Functions of Phenolic Secondary Metabolites in Lichens from Far East Russia

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Abstract

Orcinol-type depsides, polyhydroxyquinonoid pigments, melanins and usnic acid, help to protect lichens from high light intensities and the extreme climatic conditions of the Kolyma Upland and the Badjal Mountain range. The melanin isolated from the black-coloured high mountain lichen *Umbilicaria rossica* was investigated for the first time and shown to be a heterogeneous polymer, with orsellinic acid as an aromatic structural component. The multiformity of the chemical compounds in the high mountain lithophilic lichens is a response to the challenge of the severe climate. Orcinol-type lichen depsides and tridepsides may provide protection against peroxidation by blocking toxic metal ions that might otherwise initiate free radical reactions. In Far Eastern epigeous lichens, the presence of the dimeric naphthazarin pigments cuculloquinone, islandoquinone, 6,6'-bi('3-ethyl-2,7-dihydroxy-naphthazarin), and their monomeric forms may provide protection from oxidation. The similarities in the chemical structures, and antioxidant properties of the pigments in some epigeous lichens with those of the sea urchins are discussed.

Keywords: Parmeliaceae, *Umbilicaria*, usnic acid, depsides, depsidones, melanin, orsellinate depside hydrolase, quinonoid pigments, antioxidant activity

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1. Introduction

Investigations of the lichen flora of northeastern Russia (north of Far East) have shown that it is rich and varied (Rassadina, 1950; Lokinskaya, 1967). The chemical composition and especially the pigments of many of the widely distributed species are unknown. Usnic acid and ergosterol peroxide were the first secondary metabolites to be studied in lichens from this area (Sviridov and Strigina, 1978).

The great variety of lichen acids and their abundance in these northeastern lichens suggests that they are necessary substances for survival in this harsh climate but their function is not well understood. Some lichen acids depress growth of mosses and free-living fungi (Gardner and Mueller, 1981); others participate in the weathering of rocks, soil-formation and accumulation of metal cations and also anions including carbonate and phosphate. Modern concepts concerning stress tolerance suggest that this reflects the evolution of adaptations. An example of an adaptation to extreme environments in lichens is the production of large amounts of phenolic acids (Huneck and Yoshimura, 1996). Indeed, the accumulation of lichen secondary metabolites is probably one response to stresses that could include invasion by microorganisms, large fluctuations in temperature, freeze-thaw cycles, and hydration-desiccation events.

In this study we attempted to elucidate the distinctive properties of northeastern lichens including the composition and the structure of the phenolic compounds in various species. The secondary phenolic metabolites, the lichen acids and quinonoid pigments, were the primary object of our investigations. Information on the antioxidant properties of the quinonoid pigments and the orcinol-type of depsides is provided together with a characterization of the melanin from the *Umbilicaria rossica*.

2. Materials and Methods

Collection sites

The studied sites are located in two Far East regions – the Upper Kolyma Upland of the Magadan district, 62°30'N, 150°20'E, and the Urmi River head of the Badjal Mountain range of the Khabarovsk district, 50°36'N, 134°36'E. The first site was at the Biogeocenology Station "Kontakt", 5 km from the Settlement Kulu. The surveyed area comprised a reservoir of the Stokoviy Stream (the tributary of the Kulu River) at an altitude of 800–1100 m. The climate is continental with a long northern winter, snow cover up to 2.0 m and a temperature minimum in January of –50°C. There is a relatively short hot

summer with a maximum temperature of +30°C in July. The lichen flora of the Station is typical for most of the Upper Kolyma (Korolev and Tolpysheva, 1980). Epilithic lichens were collected, particularly those in the genus *Asahinea* (*A. chrysantha* (Tuck.) W. Culb. & C. Culb. and *A. scholanderi* (Llano) W. Culb. & C. Culb.) which are typical of this mountain tundra flora. *Ophioparma lapponicum* Ras. (Haematomma) is another widely distributed lichen on the bald peaks of the Kolyma Upland at a height of 1000 m. In addition the epilithic lichens *Melanelia stygia* (L.) Essl., *Arctoparmelia birulae* Elenk. grew on the northwest slopes of the mountains while the epigeous lichens *Flavocetraria cucullata* (Bell.) Kärnef. et Thell and *Cetraria islandica* (L.) Ach. were collected near the foot of the mountains and from a valley.

The second site, the Badjal Mountain Range, has a climate like the Kolyma Uplands. The average temperature in July is about +23°C with a large temperature gradient (almost 20°C) between day and night. The average January temperature is about –30°C, with a temperature gradient greater than 20°C and strong winds that blow snow off the peaks. The annual rainfall is moderate. The mean height is 900–1900 m, but the peak of Mt. Omot is 2200 m. This area is colonized by *Umbilicaria* which covers the mountain range with a black carpet. *Umbilicaria rossica* (Dombr.) Golubk. and *Parmelia stygia* (L.) Ach. were collected here.

Isolation of lichen melanin

Air-dried samples of *Umbilicaria rossica*, were first extracted with acetone and then with boiling water in order to remove gyrophoric acid (10%) and polysaccharides (18%). Melanin was then extracted from the lichen with 3.5% aqueous ammonium hydroxide (w/v 1:20) under argon at room temperature for 1 h. The procedure was repeated twice. The extract was concentrated 3.5 times in a rotary evaporator. The resultant extract had a pH of 7.5 and was acidified with 10% HCl to a pH of 3.0. The melanin precipitate was recovered by centrifugation at 3500 g, washed with distilled water at pH 7.0, and lyophilized. The yield was 2.0%. The melanin was hydrolysed with 6 M HCl for 7 h at 100°C and then for 17 h at room temperature. Both the unhydrolysed and hydrolysed melanins were characterized by ^{13}C NMR and IR spectral methods in a similar way to fungal melanins (Schnitzer and Chan, 1986; Babitskaja et al., 1996). Electron spin resonance (ESR) measurements were made using an ESR-231 spectrometer, employing a nominal operating frequency of 100 kHz. Melanin samples were analyzed by liquid-state ^{13}C NMR (34 mg in NaOD + D_2O) at 75.5 MHz spectrometer frequency on Bruker DPX-300. Infrared spectra (IR) were recorded using a Specord M 80 spectrophotometer from a melanin

suspension in petroleum jelly (Vaseline oil) (20 mg melanin were ground in one drop of Vaseline oil).

Isolation of phenolic compounds

The air dried crushed lichens *Asahinea chrysantha, A. scholanderi, Arctoparmelia birulae* were extracted with hexane and then chloroform. Usnic acid and atranorin were isolated from the hexane extracts by crystallization. Anthraquinone pigments were separated from the hexane extracts by column chromatography using silicic acid and Sephadex LH-20; alectoronic and collatolic acids, together with their β-forms, were isolated from the chloroform extracts by a similar procedure. All constituents were identified by means of thin layer chromatography (TLC), spectral methods and melting points which had been reported in previous publications (Krivoshchekova et al., 1983a, 1983b). Biruloquinone was separated from the chloroform extract of *A. birulae* by complexing it with magnesium carbonate and copper acetate. Its structure was determined by using derivatives (Krivoshchekova et al., 1983b).

The lichen acids of *Melanelia stygia* were separated by preparative TLC from an acetone extract (Mishchenko et al., 1984). The quinonoid pigment cuculloquinone from *Flavocetraria cucullata* was isolated with methanol containing 1% HCl and subsequently purified by complex formation with copper acetate (Krivoshchekova et al., 1982). Naphthaquinones from *Cetraria islandica* were isolated with ethyl ether containing 2% HCl. The 'boiled down' extract was re-extracted with benzene and separated by DCCC (droplet counter current chromatography) in a chloroform-methanol-water mixture (Stepanenko et al., 1997). Usnic and divaricatic acids were isolated from an ethyl ether extract of *Ophioparmia lapponicum* by crystallization (Maximov et al., 1990).

3. Results and Discussion

The extreme climate of the Badjal Mountain range provides a challenge to plants growing in the region. Coloured lichen substances provide protection from the solar radiation on long summer days. Most of the *Umbilicaria* species which dominate the mountain lichen flora of the Russian Far East are black due to melanin pigments. Melanins act as molecular phototraps transforming the sun's energy into the heat and they can protect the fungal cells of lichens from seasonal desiccation (Zdanova and Vasilevskaya, 1982). Melanins can also act as a sun screen for lichen photobionts as shown in *Lobaria pulmonaria* (Gauslaa and Solhaug, 2001).

The melanin of *U. rossica* was tested for its reactivity. It was decolorized by H_2O_2, bromine water and sodium bisulphite; reactions that are used to indicate

that a compound is a melanin. Information on the structural chemistry results from the studies on hydrolysed melanin of *U. rossica*. Six molar HCI removed amino acids and carbohydrate components from the melanin. The signals of the polysaccharide β-1,6-D-glucan were only present in region 50–110 ppm in the ^{13}C NMR spectrum of the unhydrolysed melanin, and they were absent in the spectrum of hydrolysed melanin. The ^{13}C NMR spectrum of the hydrolysed melanin has 3 main signal regions: aliphatic C at 14–58 ppm, aromatic C at 95–140 ppm, and signals of C in COOH at 171–178 ppm. Strong aliphatic resonances prevailed in the spectrum of melanin. They were present at 14.0, 22.7, 37.8 ppm, 29.0–30.0 ppm as the strongest signal conforming to long-chain hydrocarbons. Aromatic C-signals were present at 110.3, 115.7, 124.1 ppm and there was a resonance at 173.5 ppm, conforming to C in COOH. The ^{13}C NMR spectra revealed that lichen melanin is heterogeneous as are fungal melanins (Schnitzer and Chan, 1986). Certain signals of the lichen melanin spectrum were similar to those observed in ^{13}C NMR spectrum of orsellinic acid (23.5, 100.6, 105.0, 110.9, 142.9, 161.8, 164.5, 173.3) (Huneck and Yoshimura, 1996); and the IR spectra of the melanin before and after hydrolysis showed a band at 1720 cm^{-1} conforming to C=O in COOH. It is possible that this also belongs to an orsellinic acid fragment of melanin. Thus spectral data suggest that orsellinic acid is probably a structural component of melanin from *U. rossica*.

Bell and Wheeler (1986) reported, that fungi of genus *Aspergillus* and other genera of free-living fungi produce considerable amounts of extracellular heterogeneous melanins. These fungi accumulate a large number of phenols derived from tetraketides. Mosbach and Schultz (1971) established that the photobiont of *Lasallia pustulata* produces the enzyme orsellinate depside hydrolase, which easily hydrolyses gyrophoric acid to orsellinic acid. Another enzyme, orsellinate decarboxylase, catalyses the decarboxylation of orsellinic acid to orcinol in the mycobiont of this lichen. Thus, enzymes from both symbionts participate in the enzymic degradation of the tridepside gyrophoric acid. The main lichen compound of *U. rossica* is gyrophoric acid, which is probably also cleaved by these enzymes. Additional studies are needed to prove that orcinol is involved in melanin formation. We suggest, that orsellinic acid and orcinol may participate in the formation melanin in other species of *Umbilicaria*, which contain significant quantities of orcinol-type tridepsides such as gyrophoric acid and umbilicaric acid.

Information on the molecular weight of unhydrolyzed melanin was obtained by gelchromatography on Sephadex G-50. The melanin elution diagram revealed two main fractions; one with MW from 10 to 20 kDa (1) or more, and a second fraction with a MW of 4–6 kDa (2). There was an intermediate zone (between 1 and 2) with a MW 6–10 kDa. The amount of every fraction was about 32%. These data indicated, on polydispersion of the melanin, that it has

components of different MW, and in this respect is similar to melanins of free-living fungi (Babitskaya et al., 2000).

Melanin is stable free radical and it can also scavenge other free radicals that result from environmental stress. The free radical content of melanin can be determined by electron spin resonance spectroscopy (ESR), that detect these radicals as paramagnetic particles (Bell and Wheeler, 1986). ESR spectrum of the lichen melanin showed the characteristic slightly asymmetric singlet (g-factor = 2.0068) similar to spectra for fungal melanins, and the concentration of the paramagnetic centres was 1.7×10^{18} spin per g of substance.

Other substances which protect lichens against high light intensities in these environments include usnic acid and atranorin. They are the most widely distributed low-molecular weight metabolites found in the upper cortex of high-mountain lichens. For example the lithophilous lichen *Ophioparma lapponicum* has a light-green upper lichen surface and contains 6% of usnic acid (Maximov et al., 1990). The chemistry of the genus *Asahinea* has been little studied while that of *Arctoparmelia birulae* was unknown. These typical Far Eastern epilithic lichens proved to have a similar but peculiar chemistry (Krivoshchekova et al., 1983a, 1983b).

Table 1.   Compositions of the *Asahinea* and *Arctoparmelia birulae* extracts

Main compounds	Content (%)*		
	A. chrysantha	*A. scholanderi*	*A. birulae*
Usnic acid	1.30	0.01	2.43
Atranorin	0.09	0.02	0.90
Alectoronic acid	0.25	0.01	5.10
β-alectoronic acid	0.31	0.01	0.57
Collatolic acid	–	0.04	–
β-collatolic acid	–	0.02	–
Islandicin	1×10^{-2}	2×10^{-2}	1×10^{-3}
Cynodontin	3×10^{-3}	2×10^{-3}	–
Biruloquinone	–	–	0.01
Pentahydroxy-methylanthraquinone	3×10^{-3}	3×10^{-4}	–

*% is given on weight of air dried lichens: $\dfrac{\text{W of a compound} \times 100}{\text{W of lichen}}$

The upper surfaces of *Asahinea chrysantha* and *Arctoparmelia birulae* are respectively light-yellow and grey-green due to large amounts of usnic acid (Table 1). In contrast, *Asahinea scholanderi* has a low level of usnic acid, consequently the colour of the upper cortex varies from light-grey in shaded places to black in well-illuminated areas (Krivoshchekova et al., 1983a). Both species of *Asahinea* have a glossy-black lower thallus surface shading to brown at the margins of the lobes. The black colour is due to polyhydroxy-anthraquinone pigments, which have been studied previously in these lichens (Mishchenko et al., 1980). Islandicin, cynodontin, pentahydroxy-methyl-anthraquinone are dominant among these pigments (Huneck and Yoshimura, 1996). The violet colour of the lower cortex in *Arctoparmelia birulae* is caused by the blue-violet pigment biruloquinone, a hydroxy derivative of ortho-phenanthrenequinone (Krivoshchekova et al., 1983b; Huneck and Yoshimura, 1996). The structure of these various pigments is shown in Fig. 1.

The epilithic lichens *Asahinea* sp. and *Arctoparmelia birulae* contain unusual medullary lichen acids. In addition to the orcinol-type depsidones alectoronic and collatolic acids, they contain diphenylethers (Fig. 2), where substitution patterns correspond to those of β-alectoronic and β-collatolic acids (Krivoshchekova et al., 1983a; Huneck and Yoshimura, 1996). The accumulation of substantial quantities of the diphenylethers is believed to have been caused by the presence of an orsellinate depside hydrolase.

The Badjal mountain range samples of the epilithic lichen *Parmelia stygia* have a chemical composition which is different from that of the same lichens found in other regions. The Badjal samples are characterized by the presence of fumarprotocetraric acid, which is a β-orcinol-type of depsidone and caperatic acid (an aliphatic acid), as well as by significant amount of orcinol-type depsidones, namely lobaric acid (0.9%) and colensoic acid (1.3%) (Mishchenko et al., 1984). This complex mixture of depsidones of the orcinol-type and β-orcinol-type in *P. stygia* probably reflects an adaptation to the extreme conditions of high mountain habitats

Lichens are hypothesized to respond to extreme changes in their environment by the synthesis of large amounts of stress metabolites (Huneck and Yoshimura, 1996). Our investigations confirm this because northeastern lichens contain large amounts of lichen substances; for example, *Umbilicaria rossica* has 10% gyrophoric acid and *Ophioparma lapponicum* 12.7% divaricatic acid. The complex mixture of lichen acids and quinonoid pigments in the mountain epilithic lichens of *A. chrysantha, A. scholanderi*, and *Arctoparmelia birulae* (Table 1), and the mixture of orcinol- and β-orcinol-type depsidones in high mountain *M. stygia* thalli, probably reflect adaptations to extreme cold and to large fluctuation in temperature, moisture, and insulation.

We have also examined the chemical composition of the widely distributed epigeous lichens *Flavocetraria cucullata* and *Cetraria islandica*. A
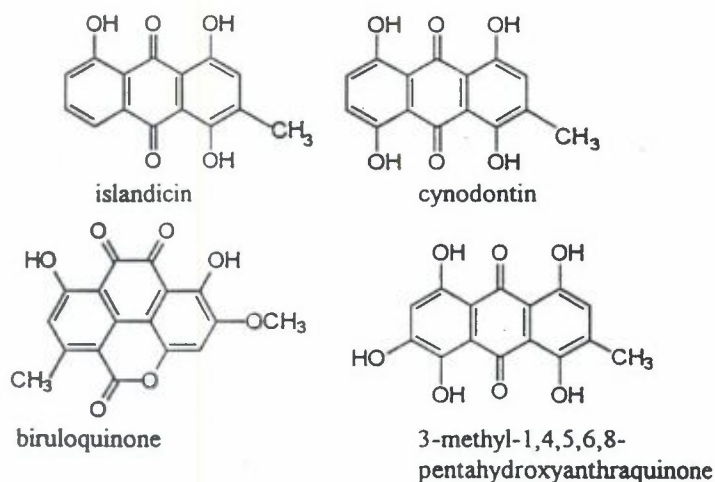
islandicin

cynodontin

biruloquinone

3-methyl-1,4,5,6,8-
pentahydroxyanthraquinone

Figure 1.   Anthraquinones from lichens *Asahinea chrysantha, A. scholanderi* and *Arctoparmelia birulae.*

R=H   alectoronic acid
R=CH₃ collatolic acid
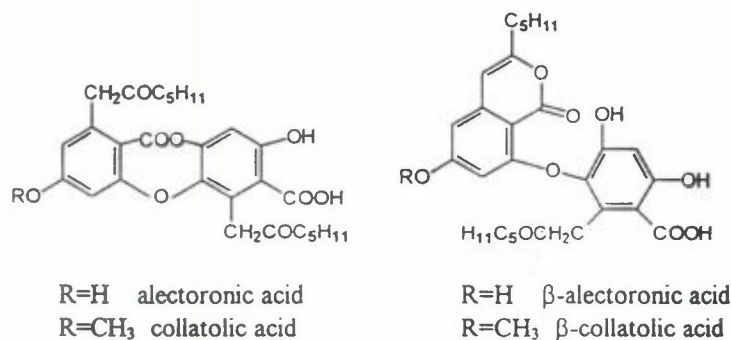
R=H   β-alectoronic acid
R=CH₃ β-collatolic acid

Figure 2.   Alectoronic and collatolic acids and the β-forms from *Asahinea chrysantha, A. scholanderi* and *Arctoparmelia birulae.*

characteristic of these species is their bright red or orange-red thallus tips. The morphology and chemistry of *C. islandica* is variable and the Far Eastern samples are distinctly different from samples of other regions. Rassadina (1950) established that *C. islandica* var *polaris* is the predominant taxon of this species in the north of the Russian Far East. The principal chemical difference of this variant is the absence of lichesterinic acids which are characteristic of European collections of *C. islandica* (Stepanenko et al., 1996).

The bright-red thallus tips of these lichens are due to an accumulation of polyhydroxynaphthaquinonoid pigments (Fig. 3). Cuculloquinone, 7,7'-
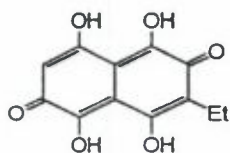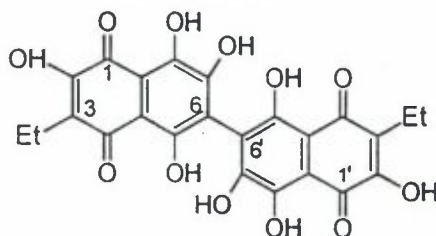
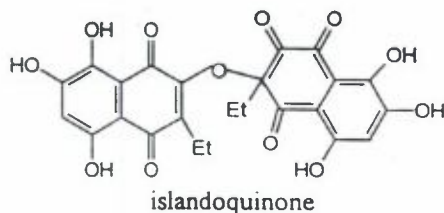1,4,5,8- tetrahydroxy-
3-ethylnaphtha- 2,6-quinone
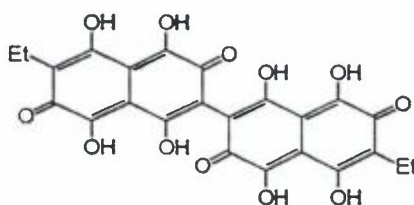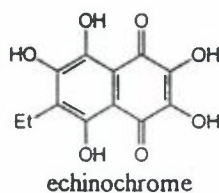
cuculloquinone
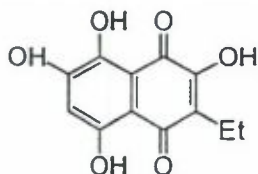
3-ethyl-2,7-dihydroxynaphthazarin

6,6'-bi(3-ethyl-2,7-dihydroxynaphthazarin)

islandoquinone

echinochrome

Figure 3.   Naphthaquinonoid pigments from *Flavocetraria cucullata* and *Cetraria islandica* var *polaris*.

bis(1,4,5,8-tetrahydroxy-3-ethylnaphta-2,6-quinone) and its monomeric form were isolated from the lichen *Flavocetraria cucullata* (Krivoshchekova et al., 1982; Huneck and Yoshimura, 1996). Three other pigments, monomeric 3-ethyl-2,7-dihydroxynaphthazarin and its dimers, islandoquinone and 6,6'-bi(3-ethyl-2,7-dihydroxy-naphthazarin, were isolated from *C. islandica* (Stepanenko et al., 1997). The structures of these pigments (Fig. 3) reveals that they are similar to the naphthazarin pigments found in the spinochromes of sea urchins (Thomson, 1971). The last-mentioned dimeric pigment has also been found in the deep sea holothuroids (Thomson, 1987).

The amount of the naphthazarin pigments in these lichens depends on the moisture content of the substrates. The greatest quantities of these pigments are found under conditions of the stable moderate moisture regimes in places of

prolonged snow cover, adjacent to crusts of ice, or among mosses. In our study, lichen thalli growing among mosses were preferred for isolation of the pigments, since they were the largest, 20–25 cm tall, and their red tips were about 8 cm long (i.e. 1/3 of the thalli). They also had big apothecia. In contrast, thalli from dry places possessed coloured tips which were small (1–2 mm) and nearly black in colour.

The naphthazarin pigments from the large thallus tips had structures analogous to the typical sea urchin pigment echinochrome. The red pigment cuculloquinone was selected for a study of its antioxidant activity and this was compared with that of echinochrome. Boguslavskaya et al. (1985) determined that echinochrome possesses a high level of antioxidant activity. Cuculloquinone proved to have antiradical activity when tested using a standard method involving the decolorization of the diphenylpicrylhydrazyl (DPPH) free radical (λ_{max} 530 nm) (Papariello and Janish, 1966). The coloured DPPH radical takes away hydrogen atoms from unbound hydroxy groups of phenols forming decoloured diphenylpicryl-hydrazin. Cuculloquinone inactivated DPPH to an extent of 80% while echinochrome induced 95% inactivation within the one hour reaction time. By comparison, the standard antioxidant ionol (BHT) only caused a 40% decolorization of DPPH.

Cuculloquinone also showed activity using the standard ferro-ascorbate model with egg phosphatidylcholine (Mishchenko, 1986). The addition of it in equimolar amounts (1:1) in relation to the ferro-ascorbate initiator inhibited oxidation of phosphatidylcholine. A 6–7-fold excess of echinochrome was necessary for inhibition, while ionol did not inhibit even at a 100-fold excess. These results show the substantial complexing properties of cuculloquinone which forms a stable complex with ferrous ions (Mishchenko, 1986). The naphthazarinic pigments also form strong complexes with metals, which are only broken by treatment with hydrochloric acid. Other quinonoid pigments in lichens such as hydroxy derivatives of naphta-, anthra-, phenantrenequinones probably also form complexes with metal ions. This may account for the location of these pigments on the lower surface of the epilithic lichens and on the thallus tips of the epigeous species.

In sea urchins, spinochromes may react with lipid peroxide radicals (Boguslavskaya et al., 1985) and interact with superoxide radicals (Lebedev et al., 1999). The scavenging of superoxide radicals by echinochrome protects the cell lipids and biopolymers of sea urchins from peroxidation or oxidation damage. Cuculloquinone demonstrates high antioxidant activity, and so it probably protects lichen cells in *Flavocetraria cucullata* from peroxidation. The naphthazarinic pigments of *Cetraria islandica* may play a similar role.

The high level of survival of sea urchin embryos following treatment with echinochrome during cryopreservation emphasizes another important role for such compounds (Naidenko and Koltsova, 1998). Echinochrome has a protective

effect on embryos during deep freezing and following thawing. It is logical to assume that naphthazarin lichen pigments may contribute to the adaptation of epigeous lichens to extremely low temperature and permafrost of the Kolyma Uplands.

The phenolic metabolites of the Far Eastern lichens play a part in their adaptation to the rigorous climate, although the lichen substances vary in each lichen species. Melanins may be essential for protection of high-mountain lichens against UV radiation, desiccation and temperature extremes. Usnic acid and atranorin is located in upper cortex of many lichen species and may protect the lichens from high insolation. Quinonoid pigments located on the lower thallus surface protect lichens from oxidation and winter-killing. The quinonoid pigments of lichen apothecia probably have similar protective functions. The orcinol-type of lichen depsides and tridepsides show antiradical activity using the DPPH model, and also complex iron using the ferro-ascorbate model (Mishchenko, 1986). Other orcinol-type depsides and tridepsides may have similar activities. These lichen acids may, in nature, provide protection against peroxidation by blocking toxic metal ions, that initiate of free radical processes. The large quantities of these compounds found in lichens from the Far East indicate that the compounds have a biological importance, especially for high mountain lichens such as *Umbilicaria*.

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