Review

# Current Results on Biological Activities of Lichen Secondary Metabolites: a Review

Katalin Molnár[a,*] and Edit Farkas[b]

[a] Duke University, Department of Biology, Durham, NC 27708-0338, USA.
Fax: +1 91 96 60 72 93. E-mail: kmcz100@gmail.com
[b] Institute of Ecology and Botany, Hungarian Academy of Sciences, H-2163 Vácrátót, Hungary

* Author for correspondence and reprint requests

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Lichens are symbiotic organisms of fungi and algae or cyanobacteria. Lichen-forming fungi synthesize a great variety of secondary metabolites, many of which are unique. Developments in analytical techniques and experimental methods have resulted in the identification of about 1050 lichen substances (including those found in cultures). In addition to their role in lichen chemotaxonomy and systematics, lichen secondary compounds have several possible biological roles, including photoprotection against intense radiation, as well as allelochemical, antiviral, antitumor, antibacterial, antiherbivore, and antioxidant action. These compounds are also important factors in metal homeostasis and pollution tolerance of lichen thalli. Although our knowledge of the contribution of these extracellular products to the success of the lichen symbiosis has increased significantly in the last decades, their biotic and abiotic roles have not been entirely explored.

*Key words:* Lichens, Secondary Compounds

## Introduction

Biochemical research of lichenized fungi went through "exponential" development as it was summarized in a review by Culberson and W. L. Culberson (2001) who forecasted development in various directions. They greatly contributed to the development of this field by establishing new methods of chemical analysis (Culberson, 1972a, 1974; Culberson and Kristinsson, 1970), compiling known compounds and structures (Culberson, 1969a, 1970; Culberson *et al.*, 1977a), and continuing research over decades (from W. L. Culberson, 1955, 1957, 1958; Culberson 1963a, b; W. L. Culberson and Culberson, 1956; Culberson and W. L. Culberson, 1958; to Brodo *et al.*, 2008). They emphasized that, while most of this research concerned the discovery and study of new substances, that knowledge was incomplete, even with the development of analytical methods.

However, substantial changes are expected in this field with the exploration of the biological/ecological role of lichen substances, along with increased use and importance of lichens. Molecular biological research on fungi (Fehrer *et al.*, 2008; Lutzoni *et al.*, 2004; Nelsen and Gargas, 2008, 2009; Nordin *et al.*, 2007; Stenroos *et al.*, 2002; Zhou *et al.*, 2006) and experimental techniques (*e.g.*, culturing: Brunauer *et al.*, 2006, 2007; Culberson and Armaleo, 1992; Hager and Stocker-Wörgötter, 2005; Hamada, 1989; Joneson and Lutzoni, 2009; Stocker-Wörgötter, 2001) are becoming more easily and widely adaptable to lichenology. These techniques have already revolutionized research on the use of lichen substances. This paper focuses on recent studies done since previous reviews (Boustie and Grube, 2005; Lawrey, 1986; Romagni and Dayan, 2002; Rundel, 1978), and shows various new possible applications for currently more than a thousand known lichen substances.

## The Lichens: Lichenized Fungi

A lichen is a stable, ecologically obligate, self-supporting mutualism between an exhabitant fungus (the mycobiont) and one or more inhabitant, extracellulary located unicellular or filamentous photoautotrophic partners (the photobiont: alga or cyanobacterium) (after Hawksworth and Honegger, 1994). Lichen thalli are complex ecosystems rather than organisms (Farrar, 1976;

Lumbsch, 1998). According to recent estimations, lichens comprise about 18 500 species (Boustie and Grube, 2005; Feuerer and Hawksworth, 2007; Kirk *et al.*, 2008). Since 1983, the name of a lichen refers to its mycobiont (Voss *et al.*, 1983). Names of lichens in this paper follow the online database www.indexfungorum.org, the names originally used in the cited papers are in brackets.

The fungal partners are mostly (98%) Ascomycota (Gilbert, 2000; Honegger, 1991) and the others belong to the Basidiomycota and anamorphic fungi. Approximately 21% of all fungi are able to act as a mycobiont (Honegger, 1991), thus lichens form the largest mutualistic group among fungi. Only 40 genera are involved as photosynthetic partners in lichen formation: 25 algae and 15 cyanobacteria (Kirk *et al.*, 2008). The photobionts in approximately 98% of lichens are not known at the species level (Honegger, 2001).

Lichenized fungi occur in a wide range of habitats: from arctic to tropical regions, from the plains to the highest mountains (Müller, 2001), and from aquatic to xeric conditions. Lichens can be found on or within rocks, on soil, on tree trunks and shrubs, on the surface of living leaves, on animal carapaces, and on any stationary, undisturbed man-made surface such as wood, leather, bone, glass, metal, concrete, mortar, brick, rubber, and plastic (Brightman and Seaward, 1977; Seaward, 2008). Lisická (2008) reported 18 lichen species on an acrylic-coated aluminum roof. Most lichens are terrestrial, but a few species occur in freshwater streams and others in marine intertidal zones (Nash, 2008). Lichens are able to survive in extreme environmental conditions; they can adapt to extreme temperatures, drought, inundation, salinity, high concentrations of air pollutants, and nutrient-poor, highly nitrified environments (Nash, 2008), and they are the first colonizers of terrestrial habitats (pioneers). In addition, both fungal and algal cells in the lichen thallus are known for their ability to survive in space (Sancho *et al.*, 2007). Interactions between the symbiotic partners partially explain this spectacular success of lichens in unusual environments (Bačkor and Fahselt, 2008). Nevertheless, many lichens are very sensitive to various air pollutants, especially nitrogen-, sulfur- and heavy metal-based compounds; therefore they are widely used as bioindicators (Fernández-Salegui *et al.*, 2007; Glavich and Geiser, 2008; Gries, 1996; Sheppard *et al.*, 2007 – only a few of many studies).

## The Lichen Substances: Secondary Metabolic Products

Lichens produce a great variety of secondary metabolites, and most of them are unique to lichen-forming fungi. These chemically diverse (aliphatic and aromatic) lichen substances have relatively low molecular weight (Türk *et al.*, 2003). They are produced by the mycobiont (Elix, 1996; Huneck, 1999), and accumulate in the cortex (such as atranorin, parietin, usnic acid, fungal melanins) or in the medullary layer (such as physodic acid, physodalic acid, protocetraric acid) as extracellular tiny crystals on the outer surfaces of the hyphae (Figs. 1, 2). The photobiont might also have an influence on the secondary metabolism of the mycobiont (Brunauer *et al.*, 2007; Yamamoto *et al.*, 1993; Yoshimura *et al.*, 1994).

Approximately 1050 secondary compounds have been identified to date (Stocker-Wörgötter, 2008). This number is much higher than that found in previous literature sources (*e.g.*, Culberson and Elix, 1989; Elix, 1996; Elix and Stocker-Wörgötter, 2008; Galun and Shomer-Ilan, 1988; Huneck, 1999; Huneck and Yoshimura, 1996; Lumbsch, 1998). The large increase is due to the fact that, previously, only "natural" substances oc-

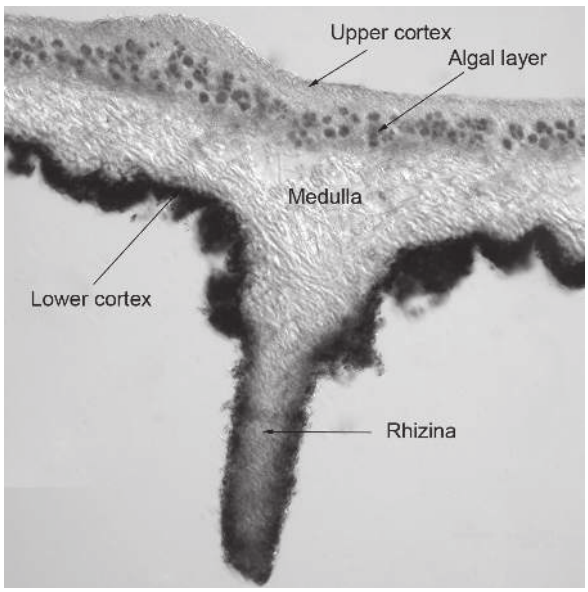


Fig. 1. Cross-section of the stratified foliose thallus of *Umbilicaria mammulata*. (Micrograph by K. Molnár.)

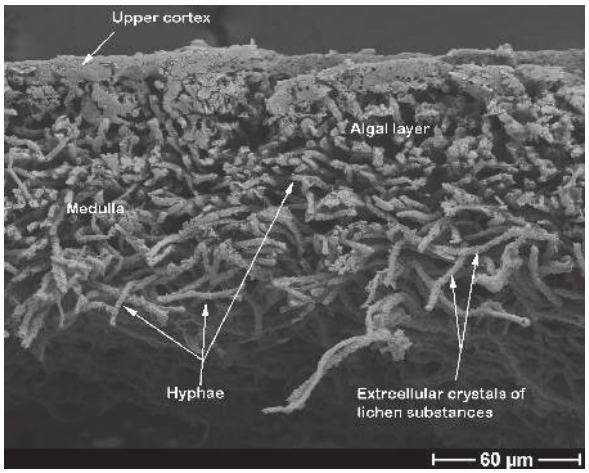


Fig. 2. Cross-section of the foliose thallus of *Hypogymnia physodes*. Hyphae are covered by the extracellular crystals of secondary metabolites. (SEM micrograph by K. Bóka and K. Molnár.)

curring in intact lichen thalli were counted, but now, substances identified from cultures are also being included. Mycobionts grown without their photobionts synthesize specific secondary lichen compounds under certain conditions (Culberson and Armaleo, 1992; Fazio *et al.*, 2007; Hager *et al.*, 2008; Mattsson, 1994; Stocker-Wörgötter and Elix, 2002), but can also produce substances that are different from the metabolites found in symbiosis (Brunauer *et al.*, 2007; Yoshimura *et al.*, 1994). Each lichen mycobiont prefers specially adapted culture conditions (such as nutrient medium, added sugars or polyols, pH, temperature, light, stress) to produce the specific secondary metabolites (Hager *et al.*, 2008). Similarly, lichen "tissue" cultures, in many cases, can produce secondary substances (Yamamoto *et al.*, 1985, 1993), but the chemistry is usually different from the chemosyndrome of the corresponding natural lichen thalli (Yamamoto *et al.*, 1993). Lichenized Basidiomycota do not contain lichen substances (Lumbsch, 1998).

Lichen products are restricted to specific areas of the thallus (Feige and Lumbsch, 1995; Lawrey, 1995; Nybakken and Gauslaa, 2007), which correlate with the different functions of lichen metabolites. These patterns are consistent within certain taxonomic units (Lawrey, 1995). Hyvärinen *et al.* (2000) reported that the concentrations of secondary compounds in the foliose lichens *Hypogymnia physodes*, *Vulpicida pinastri*, and *Xanthoria parietina* are higher in sexual (apothecia of *X. parietina*) and asexual (soredia of *H. physodes* and *V. pinastri*) reproductive structures than in the vegetative parts of the thallus. This pattern is concordant with the optimal defense theory (ODT), which states that the structures most important for fitness should be chemically better defended.

Fluorescence microscopy is used to determine the location of fluorescent substances in lichen thalli (Kauppi and Verseghy-Patay, 1990). Scanning electron microscopy (SEM) and laser microprobe mass spectrometry (LMMS), together with fluorescence microscopy and transmission electron microscopy (TEM), have also been used to locate compounds (Elix, 1996; Elix and Stocker-Wörgötter, 2008). Additionally, FT-Raman spectroscopy is a non-destructive analytical method used to identify lichen substances spatially in the intact lichen thallus (Edwards *et al.*, 2005). Lichens may contain substantial amounts of secondary metabolites, usually between 0.1–10% of the dry weight, but sometimes up to 30% (Galun and Shomer-Ilan, 1988; Solhaug *et al.*, 2009; Stocker-Wörgötter, 2008).

The distribution patterns of secondary metabolites are usually taxon-specific and, therefore, have been widely used in lichen taxonomy and systematics (Carlin, 1987; W. L. Culberson, 1969b; Fehrer *et al.*, 2008; Hawksworth, 1976; Nelsen and Gargas, 2008; Nordin *et al.*, 2007; Nylander, 1866; Piercey-Normore, 2007; Schmitt and Lumbsch, 2004). However, it has been shown that the production of lichen compounds can be homoplasious and, therefore, similarities in secondary chemistry may not necessarily indicate close phylogenetic relationships (Nelsen and Gargas, 2008). The production of secondary compounds is genetically controlled (Culberson and W. L. Culberson, 2001), and in some instances is correlated with morphology and geography in individuals at the species and genus levels (Egan, 1986; Zhou *et al.*, 2006).

Asahina and Shibata (1954) published a classification of about 80 lichen substances based on their chemical structures and biosynthetic pathways. This system was modified from time to time, as more was known about lichen chemistry through improved analytical methods. Lichen substances were reclassified by Culberson and Elix (1989) according to their biosynthetic origins and chemical structural features. Most secondary

lichen metabolites are derived from the acetyl-polymalonyl pathway (including the polyketide pathway), while others originate from the mevalonic acid and shikimic acid pathways.

## The Development of Analytical Methods (and their Application in Lichenology)

Nylander (1866) was the first lichenologist to use chemistry for taxonomical purposes. He detected the presence of various lichen substances by color spot tests. In the early 20th century, Zopf (1907) and Hesse (1912) described numerous lichen compounds, mostly without their structural characterization, as organic chemistry was in its infancy (Shibata, 2000). Asahina developed the microcrystallization technique to identify lichen metabolites (Asahina, 1936–1940). This simple and rapid technique allowed lichenologists to identify the major constituents in hundreds of lichen species, but it was not useful for detecting minor components and analyzing mixtures of lichen substances. In 1952, Wachtmeister introduced paper chromatography for the separation and characterization of lichen substances. Mitsuno (1953) explained the relationship between the chemical structures of lichen compounds and their paper chromatographic Rf values. Since paper chromatography could not always separate individual compounds, Ramaut (1963a, b) began using thin layer chromatography (TLC) with Pastuska's solvent phase for depsides and depsidones. According to Lumbsch (1998), the vast majority of lichen secondary metabolites, especially substances which are unique to lichens, belong to these two groups.

TLC has been used to study specific groups of lichen products (Bendz *et al.*, 1965, 1966, 1967; Santesson, 1965, 1967a, b). Different authors used different solvent systems and chromatographic conditions, making it impossible to compare their results. This problem was solved when a standardized method was developed by Chicita F. Culberson and Hör-Dur Kristinsson in 1970. They introduced Rf classes, which depend only on the relative order of spots, and which are more reliably constant. This standardized method has been used for routine analyses of lichen products in chemotaxonomic and phytochemical studies, with various updates over time (Culberson, 1972b, 1974; Culberson and Johnson, 1976, 1982; Culberson *et al.*, 1981). Later the use of high-performance thin layer chromatography (HPTLC) in screening lichen substances was developed (Arup *et al.*, 1993). HPTLC is more sensitive, allows the running of more samples in a shorter period of time, and requires smaller amounts of solvent. Because of its simplicity, this technique has become the most widely used microchemical method for identifying lichen substances (Fig. 3).

The first use of high-performance liquid chromatography (HPLC) on crude lichen extracts was tried by Culberson (1972a), because most of the secondary natural products of lichens have low volatility and low thermal stability, and thus gas chromatography is not able to analyze them. She used normal-phase silica columns and isocratic elution with mobile phases of mixtures of hexane, isopropyl alcohol and acetic acid. Reverse-phase HPLC was first used for the separation of orcinol and $\beta$-orcinol depsides and depsidones on a C18 column and with a water/methanol/acetic acid mobile phase (Culberson and W. L. Culberson, 1978a; W. L. Culberson and Culberson, 1978b).

Although these isocratic methods yielded excellent results for the separation and identifica-

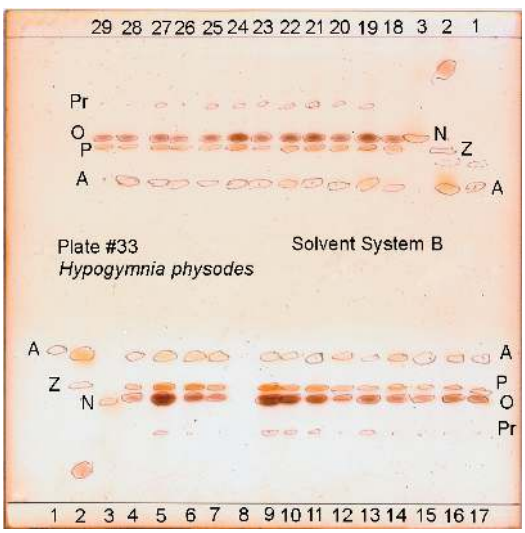


Fig. 3. Lichen substances on an HPTLC plate developed in solvent system B (cyclohexane/methyl *tert*-butyl ether/formic acid, 6.5:5:1) after being treated with sulfuric acid (according to Arup *et al.*, 1993). A, atranorin (control); Z, zeorin (control); N, norstictic acid (control); P, physodic acid; O, oxyphysodic (= 3-hydroxyphysodic) and physodalic acids; Pr, protocetraric acid. (Scanned image.)

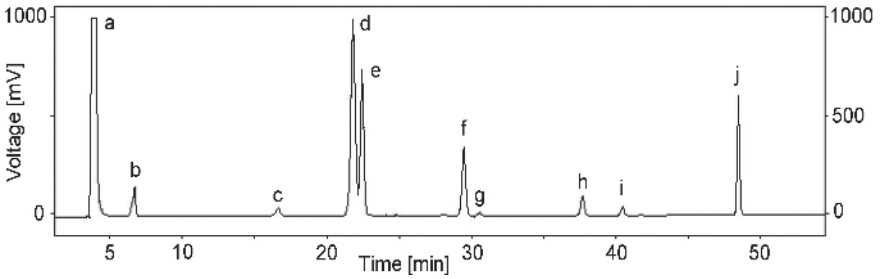


Fig. 4. HPLC chromatogram of the acetone extract of *Hypogymnia physodes* (collected on the mountain Látóhegy, Budapest, Hungary, collection no. 208/a) at 245 nm. Peaks: a, acetone; b, benzoic acid (internal standard); c, protocetraric acid; d, 3-hydroxyphysodic acid; e, physodalic acid; f, 2'-*O*-methylphysodic acid; g, physodic acid; h, atranorin; i, chloroatranorin; j, bis-(2-ethylhexyl)-phthalate (internal standard).

tion of lichen substances, gradient elution is more effective for HPLC analysis of crude lichen extracts, which frequently contain compounds of wide-ranging hydrophobicities (Culberson and Elix, 1989). Gradient elution was introduced in lichenology by Strack *et al.* (1979), who separated 13 phenolic lichen products, including examples of depsides, depsidones, dibenzofurans and pulvinic acid derivatives, using an RP-8 column with a 70-min linear gradient from water containing 2% acetic acid (solvent A) to 100% methanol (solvent B). Huovinen (1987) developed a standard HPLC method for the identification and accurate quantification of aromatic lichen compounds on three different reverse-phase columns (RP-8, RP-18 and RP-phenyl) using gradient elution with methanol and orthophosphoric acid, as well as two internal standards: benzoic acid (low retention time) and bis-(2-ethyl-hexyl)-phthalate (high retention time) (Fig. 4). Retention indices (R.I.) in relation to the internal standards were defined, which are more consistent markers than retention times. Later the standard method was improved by Feige *et al.* (1993), using benzoic acid and solorinic acid [more hydrophobic compound than bis-(2-ethyl-hexyl)-phthalate] as internal standards, making the method suitable for the identification of lichen extracts containing chloroxanthones or long-chain depsides as well.

The use of $^{1}H$ and $^{13}C$ NMR spectroscopy, mass spectrometry and X-ray crystal analysis in structural elucidation have also increased the number of known lichen metabolites (Culberson and Elix, 1989).

## The Significance of Lichen Substances

Secondary metabolites are not absolutely essential for the survival and growth of lichens (Bentley, 1999), nevertheless, their study has revealed many possible advantages. We know more about these substances through experimental studies, but the functions of these compounds in the lichen symbioses are still poorly understood (Hager *et al.*, 2008). They may impact biotic and abiotic interactions of lichens with their environment. They may help to protect the thalli against herbivores, pathogens, competitors and external abiotic factors, such as high UV irradiation. Many of them exhibit multiple biological activities, such as the dibenzofuran usnic acid (*e.g.*, antimicrobial and larvicidal effects, anticancer activities, known also for its UV absorption) (Fig. 5). When we analyze the biological activities of lichen substances, we must consider and observe their role in natural processes, but also study their role in special circumstances seldom occurring in nature, *e.g.*, in experimental situations and with their use as medicines in humans or animals.

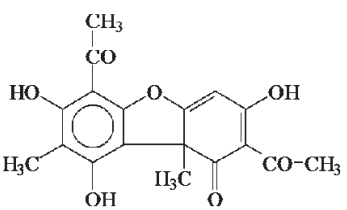


Fig. 5. Chemical structure of usnic acid.

Structurally closely related metabolites often have essentially different biological actions. Hager *et al.* (2008) reported that barbatic and diffractaic acids, which differ in only one functional unit, have diverging biological effects. Barbatic acid (extracted from a metabolite-forming *Heterodea muelleri* mycobiont culture) strongly inhibits the growth of *Trebouxia jamesii* (the photobiont in *H. muelleri*) and slows down the mitosis rate of the alga at a concentration comparable to the quantity found in the lichen thallus (in nature). It can cause cell death in higher concentrations. At the same time, diffractaic acid (from a mycobiont culture, as before) has no effect on algal growth at all. On the basis of this result, barbatic acid may regulate algal growth and mitosis in the lichen thalli.

**Antioxidant Activity**

Free radicals (reactive oxygen species, such as the hydroxyl radical, superoxide anion, and hydrogen peroxide, and reactive nitrogen species, such as nitric oxide) play an important role in many chemical processes in the cells, but they are also associated with unwanted side effects, causing cell damage. They attack proteins and nucleic acids, as well as unsaturated fatty acids in cell membranes. Food deterioration, aging processes and several human chronic diseases, such as Alzheimer's disease, atherosclerosis, emphysema, hemochromatosis, many forms of cancer (for example, melanoma), Parkinson's disease, and schizophrenia, may be related to free radicals. Oxidative stress occurs also in lichen thalli, and secondary compounds afford protection against free radicals generated by UV light (Marante *et al.*, 2003).

The damaging effects of free radicals can be ameliorated by free radical scavengers and chain reaction terminators – enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, as well as antioxidants such as glutathione, polyphenols (lignins, flavonoids), carotenoids, melanins, and vitamins E and C.

Since synthetic antioxidants are often carcinogenic, finding natural substitutes is of great interest. Lichens have been found to contain a variety of secondary lichen substances with strong antioxidant activity. These are substances which have high ability to scavenge toxic free radicals due their phenolic groups. Hidalgo *et al.* (1994) reported the antioxidant activity of some depsides, such as atranorin (isolated from *Placopsis* sp.) and divaricatic acid (isolated from *Protousnea malacea*), and depsidones, such as pannarin (isolated from *Psoroma pallidum*) and 1'-chloropannarin (isolated from *Erioderma chilense*). All of these secondary compounds inhibited rat brain homogenate auto-oxidation and $\beta$-carotene oxidation, and depsidones were found to be the most effective. Russo *et al.* (2008) found that both sphaerophorin (depside) and pannarin (depsidone) inhibited superoxide anion formation *in vitro*, pannarin being more efficient, confirming Hidalgo *et al.* (1994). A methanol extract of *Lobaria pulmonaria* reduced the oxidative stress induced by indomethacin in the stomachs of rats, increasing the levels of superoxide dismutase and glutathione peroxidase (Karakus *et al.*, 2009). Similarly, usnic acid was shown to be a gastroprotective compound, since it reduced oxidative damage and inhibited neutrophil infiltration in indomethacin-induced gastric ulcers in rats (Odabasoglu *et al.*, 2006). Methanol extracts of *Dolichousnea longissima* (as *Usnea longissima*) and *Lobaria pulmonaria* have been shown to have significant antioxidant effects *in vitro* (Odabasoglu *et al.*, 2004). According to Luo *et al.* (2009), the extreme conditions in Antarctica (such as low temperature, drought, winter darkness, high UV-B and solar irradiation) increase oxidative stress, consequently, antarctic lichens contain larger amounts of antioxidant substances and have higher antioxidant activity than tropical or temperate lichens. An acetone extract of *Umbilicaria antarctica* was found to be the most effective antioxidant in free radical and superoxide anion scavenging, as well as in reducing power assays among tested lichen species collected from King George Island, Antarctica. Lecanoric acid was identified as the main active compound. Methanol-water extracts of five lichens (*Caloplaca regalis*, *Caloplaca* sp., *Lecanora* sp., *Ramalina terebrata*, *Stereocaulon alpinum*) from Antarctica were screened for their antioxidant effects by Bhattarai *et al.* (2008), who found varying antioxidant success against the stable free radical diphenylpicrylhydrazyl (DPPH) on a TLC plate.

All of these studies show that lichens and lichen substances might be novel sources of natural antioxidants.

## Effect on Metal Homeostasis and Pollution Tolerance

Lichen secondary metabolites are sensitive to heavy metal accumulation and might play a general role in metal homeostasis and pollution tolerance. Their sensitivity to heavy metals is species-specific.

Remarkable changes in the levels of secondary compounds were found in *Hypogymnia physodes* thalli transplanted to areas polluted with heavy metals and acidic inorganic sulfur compounds (Białonska and Dayan, 2005). For example, the levels of atranorin, physodic acid and hydroxyphysodic acid were significantly decreased in thalli transplanted to the vicinity of a chemical plant producing chromium, phosphorous and sulfur compounds. In contrast, the level of physodalic acid was significantly increased, suggesting that this compound might be effective against pollution stress. The present authors have found similar results with the analyses of thalli growing naturally under various environmental conditions and pollution levels (Molnár and Farkas, manuscript under preparation). Hauck and Huneck (2007a) demonstrated the ion-specific increase or decrease of heavy metal adsorption at cation exchange sites (hydroxy groups) on cellulose filters coated with four lichen substances produced by *Hypogymnia physodes* (atranorin, physodic acid, physodalic acid and protocetraric acid). They used this model system to imitate lichen cell walls, which contain many hydroxy and carboxy groups as binding sites for metal cations. The alkali metal ion $Na^+$, the alkaline earth metal ions $Ca^{2+}$ and $Mg^{2+}$, and the transition metal ions $Cu^{2+}$, $Fe^{2+}$, $Fe^{3+}$ and $Mn^{2+}$ were studied. Lichen compounds significantly inhibited the adsorption of $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$ and $Mn^{2+}$, whereas they increased the adsorption of $Fe^{3+}$. The level of $Fe^{2+}$ was not affected. The depsidone physodalic acid was found to be the most effective.

Hauck and Huneck (2007b) also used cellulose filter strips to simulate cell wall surfaces. The depsidone fumarprotocetraric acid, the main lichen compound in *Lecanora conizaeoides*, has been shown to reduce $Mn^{2+}$ adsorption at cation exchange sites *in vitro*. This capability of fumarprotocetraric acid may be a key factor in the high Mn tolerance of this lichen species.

Similar results have been found by Hauck (2008) using lichen thalli instead of an artificial system. The intracellular uptake of $Cu^{2+}$ and $Mn^{2+}$ was significantly lower in intact *Hypogymnia physodes* thalli containing a set of seven lichen metabolites compared to lichens treated with acetone. The intracellular uptake of $Fe^{2+}$ and $Zn^{2+}$ was not affected by the lichen substances. These impacts are consistent with the ecology of *Hypogymnia physodes*, *i.e.*, $Cu^{2+}$ and $Mn^{2+}$ might be toxic in ambient concentrations on acidic bark (the preferred substrate of *H. physodes*), but $Fe^{2+}$ and $Zn^{2+}$ have never been found to limit the survival of this species.

The dibenzofuran usnic acid and the depside divaricatic acid were both found to significantly increase the intracellular uptake of $Cu^{2+}$ in *Evernia mesomorpha* and in *Ramalina menziesii* (usnic acid only) originating from nutrient-poor habitats (Hauck *et al.*, 2009). At the same time, the intracellular uptake of $Mn^{2+}$ was reduced. Since $Cu^{2+}$ is one of the rarest micronutrients in acidic tree bark and $Mn^{2+}$ often reaches toxic concentration, the influence of the compounds facilitates the survival of the two lichen species.

These results show that lichen metabolites control metal homeostasis in lichens by promoting the uptake of certain metal cations, reducing the adsorption of others, thereby enhancing the tolerance of lichens to heavy metals in polluted areas.

## Photoprotection

Lichens use a number of strategies to protect the light-sensitive algal symbionts against high levels of light and the damaging effects of UV radiation, mainly the xanthophyll cycle in the algal thylakoid membranes, as well as light screening and UV-B protection by lichen compounds.

The light-screening theory was formulated by Ertl (1951), who found that cortical lichen compounds increase the opacity of the upper cortex, and thus decrease high incident irradiance reaching the algal layer.

Light-screening pigments (such as parietin, usnic acid, vulpinic acid) regulate the solar irradiance reaching the algal layer (Galloway, 1993; Rao and LeBlanc, 1965; Rundel, 1978; Solhaug and Gauslaa, 1996) by absorbing much of the incident light and thus protecting the photosynthetic partner from intense radiation (Rao and LeBlanc, 1965).

UV-B light inhibits photosynthesis and damages DNA. Several lichen secondary metabolites

(including atranorin, calycin, pinastric acid, pulvinic acid, rhizocarpic acid, usnic acid, vulpinic acid) have strong UV absorption abilities and might function as filters for excessive UV-B irradiation (Galloway, 1993; Rundel, 1978; Solhaug and Gauslaa, 1996). UV-B light might be essential for the synthesis of UV-B absorbing pigments (Nybakken and Julkunen-Tiitto, 2006; Nybakken *et al.*, 2004). Rao and LeBlanc reported (1965) that the fluorescence spectrum of the cortical depside atranorin coincides with the absorption spectrum of algal chlorophyll; therefore, the light emitted by atranorin can be used in photosynthesis.

## Allelopathy

Lichen secondary metabolites can function as allelopathic agents (called allelochemicals), *i.e.*, they may affect the development and growth of neighboring lichens, mosses and vascular plants, as well as microorganisms (Kershaw, 1985; Lawrey, 1986, 1995; Macías *et al.*, 2007; Romagni *et al.*, 2004; Rundel, 1978). Allelopathic compounds are released into the environment and might influence other organisms' photosynthesis, respiration, transpiration, protein and nucleic acid synthesis, ion membrane transport, and permeability (Chou, 2006; Macías *et al.*, 2007).

Culberson *et al.* (1977b) reported that *Lepraria* sp. had a non-random distribution on two morphologically similar but chemically very different *Xanthoparmelia* species, which were growing together. The lichenicolous *Lepraria* sp. occurred commonly on 73% of the thalli of *Xanthoparmelia verruculifera* (as *Parmelia verruculifera*) examined. In contrast, only 13% of *Xanthoparmelia loxodes* (as *Parmelia loxodes*) specimens served as a host for the same species of *Lepraria*. The lichen substances presumably had allelopathic effects on *Lepraria*, and the secondary metabolites of *X. loxodes* were more detrimental to the growth of *Lepraria*. Whiton and Lawrey (1984) found that vulpinic and evernic acids severely inhibited ascospore germination of the crustose lichens *Graphis scripta* and *Caloplaca citrina*. Atranorin had an inhibitory effect only on *C. citrina*, completely eliminating its spore germination. Neither species was affected by stictic acid. Spore germination of *Cladonia cristatella* was also inhibited by vulpinic acid, but not by evernic and stictic acids (Whiton and Lawrey, 1982).

Competition occurs between lichen thalli for space and light on a variety of substrates, and plays important roles in determining the structure of lichen communities and the distribution of individual species (Armstrong and Welch, 2007). Lichen secondary chemistry might play a role in this competition (Armstrong and Welch, 2007).

Populations of mosses and lichens frequently occur together on rocks, soil, and trees, and they compete for light, substrate, nutrients, and water (Lawrey, 1977). Lichen substances also have inhibitory effects against other cryptogams in overlapping niches, such as mosses, and might significantly influence the competitive interactions in cryptogam communities. In the Great Smoky Mountains of the eastern United States, Heilman and Sharp (1963) observed that the lichen *Thelotrema petractoides* (as *Ocellularia subtilis*) was inhibiting and overgrowing a colony of *Frullania eboracensis* on the bark of *Aesculus octandra*. Similarly, the saxicolous lichen *Lecidea albocaerulescens* inhibited a community of bryophytes on greywacke boulders (including *Anomodon attenuatus*, *Hedwigia ciliata*, *Porella platyphylla*, and *Sematophyllum* sp.). The 4-*O*-methylated depsides evernic and squamatic acids retarded spore germination and protonemal growth of three common moss species occurring with the lichens: *Ceratodon purpureus*, *Funaria hygrometrica* and *Mnium cuspidatum* (Lawrey, 1977).

Lichens have also long been known to inhibit or greatly retard the growth of higher plants (Pyatt, 1967). *Cladonia stellaris* (as *C. alpestris*) and *C. rangiferina*, two common species in boreal forests, have been shown to have allelopathic effects on jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) (Fisher, 1979). Lichen mulch containing both species significantly reduced the growth as well as N and P concentrations of both seedlings and transplants of these coniferous trees. Compared to control plants, the roots of the seedlings treated with lichen mulch were longer, but less massive, and have significantly less mycorrhizae. Marante *et al.* (2003) reported that twelve lichen substances identified in "Letharal," the phenolic fraction of *Lethariella canariensis*, showed allelopathic activity against the seeds of common garden plants, and inhibited the germination process of cabbage, lettuce, pepper, and tomato. It was also demonstrated that rainwater carries the lichen compounds into the soil by lixiviation.

Lichen substances were found to inhibit mycorrhizal fungi and their plant hosts (Fisher, 1979; Lawrey, 1995; Rundel, 1978). Henningsson and Lundström (1970) stated that the epiphytic lichen *Hypogymnia physodes* had a fungistatic effect on various wood-decaying fungi, and in this way lichens can protect their substrates from decay.

## Antimicrobial Activity

Lichens produce antibiotic secondary metabolites that provide defense against most of the pathogens in nature. Several examples (from the species indicated) are described below.

Atranorin (from *Physcia aipolia*), fumarprotocetraric acid (from *Cladonia furcata*), gyrophoric acid (from *Umbilicaria polyphylla*), lecanoric acid (from *Ochrolechia androgyna*), physodic acid (from *Hypogymnia physodes*), protocetraric acid [from *Flavoparmelia caperata* (as *Parmelia caperata*)], stictic acid [from *Xanthoparmelia conspersa* (as *Parmelia conspersa*)] and usnic acid (from *Flavoparmelia caperata*) showed relatively strong antimicrobial effects against six bacteria and ten fungi, among which were human, animal and plant pathogens, mycotoxin producers and food-spoilage organisms (Ranković and Mišić, 2008; Ranković *et al.*, 2008). Usnic acid was found to be the strongest antimicrobial agent (comparable to streptomycin), and physodic and stictic acids the weakest.

According to Schmeda-Hirschmann *et al.* (2008), dichloromethane and methanol extracts of *Protousnea poeppigii* had strong antifungal effects against the fungal pathogens *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum*. The extracts were also active against the yeasts *Candida albicans*, *C. tropicalis*, *Saccharomyces cerevisiae* and the filamentous fungi *Aspergillus niger*, *A. flavus* and *A. fumigatus*, but with much higher strength. Isodivaricatic acid, divaricatinic acid and usnic acid, the main lichen metabolites in *Protousnea poeppigii*, also displayed antifungal action against *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum*, usnic acid being less active. In the same assay, extracts of *Usnea florida* also showed strong antifungal properties.

Methanol extracts of five lichens from Antarctica (*Caloplaca regalis*, *Caloplaca* sp., *Lecanora* sp., *Ramalina terebrata*, *Stereocaulon alpinum*) exhibited target-specific antibacterial activity, especially strong against Gram-positive bacteria, compared to previously described lichen compounds (Paudel *et al.*, 2008).

Whiton and Lawrey (1982) reported that ascospore germination of *Sordaria fimicola* was significantly inhibited by evernic and vulpinic acids.

Aqueous, ethanol and ethyl acetate extracts of *Alectoria sarmentosa* and *Cladonia rangiferina* were found to have moderate antifungal action against different species of fungi, including human pathogens (Ranković and Mišić, 2007), ethanol extracts showing the highest activity.

Halama and Van Haluwin (2004) reported that acetone extracts of *Evernia prunastri* and *Hypogymnia physodes* showed a strong inhibitory effect on the growth of some plant pathogenic fungi, *i.e.*, *Phytophthora infestans*, *Pythium ultimum*, and *Ustilago maydis*.

Since microorganisms have developed resistance to many antibiotics, pharmacologists need to pursue new sources for antimicrobial agents. All these results suggest that lichens and their metabolites yield significant new bioactive substances for the treatment of various diseases caused by microorganisms.

Lichen compounds can provide protection against lichenicolous fungi, but some of these fungi are tolerant of the lichen metabolites. Lawrey (2000) showed that *Fusarium* sp., a lichen inhabitant, enzymatically degrades lecanoric acid in *Punctelia subflava* (as *Punctelia rudecta*), thus permiting *Nectriopsis parmeliae* (as *Nectria parmeliae*), an obligate lichenicolous fungus, to colonize the lichen thallus.

## Antiherbivore and Insecticidal Activity

Lichens are grazed by herbivores, *e.g.*, insects, mites, snails, slugs, lepidopteran larvae, caribou, and reindeer. However, herbivory on lichens seems to be rare, presumably due to their low nutritional quality, specific structural features (for example, the gelatinous sheath in Collemataceae, thick cortex), and the production of defense compounds (Lawrey, 1986; Rundel, 1978). Zukal (1895) first proposed that secondary compounds might protect lichens from herbivory, and this idea was later supported by strong experimental evidence (*e.g.*, Asplund and Gauslaa, 2007, 2008; Gauslaa, 2005; Nimis and Skert, 2006; Pöykkö *et al.*, 2005). Lichen secondary compounds also play an important role in the food preference of her-

bivores (Baur *et al.*, 1994; Pöykkö and Hyvärinen, 2003; Reutimann and Scheidegger, 1987).

Both enantiomers of usnic acid, a widespread cortical dibenzofuran, exhibited strong larvicidal activity against the third and fourth instar larvae of the house mosquito (*Culex pipiens*), and larval mortality was dose-dependent (Cetin *et al.*, 2008). Antifeedant activity and acute toxicity (injected into the larval haemolymph) of (–)- and (+)-usnic acids and vulpinic acid against the polyphagous larvae of the herbivorous insect *Spodoptera littoralis* have also been reported (Emmerich *et al.*, 1993). All three lichen compounds caused severe growth retardation at concentrations comparable or even below those present in lichens, as well as increased the larval period (delayed the pupation) in a dose-dependent manner.

It is known that natural plant-derived products have a less detrimental impact on the environment than synthetic chemicals, and thus lichen substances could be good candidates for new pesticides (Cetin *et al.*, 2008; Dayan and Romagni, 2001; Fahselt, 1994; Romagni and Dayan, 2002).

Harmful effects of lichen substances on vertebrate herbivores have also been reported. Poisoning and subsequent death of an estimated 400–500 elk (*Cervus canadensis*) was reported in Wyoming during the winter of 2004 (Cook *et al.*, 2007; Dailey *et al.*, 2008), putatively due to ingestion of the lichen *Xanthoparmelia chlorochroa*. This lichen was found in the area and in the rumen of elks as well (Cook *et al.*, 2007). Clinical signs were red urine, ataxia, and muscular weakness, which rapidly progressed to recumbency and myodegradation. To identify the toxin, ewes were dosed with (+)-usnic acid extracted from *X. chlorochroa*. It was shown that high doses caused selective skeletal muscle damage in these animals. Since the toxic dose was very high, other lichen substance(s), in addition to (+)-usnic acid, may have interacted to cause the poisoning in elks. This sort of poisoning takes place periodically in western North America, when elks have to leave their regular winter habitats and move to lower elevations due to harsh weather conditions (Elix and Stocker-Wörgötter, 2008).

## Effects on Human Organisms

### *Cytotoxic, antitumor, and antiviral activity*

Many lichen secondary metabolites exhibit cytotoxic and antiviral properties and could be potential sources of pharmaceutically useful chemicals.

The cytotoxic activity of eight lichens [*Cladonia convoluta*, *C. rangiformis*, *Evernia prunastri*, *Flavoparmelia caperata* (as *Parmelia caperata*), *Parmotrema perlatum* (as *Parmelia perlata*), *Platismatia glauca*, *Ramalina cuspidata*, *Usnea rubicunda*] on two murine and four human cancer cell lines was reported by Bézivin *et al.* (2003). The lichens were extracted with three solvents (*n*-hexane, diethyl ether, and methanol). Only three of the 24 extracts were not cytotoxic against any of the tested cell lines (diethyl ether extracts of *E. prunastri* and *P. glauca*, and methanolic extract of *U. rubicunda*). The *n*-hexane extracts were usually the most active and methanolic fractions were generally less selective. *C. convoluta* (diethyl ether fraction), *C. rangiformis* (diethyl ether fraction), and *F. caperata* (*n*-hexane fraction) were the most active species. Diethyl ether and methanolic extracts of *C. convoluta* and *C. rangiformis* showed the highest selectivity on various cell lines.

(+)-Usnic acid was found to be a strong hepatotoxic agent against monogastric murine hepatocytes, due to its ability to uncouple and inhibit the electron transport chain in mitochondria and induce oxidative stress in cells (Han *et al.*, 2004). The (–)-enantiomer of usnic acid (isolated from *Cladonia convoluta*) induced apoptotic cell death in murine lymphocytic leukemia cells and was moderately cytotoxic to various cancer cell lines, such as murine Lewis lung carcinoma, human chronic myelogenous leukemia, human brain metastasis of a prostate carcinoma, human breast adenocarcinoma and human glioblastoma (Bézivin *et al.*, 2004). Usnic acid also decreased proliferation of human breast cancer cells and human lung cancer cells without any DNA damage (Mayer *et al.*, 2005). Finding cancer therapies that do not have DNA-damaging effects and that do not cause the development of secondary malignancies later in life, is of great interest. Accordingly, usnic acid may represent a novel source for a natural non-genotoxic anticancer drug (chemotherapeutic agent).

Russo *et al.* (2008) reported that the depside sphaerophorin (isolated from *Sphaerophorus globosus*) and the depsidone pannarin [isolated from *Psoroma pholidotoides* (as *Psoroma reticulatum*), *P. pulchrum*, and *P. pallidum*] inhibited the growth of M14 human melanoma cells, triggering apoptotic cell death. The anticancer activities of these

lichen metabolites are promising in the treatment of this aggressive, therapy-resistant skin tumor.

An ethyl acetate-soluble fraction (ET4) of the crude methanolic extract of *Ramalina farinacea* was found to be a broad-spectrum antiviral agent against RNA (respiratory syncytal virus and HIV-1) and DNA (adenovirus and herpes simplex virus type 1) viruses (Esimone *et al.*, 2009). Anti-HIV effects of ET4 target both entry and post-entry stages in the viral replication cycle.

Usnic acid (isolated from the aposymbiotic mycobionts of *Ramalina celastri*) exhibited specific antiviral activity against the Junin virus (Arenaviridae), which is the agent of Argentine hemorrhagic fever in humans, as well as against Tacaribe virus, a non-pathogenic arenavirus (Fazio *et al.*, 2007). Parietin (isolated from the aposymbiotic mycobionts of *Teloschistes chrysophthalmus*) showed virucidal effects against the same viruses.

*Allergy to lichen substances*

Lichens and lichen substances can be contact allergens in people who are susceptible. They can cause occupational allergic contact dermatitis in forestry and horticultural workers ("woodcutter's eczema"), and in lichen harvesters, as well as cause non-occupational allergic dermatitis during all kinds of outdoor activities, such as cutting and handling firewood, picking berries, hunting, and using cosmetics (perfumes, after-shave lotions, deodorants, and sunscreen products) that contain lichen metabolites (Aalto-Korte *et al.*, 2005); see data for 11 lichen substances that cause allergic reactions (Table I).

Contact dermatitis seems to be immunologically specific, inasmuch as the person is sensitive to only a single lichen compound or to a group of structurally similar compounds (Mitchell and Champion, 1965). Various skin and respiratory symptoms have been observed, such as erythema, itching, scaling, contact urticaria, rhinitis, and asthma (Aalto-Korte *et al.*, 2005; Mitchell and Champion, 1965). Several lichen compounds (such as atranorin and stictic acid) are able to photosensitize human skin causing photocontact dermatitis, where the exposure to sunlight leads to an aggravation of symptoms (Elix and Stocker-Wörgötter, 2008; Thune and Solberg, 1980).

*Candidates for antipyretic and analgesic drugs*

Some lichen substances have been shown to relieve pain effectively or reduce fever and inflammation in various mammals, and it is reasonable to assume that these compounds also could be effective in humans. Vijayakumar *et al.* (2000) reported that (+)-usnic acid, isolated from *Roccella montagnei*, showed significant, dose-dependent anti-inflammatory activity in rats, reducing carrageenin-induced paw edema. Diffractaic and usnic acids have an analgesic effect in mice *in vitro* (Okuyama *et al.*, 1995), and usnic acid also is an antipyretic against lipopolysaccharide-induced fever.

Table I. Literature sources mentioning data for 11 lichen substances responsible for allergic reactions to lichens.

| Lichen substance | Reference |
|---|---|
| Atranorin | Dahlquist and Fregert, 1980; Thune and Solberg, 1980; Gonçalo *et al.*, 1988; Hausen *et al.*, 1993; Stinchi *et al.*, 1997; Aalto-Korte *et al.*, 2005; Cabanillas *et al.*, 2006 |
| Diffractaic acid | Thune and Solberg, 1980 |
| Evernic acid | Dahlquist and Fregert, 1980; Thune and Solberg, 1980; Gonçalo *et al.*, 1988; Hausen *et al.*, 1993; Aalto-Korte *et al.*, 2005; Cabanillas *et al.*, 2006 |
| Fumarprotocetraric acid | Dahlquist and Fregert, 1980; Thune and Solberg, 1980; Gonçalo *et al.*, 1988; Hausen *et al.*, 1993 |
| Lobaric acid | Thune and Solberg, 1980 |
| Perlatolic acid | Hausen *et al.*, 1993 |
| Physodalic acid | Thune, 1977; Thune and Solberg, 1980 |
| Physodic acid | Thune, 1977; Thune and Solberg, 1980 |
| Salazinic acid | Thune and Solberg, 1980 |
| Stictic acid | Thune and Solberg, 1980; Hausen *et al.*, 1993 |
| Usnic acid | Mitchell and Champion, 1965; Thune and Solberg, 1980; Gonçalo *et al.*, 1988; Hausen *et al.*, 1993; Stinchi *et al.*, 1997; Aalto-Korte *et al.*, 2005; Cabanillas *et al.*, 2006 |

## Conclusions

More than 1000 secondary products have been identified to date in lichens, and new compounds will certainly be found from poorly studied or newly discovered lichens, especially from the under-collected tropics. Here we have shown that lichen secondary substances exhibit a huge array of remarkable biological activities, and many of them have important ecological roles. Some of the activities already mentioned (*e.g.*, photoprotection, reaction to pollution) should be thoroughly studied. Furthermore, the properties of lichen substances make them possible pharmaceuticals. At the same time, we have to be aware that lichens are slow-growing ecosystems, and exploitation of their secondary products could threaten their survival. However, improved culture methods and varied growing conditions can positively influence secondary metabolite production in aposymbiotically grown mycobionts (Stocker-Wörgötter, 2008) and in cultured lichens (Behera *et al.*, 2009), without having to harvest and put at risk the extinction of natural communities.

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