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ANTIOXIDANT ACTIVITY OF AROMATIC ALKALOIDS FROM THE MARINE SPONGES *Aaptos aaptos* AND *Hyrtios* SP.

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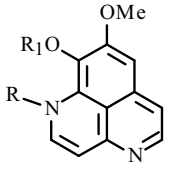
*Aaptamine (**1**) and isoaaptamine (**2**) were isolated from the marine sponge Aaptos aaptos; 6-bromo-2′-de-*N*-methylaplysinopsin (**3**) from the marine sponge* Hyrtios *sp. Alkaloids **1**–**3** were tested for the ability to trap 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, to reduce Folin–Ciocalteau reagent (FCR), and to inhibit oxidation of linoleic acid (LA) induced by peroxide radicals. Compounds **1** (IC_{50} 18 μM), **2** (IC_{50} 16 μM), and **3** (IC_{50} 18 μM) reacted strongly with DPPH, comparable with trolox (IC_{50} 16 μM) and showed high reducing ability for FCR. The inhibition of LA oxidation by **1**–**3** was comparable with that of ionol (BHT). It was shown that the antioxidant activity of **1**–**3** was related to their ability to release both electrons and H atoms.*

Key words: marine sponges, aromatic alkaloids, aaptamines, aplysinopsins, antioxidants.

Research on marine natural compounds, which began about 60 years ago, has shown that almost every class of marine organisms synthesizes a variety of molecules with unique structures that are not observed among natural compounds from terrestrial sources owing to the physical and chemical conditions of the marine environment [1, 2]. Marine sponges are one of the most ancient classes of animals. The class Demospongiae contains about 95% of living species and is represented around the world by about 14,000–15,000 species [3]. Marine sponges, from which more than 50% of marine natural compounds have been isolated [2], are a rich source of compounds with cytotoxic and antitumor properties [4, 5]. The antioxidant activity of compounds isolated from marine sponges is relatively little studied [6–9]. Avarol from the Mediterrean sponge *Disydea avara* is the only example of a well studied antioxidant from sponges. It is currently manufactured as a preparation against psoriasis [10].

In continuation of the search for antioxidants from marine organisms, we investigated marine sponges *Aaptos aaptos* (Hadromerida, Suberitidae) and *Hyrtios* sp. (Dictyoceratida, Thorectidae), the alcohol extracts of which acted as strong traps for 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Our goal was to isolate the active metabolites from these species of marine sponges and study the antioxidant activity and mechanism of antioxidant activity of the obtained compounds.

Fractionation of the extract from *A. aaptos* isolated compounds that were active traps for DPPH. These were aaptamine (**1**), isoaaptamine (**2**), and their HCl salts. They were identified by comparison of their spectral properties with the literature [11, 12]. Fractionation of the extract from *Hyrtios* sp. isolated the active compound 6-bromo-2′-de-*N*-methylaplysinopsin (**3**), which was identified by comparison of spectral data with those published [13].



1, 2

1: R = H, R_1 = Me
2: R = Me, R_1 = H

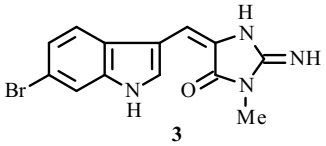


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TABLE 1. Antioxidant Activity of Aaptamine (**1**), Isoaaptamine (**2**), and 6-Bromo-2′-de-*N*-methylaplysinopsin (**3**) in Various Methods

Compound	DPPH[a]	FCR[b]	IOLA[c]
	IC_{50}, μM	GAE,[d] mmol/L	rate, μm/min
1	18	0.50	0.91
2	16	0.52	0.78
3	18	0.48	0.84
Trolox	16	0.70	–
BHT	360	–	0.84
Control[e]			12.21

[a]DPPH is 2,2-diphenyl-1-picrylhydrazyl radical; [b]FCR, Folin–Ciocalteau reagent; [c]IOLA, induced oxidation of linoleic acid; [d]GAE, gallic acid equivalent; [e]control, oxidation of linoleic acid without antioxidants.

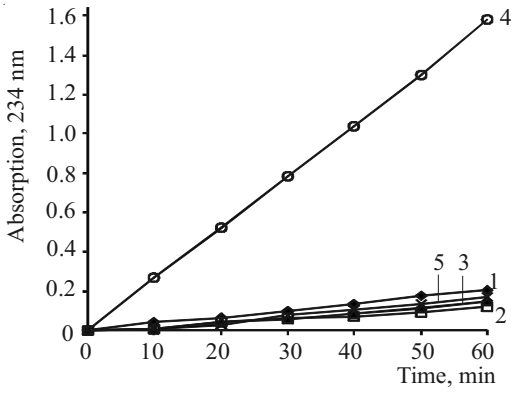

Fig. 1. Oxidation of linoleic acid induced by AAPH without antioxidant (control, **4**) and with aaptamine (**1**), isoaaptamine (**2**), 6-bromo-2′-de-*N*-methylaplysinopsin (**3**), and ionol (BHT, **5**).

We used three methods to study the antioxidant properties of **1–3**. These were bleaching of an alcohol solution of DPPH, reduction of Folin–Ciocalteau reagent (FCR), and inhibition of induced peroxyl radical oxidation of linoleic acid (IOLA).

Table 1 gives results of the DPPH test expressed in IC_{50} values. The antiradical activities of **1** (IC_{50} 18 μM), **2** (IC_{50} 16 μM), and **3** (IC_{50} 18 μM) were comparable with that of trolox (IC_{50} 16 μM).

It is known that one pathway for trapping DPPH radicals is based on transfer of one electron from the antioxidant to the radical [14]. Thus, the reducing ability of a compound is an important parameter that reflects its antioxidant action. A method based on the use of FCR that is usually used to determine the total phenol content was used to estimate the reducing ability of **1–3** [15]. The FCR method is based on electron transfer and indicates the reducing ability of the compounds [14, 16]. Absorption of the resulting blue products at 750 nm is directly related to the reducing power of the compounds [16]. Table 1 gives the reducing ability of **1**–**3** expressed in gallic acid equivalents (GAE, mmol/L), which was based on a standard curve for gallic acid.

The reducing ability of the studied compounds in the FCR method was about 71% (**1**), 74% (**2**), and 68% (**3**) of the trolox activity. The presence of reducing properties in **1–3** indicated that the compounds were electron donors. They could react with free radicals, converting them into more stable products and thereby terminating radical chain reactions. These results agreed with those of the DPPH test. Compounds **1–3** were strong reductants of FCR and were highly active traps of DPPH radicals.

The principal role in lipid oxidation in biological systems is played by peroxyl radicals. The key step in progression of the radical chain reaction is H-atom transfer from the lipid to peroxyl radical [14]. Compounds capable of donating a H-atom to peroxyl radicals and thereby inhibiting lipid oxidation induced by peroxyl radicals can act as antioxidants. We used

oxidation of linoleic acid induced by peroxyl radicals formed by thermal decomposition of the azo-initiator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) to estimate the inhibitory activity of **1–3** [17]. The oxidation process was followed by monitoring absorption of linoleic acid diene hydroperoxides formed during the oxidation. Figure 1 shows typical curves of AAPH-induced control oxidation of linoleic acid and oxidation in the presence of antioxidants.

The concentration of linoleic acid hydroperoxides was calculated using the known molar extinction coefficient [ε(234 nm) 27,000 $M^{-1}cm^{-1}$] in order to estimate the rate of oxidation [17]. The results for inhibition of IOLA (Table 1) showed that **1–3** were active antioxidants in this method and that their activities were comparable with that of the antioxidant standard ionol.

Aaptamines are a group of marine benzo[*de*]-1,6-naphthyridine alkaloids. Several types of activity are known for them including α-adrenoblocking [18], anti-HIV-1 [19], antimicrobial [19], antiherpetic [20], inhibition of monoamine oxidase A [21], inhibition of sortase A [22], and anticancer [19, 23]. The mechanism of the antioxidant activity of **1** and **2** was studied for the first time although antioxidant activity had been observed for **1** and **2** previously [7].

Aplysinopsins are a class of indole alkaloids that exhibit various biological activities including inhibition of neuronal synthase of nitric oxide [24] and serotonin 5-HT2 receptors [25]. Antioxidant activity had not been previously reported for these metabolites. The antioxidant activity of **3** was studied for the first time.

The antioxidant activity of marine metabolites representing two different classes of aromatic alkaloids was estimated using three different methods. The results showed that marine sponges are a potential source of antioxidants with unique structures that act as reductants by donating electrons and as terminators of oxidation chains by donating protons.

EXPERIMENTAL

We used silica gel (Sorbpolimer, Krasnodar, Russia) and Sephadex LH-20 (Pharmacia Fine Chemicals, Sweden) for column chromatography. Absorption spectra were measured on a UV-mini 1240 (Shimadzu) spectrophotometer. We used 2,2-diphenyl-1-picrylhydrazyl (DPPH, Fluka); trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma–Aldrich), 2,6-di-*t*-butyl-4-methylphenol (ionol, BHT, Olaine); linoleic acid (99%, Sigma—Aldrich), and 2′-azobis(2-amidinopropane) dihydrochloride (AAPH, Sigma—Aldrich).

Isolation of Antioxidants. The sponge *Aaptos aaptos* was collected during the fifth scientific cruise of the RS Academic Oparin in the waters of Viet Nam, Ku Lao Re island, at a depth of 3–6 m; *Hyrtios* sp., during its third scientific cruise in Seychelles waters at a depth of 3 m. Sponges were freeze dried and stored at –20°C until used.

Marine sponge *A. aaptos* (8 g) was extracted exhaustively with acetone at room temperature. The acetone extract was concentrated and separated over a column of Sephadex LH-20 using $CHCl_3$:EtOH (3:1) to produce two colored fractions. The first yellow fraction was separated over a column of Sephadex LH-20 using $CHCl_3$:EtOH (2:1) to produce aaptamine hydrochloride (**1**) (21.7 mg, 0.27%) and aaptamine (**1**) (10 mg, 0.13%). The second yellow fraction was also separated over a column of Sephadex LH-20 using $CHCl_3$:EtOH (2:1) to produce isoaaptamine hydrochloride (**2**) (20 mg, 0.25%) and isoaaptamine (**2**) (4 mg, 0.05%).

Marine sponge *Hyrtios* sp. (100 g) was extracted exhaustively with EtOH. The extract was concentrated and chromatographed over a column of silica gel using EtOH:$CHCl_3$ (1:6) to isolate fractions that were active in the DPPH test. The final purification of this fraction over a column of Sephadex LH-20 using MeOH gave 6-bromo-2′-de-*N*-methylaplysinopsin (**3**) (4 mg, 0.004%), yellow crystals, mp 186–188°C, high-resolution mass spectrum $[M]^+$ *m*/*z* 318.0119/320.0120 (calcd for $C_{13}H_{11}N_4OBr$, 318.0116/320.0116) [13].

Determination of Antiradical Activity (DPPH method). We used a slight modification of the literature method [26]. A solution of the compounds (1.3 mL) of various concentrations (5, 10, 20, 50, and 100 μM) in MeOH was treated with DPPH (6 mg/50 mL, 0.1 mM final concentration) in MeOH (0.7 mL) to total volume 2 mL. Absorption at 517 nm was measured after 30 min. The percent inhibition was calculated using the formula: inhibition (%) = $100 - (A_{samp} \times 100/A_{control})$. The IC_{50} value was obtained from a curve of the percent inhibition as a function of concentration. The IC_{50} value signifies the concentration of the compound required for trapping of 50% of the DPPH radicals. Trolox and ionol (BHT) were used as standard controls. Measurements were made in triplicate for each compound.

Reducing ability (FCR method) was determined by a slight modification of the reported procedure [15]. A solution of the studied compound (20 μL, 20 mM, 1.0 mM final concentration) was treated with distilled water (300 μL) and FCR

(20 μL). The mixture was treated after 1 min with Na_2CO_3 solution (60 μL, 20%) and left at room temperature for 1 h. Then, absorption at 750 nm was measured. The reducing ability of the compounds was expressed in gallic acid equivalents (GAE, mmol/L) based on a standard curve of absorption at 750 nm as a function of gallic acid concentration. The GAE signifies the concentration of gallic acid with reducing ability (absorption at 750 nm) equivalent to a solution of the studied compound (1.0 mM). Measurements were made in triplicate for each compound.

Inhibition of Induced Oxidation of Linoleic Acid (IOLA Method). We used a slight modification of the known methods [17, 27]. Starting phosphate buffer solution (0.1 M, pH 7.4) was stored in a plastic bottle for less than two weeks. Starting Tween-40 micelle solution (0.1 M, 3.5 mL) in phosphate buffer (0.1 M, 30 mL) was used for one week. Starting linoleic acid micelle solution (0.1 M) in Tween-40 solution (0.1 M), starting AAPH solution (0.5 M) in phosphate buffer (0.1 M), and starting antioxidant solutions (20 mM) in MeOH were freshly prepared.

Oxidation was carried out in a glass beaker (15 mm diameter, 40 mm height). The reaction mixture contained phosphate buffer (3.58 mL, 0.1 M), a solution of the test compound (20 μL, 20 mM, 0.1 mM final concentration) or MeOH as a control (20 μL), and linoleic acid micelle solution (200 μL, 0.1 M, 5.0 mM final concentration). The reaction mixture was dispersed in an ultrasonic bath (Elmasonic Model S10 H) at 50°C for 3 min until a transparent micelle solution was obtained. Oxidation was induced by adding AAPH solution (200 μL, 20 mM, 1.0 mM final concentration). The reaction mixture (total volume 4 mL) was held at constant (50°C) temperature in the ultrasonic bath for 1 h, taking a sample (400 μL) every 10 min for the absorption measurement. Absorption was measured at 234 nm in a 1-mm cuvette and corrected for absorption at the start of oxidation.

The rate of formation of diene hydroperoxides was calculated using the formula: oxidation rate $(\mu M/min) = (A_a - A_0)/(T_a - T_0) \times \varepsilon^{-1} b^{-1}$, where ε is the molar extinction coefficient of the linoleic acid diene hydroperoxides at 234 nm ($\varepsilon = 27{,}000\ M^{-1}cm^{-1}$) [17]; b = 0.1 cm (cuvette thickness); A_0, absorption at the start of oxidation; A_a, absorption at the given oxidation time. Measurements were made in triplicate for each compound.

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