 (1H, dd, J = 17.4, 0.9 Hz), and 5.15 (1H, dd, J = 10.7, 0.9 Hz), a methoxy group at δ_H 3.77 (3H, s), two equivalent *N*-methyl groups at δ_H 2.26 (6H, s), and four methyl groups at δ_H 1.70 (3H, br s), 1.20 (3H, s), 1.18 (3H, s), and 0.96 (3H, d, J = 6.7 Hz). The coupling constants and COSY correlations of the three olefinic proton signals at δ_H 5.98, 5.19, and 5.15 suggested the presence of a vinyl group. In the ^{13}C NMR spectrum, a ketone carbonyl signal (δ_C 216.1) and 10 aromatic/olefinic signals were observed. The COSY correlation between the methylene protons at δ_H 2.35 (2H, m) and 2.91 (2H, m), together with the HMBC cross-peak from the *N*-methyl groups at δ_H 2.26 to the methylene carbon at δ_C 59.5 indicated the presence of an *N*,*N*-dimethylethylamino moiety (Figure 3). The HMBC correlations from the vinyl protons at $\delta_{H\text{-}19a}$ 5.19 and $\delta_{H\text{-}19b}$ 5.15, and two methyl groups at $\delta_{H\text{-}20}$ 1.20 and $\delta_{H\text{-}21}$ 1.18 to a quaternary carbon at $\delta_{C\text{-}17}$ 51.8 suggested the presence of a reverse-prenyl group. Additionally, the key 2D NMR correlations of the aliphatic region signals suggested the presence of a C15 aliphatic chain moiety with *E*-geometry at C-10. The positions of the substituent groups on the phenol ring, including bromine, methoxy, and *N*,*N*-dimethylethylamino groups, were also the same as **1** and **2**, which were determined from 2D NMR correlations and comparison of the chemical shifts of ^1H and ^{13}C NMR spectra.[7] Since the major structural difference between **1** and **3** was the terminal prenyl moiety at the C15 chain, **3** was named isoaplaminone.

Aplaminol A (**4**) was obtained as a colorless oil with a molecular formula of $C_{26}H_{42}BrNO_3$, as determined by HRESIMS (*m/z* 496.2421 $[M+H]^+$ calcd for $C_{26}H_{43}{}^{79}BrNO_3{}^+$, 496.2421). The index of hydrogen deficiency was six, which was one less than those of **1** and **3**. The ^1H and ^{13}C NMR spectra were akin to the authentic data of **1**[7] except for the oxymethine signal at δ_H 3.39 (1H, td, J = 8.2, 4.6 Hz), suggesting that **4** has a hydroxy group at C-16 instead of a ketone group. The key 2D NMR correlations around the C-16 hydroxy group including an extended ^1H–^1H spin system from H-15 to H-18 and the HMBC cross-peaks from the methyl protons Me-22 (δ_H 0.90) to the carbons C-14 to C-16 (δ_C 32.2, 39.1, and 76.1) confirmed this structure. Thus, the planar structure of **4** was established as shown in Figure 1. Aplaminol B (**5**) was also obtained as a colorless oil, and the molecular formula $C_{26}H_{42}BrNO_3$ was determined by HRESIMS (*m/z* 496.2403 $[M+H]^+$ calcd for $C_{26}H_{43}{}^{79}BrNO_3{}^+$, 496.2421). The ^1H NMR spectrum was almost identical to that of **4**, except for the chemical shifts and coupling constants around H-16 [δ_H 3.50 (1H, td, J = 6.6, 3.3 Hz)], which suggested that **5** is the C-16 epimer of **4**.

The absolute configurations at C-16 of **4** and **5** were assigned to be *S* and *R*, respectively, by a modified Mosher's method (Figure S3). On the other hand, the relative configurations of **4** and **5** (the *syn*/*anti*-relationship between Me-22 and C-16-OH) were not established due to the mid-range coupling constants of the vicinal protons ($^3J_{15\text{-}16}$ = 4.6 and 6.6 Hz for **4** and **5**, respectively). In addition, comparison of the chemical shifts to those of structurally related compounds[41–44] and chemical shift calculations of *syn*- and *anti*-diastereomers using model compounds were also unsuitable for determining the stereochemistry.

Dihydroaplaminone (**6**) was obtained as a colorless oil with a chemical formula of $C_{26}H_{42}BrNO_3$, as deduced by HRESIMS (*m/z* 496.2407 $[M+H]^+$ calcd for $C_{26}H_{43}{}^{79}BrNO_3{}^+$, 496.2421). The ^1H NMR spectrum and COSY analysis showed two terminal methyl groups at H-20 and 21, which appeared as equivalent doublet signals at δ_H 0.86 (J = 6.6 Hz). This indicated that the planar structure of **4** was an analog of **1** in which the olefin moiety at C-18 was reduced. The 2D NMR correlations including HSQC, HMBC, and NOESY cross-peaks shown in Figure 3 also supported this structure.

Aplaminene (**7**) was obtained as a colorless oil, and the chemical formula was determined to be $C_{25}H_{39}BrNO_2$ by HRESIMS (*m/z* 464.2150 $[M+H]^+$ calcd for $C_{25}H_{40}{}^{79}BrNO_2{}^+$, 464.2159). The ^1H NMR spectrum measured in acetone-d_6 showed that an aromatic proton at δ_H 6.75 (s) and three olefinic protons at δ_H 5.23 (m), 5.14 (m), and 5.09 (m), a methoxy group at δ_H 3.76 (s), an *N*-methyl group at δ_H 2.39 (s), and four deshielded methyl groups at δ_H 1.73 (s), 1.64 (br s), 1.60 (s), and 1.57 (s). The key COSY correlations shown in Figure 3 suggested the presence of a typical C15 farnesyl chain without any oxidation. The configurations of the C15 chain were established to be 10*E*, 14*E*, and 18*E* from the long-range COSY cross-peaks between olefinic signals and allylic methyl signals (H-10/Me-21, H-14/Me-22, and H-18/Me-23). Since the aliphatic methylene signals of the farnesyl chain overlapped the residual solvent signal in acetone-d_6, we also measured the ^1H NMR spectrum in $CDCl_3$ (Table S3). The chemical shifts of the aliphatic signals of **7** in $CDCl_3$ (δ_H 1.96–2.15) were agreed with those of structurally related aromatic compounds with a farnesyl group.[45,46] Thus, **7** was determined to be an aplaminone derivative with a typical farnesyl chain and a monomethylamino group.

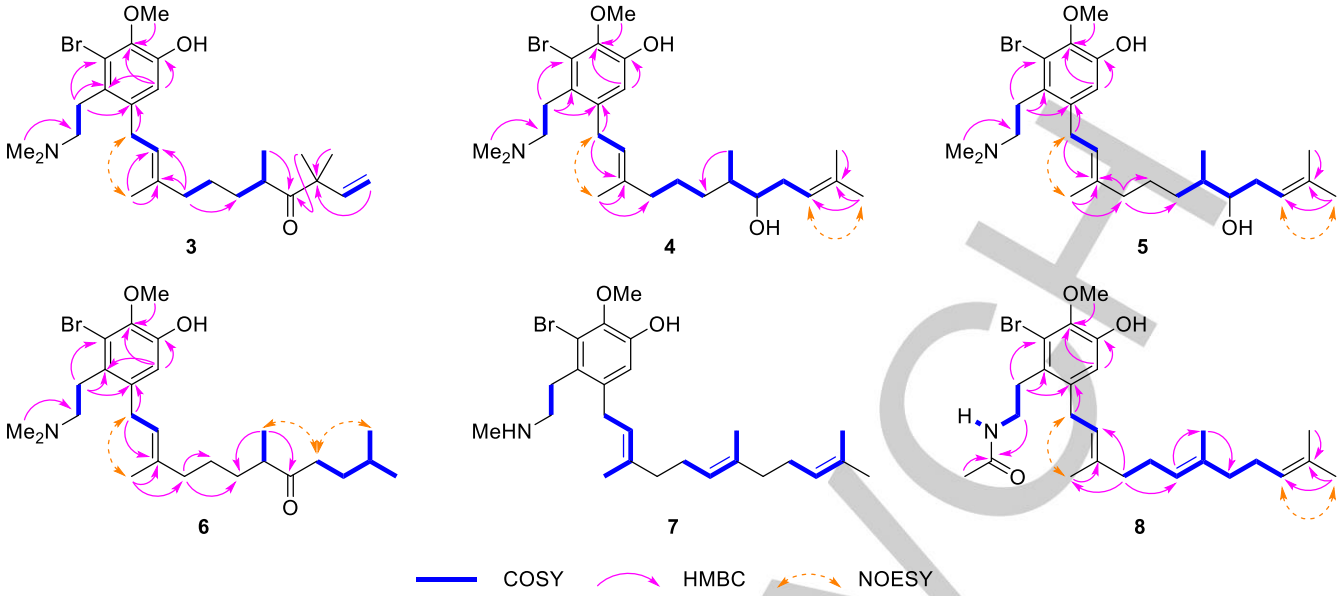


Figure 3. Key 2D NMR correlations of **3**–**8**.

N-Acetyl-*N*-demethylaplaminene (**8**) was obtained as a colorless oil, and the molecular formula $C_{26}H_{38}BrO_3$ was determined by the HRESIMS *m/z* 514.1930 $[M+Na]^+$ (calcd for $C_{26}H_{38}{}^{79}BrNaO_3{}^+$, 514.1927). The ^{1}H NMR spectrum of **8** was quite close to that of **7** except for the presence of the N-H signal at δ_H 5.60 (br s) and the methyl signal at δ_H 1.97. In the ^{13}C spectrum, a typical amide carbonyl signal was observed at δ_C 170.1. These 1D NMR data suggested that **8** is a derivative of **7** in which an *N*-methyl group was replaced by an acetyl group. The key 2D NMR correlations shown in Figure 3 also supported its structure.

Due to the scarcity of isolated compounds, the specific rotations of **3**–**6** were unavailable. Meanwhile, their ECD spectra showed positive Cotton effects around at 200–210 nm, as with neoaplaminone (**2**) (Figure S4), which suggested that these five compounds were all optically active. The absolute configurations at C-15 of aplaminone (**1**) and **2** were both previously established to be *R* by comparison with the optical rotations of their debrominated analogs prepared from natural **1** and by enantioselective synthesis.[8] Based on these data, we speculated that the absolute configuration at C-15 of the new aplaminone analogs **3**–**6** might be *R*, despite the lack of direct evidence from isolated compounds.

Cytotoxicity of Aplaminones and Their Putative Biosynthetic Pathways

We examined the cytotoxic effects of aplaminone derivatives **2**–**8** against HCT-116 human colon cancer cells using an MTT assay. All of these derivatives showed cytotoxicity with IC_{50} values of less than 10 μM, except for **8** (Table 1). Among them, **4** and **6** exhibited relatively high activity (IC_{50}: 0.99 and 0.65 μM, respectively). Of the derivatives with a farnesyl chain **7** and **8**, the cytotoxicity of **7** with an *N*-methyl group was higher than that of **8** with an *N*-acetyl group instead.

In a previous study on aplaminones,[7] the IC_{50} value of neoaplaminone (**2**) against the HeLa human cervical carcinoma cell line was reported to be 1.6×10^{-7} μg/mL (= 3.1×10^{-10} μM). Although a different cell strain was tested in this study, the IC_{50} value of **2** was much higher (3.4 μM for HCT-116 cells) than the previously reported value. During HPLC analysis using a conventional ODS column, we found that **3** was eluted at almost the same time as aplyronine A, which is mainly responsible for the cytotoxicity in *A. kurodai* extracts.[47] In fact, after silica gel column chromatography or HPLC separation, **2** and **3** still contained trace amounts of aplyronines C and A (1/390–1/590, based on the LC-MS intensity) (Figures S5 and S6), despite their high purities on ^{1}H NMR spectra. As expected, a pentabromobenzyl (PBr) HPLC column was useful for separating contaminants from aplaminones which have a bromophenol moiety capable of a dispersion force interaction. As a result, pure **2** and **3** were obtained, but their activities were 10- and 370-fold lower than before PBr column separation, respectively. Therefore, we proposed that the cytotoxicity value for **2** in the previous study might be inaccurate due to trace contamination.

Table 1. Cytotoxicity of aplaminone derivatives against HCT-116 cells

Compound	IC_{50} (μM)
1	– [b]
2	3.4
3	7.0
4	0.99
5	2.6
6	0.65
7	5.0
8	27

[a] Against HeLa cells. See ref. 7. [b] Not tested.

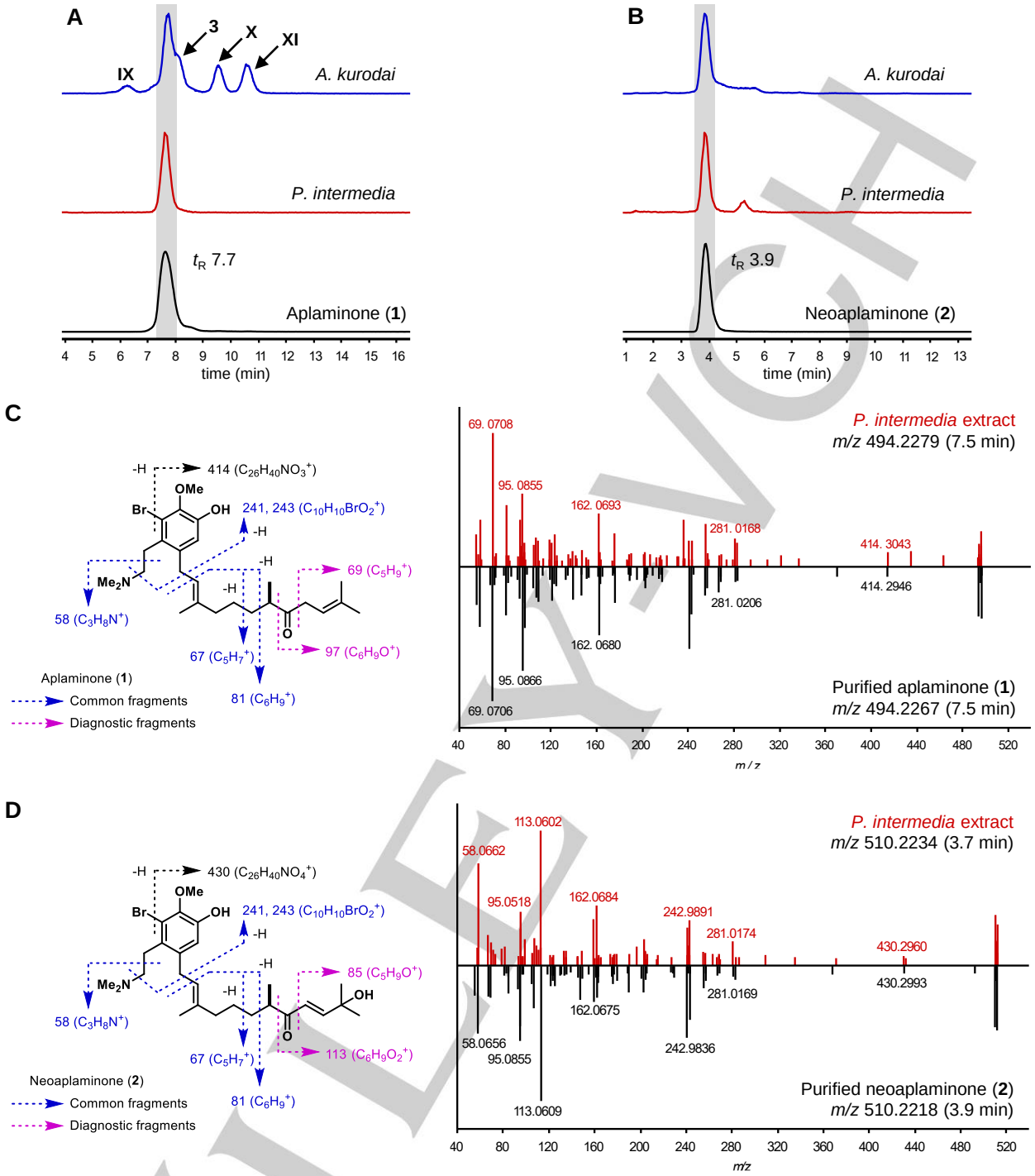


Figure 4. Detection of **1** and **2** by LC-MS/MS. (A) EIC *m/z* 494.2264 [aplaminone $[M+H]^+$ ($C_{26}H_{41}{}^{79}BrNO_3^+$), ±3 mmu dev.] of the 50% aq. EtOH fraction of *A. kurodai* (top), the EtOAc layer of *P. intermedia* (middle), and purified aplaminone (**1**, bottom). Isoaplaminone (**3**) and putative isomers of **1** (**IX**, **X**, and **XI**, see Supporting Information for detail) were also detected in the *A. kurodai* extract. (B) EIC *m/z* 510.2214 [neoaplaminone $[M+H]^+$ ($C_{26}H_{41}{}^{79}BrNO_4^+$), ±3 mmu dev.] of the 50% aq. EtOH fraction of *A. kurodai* (top), the EtOAc layer of *P. intermedia* (middle), and neoaplaminone (**2**, bottom). (C, D) MS/MS assignments and mirror plots comparing **1** and **2** detected in *P. intermedia* (top) against the standards (bottom).

Since aplaminones are brominated dopamine analogs with C15 aliphatic chains, they might be biosynthesized from tyrosine through the several key enzymatic reactions including bromination, farnesylation, *N*,*O*-methylation, and oxidation of the C15 chain. Based on the isolation yields of aplaminone analogs in this and previous studies, we propose a putative biosynthetic pathway for aplaminones from tyrosine, as shown in Figure S7. In this pathway, a series of aplaminones **1**–**8** is presumed to be derived from *N*-demethylaplaminene that possesses a primary amine and a typical farnesyl chain. Here, the reverse prenyl moiety in **3** could be explained by a skeletal rearrangement as follows (Figure S8). *N*-Demethylaplaminene would be isomerized to an exomethylene isomer at C-15. Subsequent Cope rearrangement of the terminal isoprene unit might provide the reverse prenyl structure. Finally, **3** is produced via isomerization of the double bond, *N*-methylation, and oxidative functionalization

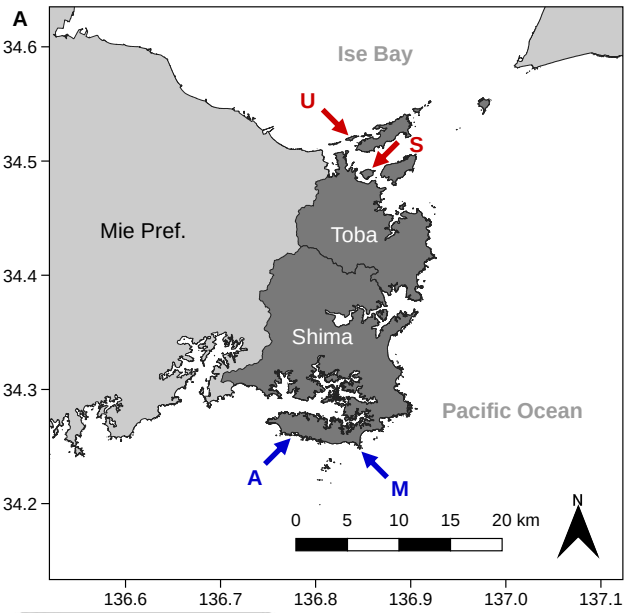
of the C15 chain as in **1**. The introduction of a reverse prenyl group by a sigmatropic rearrangement has been proposed in the biosynthesis of the fungal phenalenone herqueinone.[48] However, to our knowledge, such reaction at the terminal of the C15 aliphatic chain to give **3** is unprecedented.

Detection of Aplaminones from Sea Algae Extracts by LC-MS/MS

Since *A. kurodai* feeds on sea algae that are rich in secondary metabolites, we suspected that aplaminones might be derived from several sea algae or their associated microbes. To establish the presence of aplaminones, LC-MS/MS analysis was conducted using the EtOAc layers of 16 sea alga species belonging to the Phyla Chlorophyta, Ochrophyta, and Rhodophyta (Table S5), which were found in the habitat of the sea hare (Azuri-hama, described below). Surprisingly, among the sea algae extracts tested, both **1** and **2** were detected only in the red alga *Palisada intermedia*, which is grazed by *A. kurodai*[49] (Figures 4A, 4B, S9, and S10). The MS/MS data of **1** and **2** in the extract of *P. intermedia* well matched those of **1** and **2** from *A. kurodai*, including some common fragments ions such as *m/z* 243, 241, and 58 derived from the common structures (blue arrows in Figures 4C and 4D) and diagnostic fragment ions (*m/z* 97 and 69 for **1**, and *m/z* 113 and 85 for **2**) at the aliphatic chain moiety (purple arrows). This is the first time that aplaminones were detected in sea algae extracts, which suggested that *A. kurodai* seemed to acquire cytotoxic aplaminones from sea algae via the food chain.

Geographic Distribution of Aplaminones and Their Origin

To further investigate the presence of aplaminones in sea hare and sea algae, the CH_2Cl_2 layers of *A. kurodai*, *P. intermedia*, and its related algae belonging to the *Laurencia* complex species (*Chondrophycus undulatus* and *Laurencia okamurae*) were analyzed by LC-MS/MS, which were prepared by further partition of EtOAc layers to enrich aplaminones. These specimens were collected in April–May 2022 at four sites: Azuri-hama (**A**) and Mugisaki (**M**) on the Pacific coast of the Shima Peninsula (Shima City), and Sakatejima (**S**) and Ukishima (**U**) on the Ise Bay side of the Peninsula (Toba City) (Figure 5A). Among the samples tested, both **1** and **2** were only detected from the sea algae collected at sites **A** and **M**, but not at sites **S** or **U** (Figure S11). The contents of **1** and **2** in the sea hare collected at **A** were 1.9×10^2 and 4.3×10 ng/g wet wt, respectively, which were *ca.* 2–200 times higher than those in *C. undulatus*, *L. okamurae*, and *P. intermedia* (Figure 5B). Since *L. okamurae* and *P. intermedia* were previously found in the digestive gland of *A. kurodai*,[49] we concluded that aplaminones in *A. kurodai* would be derived from the *Laurencia* complex via the food chain.Some of the *Laurencia* complex species have been known to possess various bioactive brominated compounds that are thought to be synthesized and/or stored in a cytoplasmic vesicle called “corps en cerise (CC)”.[50] Several brominated terpenoids such as aplysin, aplysinol, and laurinterol were found commonly in both of *A. kurodai* and the *Laurencia* complex species with CC.[51–56] However, the brominated dopamine analogs aplaminones were detected in *P. intermedia* that has been considered to contain none of halogenated compounds[49,55] due to the lack of CC.[57] These results also supported the hypothesis that aplaminones could originate from microorganisms associated with the sea algae. Intriguingly, the presence/absence of aplaminones varied by the collection site, even within the same species (e.g., *A. kurodai* collected at sites **A** and **U**, and *C. undulatus* collected at sites **M** and **S** in Figure 5). Consequently, the producer of aplaminones could be microbe(s) that are specifically associated with the *Laurencia* complex species locally distributed on the Pacific side of the Shima Peninsula.



B

Site	Contents of **1** / **2** (ng per g wet wt)			
	Animal	Sea algae		
	A. kurodai	*C. undulatus*	*L. okamurae*	*P. intermedia*
A	**1.9×10^2 / 43**	n.d.	**52 / 17**	**49 / 8.0**
M	-	**26 / 22**	n.d.	**0.95 / 6.3**
S	-	n.d.	-	-
U	n.d.	-	n.d.	-

Figure 5. Geographical distribution of aplaminones **1** and **2** at the Shima Peninsula. (A) Sample collection location. Solid arrows indicate the sample collection sites. Note that the sites where **1** and **2** were detected by LC-MS/MS are shown in blue while not are shown in red. (B) The contents (ng/g wet wt) of **1** and **2** in the CH_2Cl_2 layers of *A. kurodai* and the *Laurencia* complex species quantified by LC-MS. n.d.: not detected (less than 0.1 ng/wet wt), – : sample was not available.

Ise Bay is a region with calm ocean currents and a thriving aquaculture industry. On the other hand, the Pacific coast of the Shima peninsula is where the warm and nutrient-poor Kuroshio Current flows in. Thus, despite being only 25–35 km apart, the microbiomes of the *Laurencia* complex species may be highly different between the Pacific and Ise Bay sides of the Shima Peninsula due to the dissimilar coastal environments. Sea algae are known to form holobionts with their associated microbiota,[58] and the differences in microbiota are highly dependent on the kinds of host algae and their location.[59] It is speculated that the differences in microbiota between the *Laurencia* complex distributed on the Pacific and Ise Bay sides of the Shima Peninsula might result in the geographic distribution of aplaminones.

Conclusion

Toward the discovery of new cytotoxic aplaminones from *A. kurodai*, the feature-based molecular networking analysis was conducted with aqueous fractions that have not been well investigated so far. As a result, six new aplaminone derivatives **3**–**8** were successfully isolated, and their structures and cytotoxicity were established. Notably, isoaplaminone (**3**) has a terminal reverse-prenylated chain, a rare structural feature from the viewpoint of terpenoid biosynthesis. LC-MS/MS analysis of the sea algae collected in the habitat of *A. kurodai* revealed that the major aplaminones **1** and **2** were specifically detected in the red alga *P. intermedia*, which belongs to the *Laurencia* complex species. Further investigation of the sea hare and three *Laurencia* complex species collected at four sites on the Shima Peninsula revealed the geographic distribution of aplaminones. Aplaminones were commonly found in the *Laurencia* complex species, but there were large regional differences: algae were distributed in the habitat of the toxic sea hare contained aplaminones, but not in that of the non-toxic sea hare. These data strongly suggested that aplaminones accumulate in *A. kurodai* from the *Laurencia* complex species, and that aplaminones are produced by microbe(s) that are specifically associated with these red algae located on the Pacific coast of the Shima Peninsula. Further metabolomic and genomic investigations of the *Laurencia* complex and their associated microbes are underway to elucidate the origin and biosynthetic pathways of aplaminones.

The coastal marine environment in Japan changes drastically with the seasons and ocean currents, and the sea macroalgal forests that have complex life cycles also change.[60,61] Nevertheless, aplaminones have been consistently detected in the extracts of *A. kurodai* collected from the same area as in our survey over the past 15 years. This study on the dynamism and origin of bioactive compounds should provide new chemical ecological insights into the role of natural products in relation to the marine ecosystem.

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Keywords: *Aplysia kurodai* • Sea Algae • Marine Natural Products • LC-MS/MS • Chemical Ecology

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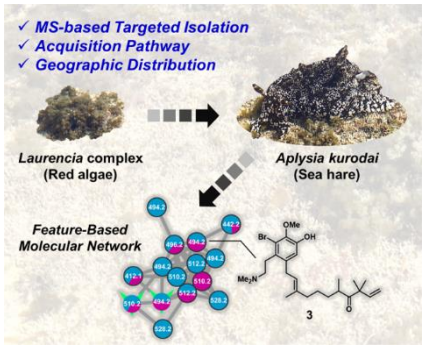
Entry for the Table of Contents



Six new cytotoxic aplaminone derivatives **3**–**8** were discovered from the herbivorous marine mollusk *Aplysia kurodai*. A comprehensive LC-MS/MS analysis of the extracts of sea algae distributing in the habitats of *A. kurodai* revealed that aplaminones could accumulate in *A. kurodai* from the red algae belonging to the *Laurencia* complex, and the presence/absence of aplaminones varied depending on the collection sites for the same species.

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