

Lichens as treasure chests of natural products

By Lucia Muggia, Imke Schmitt and Martin Grube

Lichens are self-supporting symbiotic associations formed by a fungus and one or several algal or cyanobacterial components as primary partners. Since the fungal constituent is unique in that symbiosis and usually dominates the association, lichens traditionally have been considered a type of fungus. The lichenous lifestyle is maintained by one-fifth of all fungi, including more than 40% of ascomycetes (ca. 13,500 species) (Hawksworth and Hill 1984). Lichens have diversified extensively during the past 600 million years (Yuan et al. 2005), and occur over >10% of the terrestrial surface.

Lichens were originally interpreted as single organisms and treated as their own systematic category, Lichenes, separate from fungi, mosses, and algae. During the latter half of the 19th century the Swiss botanist Simon Schwendener (1829-1919) recognized that lichens are “double-organisms” composed of algal (photobionts) and a fungal (mycobiont) partner (Schwendener 1869, also see Honegger 2000). Schwendener’s hypothesis provoked con-

siderable controversy and led to resynthesis of lichens from individually cultured partners (Stahl 1877). Culture methods have improved greatly since the 1870s, for the separated biotic partners as well as the resynthesized symbiotic associations (Stocker-Wörgötter 2002, Yoshimura et al. 2002).

Many lichen symbioses tolerate extreme environmental conditions unfavorable to the survival of the individual partners. Even under hostile circumstances, lichens can potentially achieve an age of several thousand years (Denton and Karlén 1973). In contrast to many fungi that are embedded within their substrates, most lichens expose their vegetative parts at the substrate surface, enabling the photobiont to harvest energy from solar radiation. The compact light-exposed vegetative bodies of lichens, called thalli, are among the most complex and aesthetically pleasing morphologies to have been evolved by fungi (Fig. 1).

The distinctive colors of many lichens result from the massive accumula-

tion of diverse secondary compounds, the so-called “lichen substances”, which can comprise up to 20% of a lichen’s dry weight. These compounds are predominantly small, structurally complex molecules (Huneck 2001, Huneck and Yoshimura 1996). Crystalline pigments often are deposited either extracellularly (extrolites) or in the upper surface layers of the lichen’s vegetative body, whereas colorless substances tend to accumulate in parts of the thallus not exposed to direct sunlight. Concomitant production of biogenetically related and unrelated compounds in different strata of the thallus (Fig. 2) is common, and congeners often are present within a morphological stratum of the same lichen or among varieties of the same species (chemosyndromatic variation). Structures for more than 1000 different lichen substances have been reported to date.

Experiments with algal-free mycobionts have demonstrated that lichen secondary metabolites are formed mainly by the fungal component (Ahmadjian and

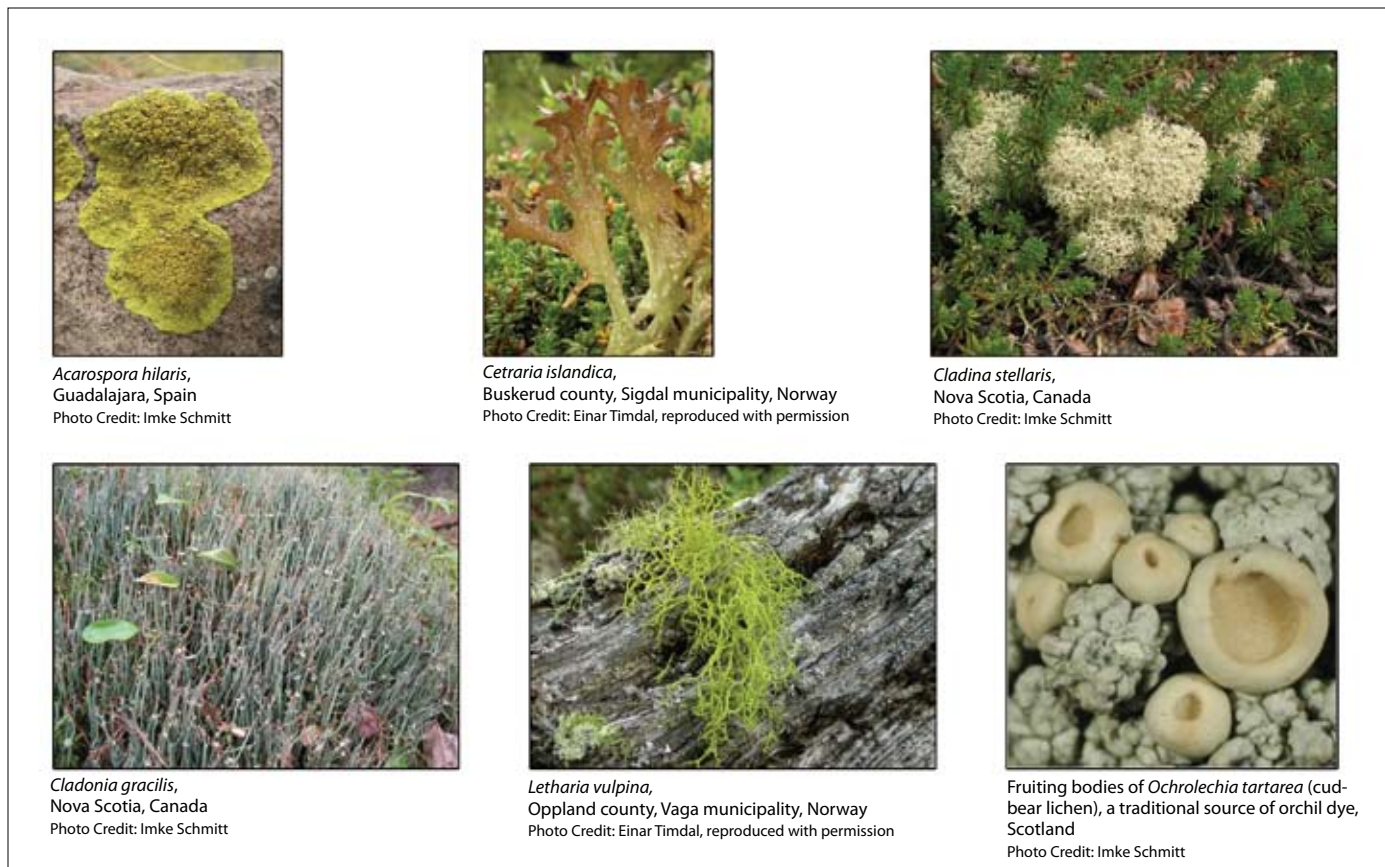


Figure 1. Lichenized fungi have a remarkable variety of growth forms.

Reynolds 1961, Culberson and Armaleo 1992); however, photobionts belonging to specific species of cyanobacteria as well as other lichen-associated microbes (e.g., bacteria), may contribute to a lichen's secondary metabolite profile. According to phylogenetic hypotheses, the capacity of ascomycete fungi to produce diverse, chemically complex compounds evolved during an early radiation which gave rise to the major groups of ascomycetes and which encompassed all major groups of ascomycete lichens (Lutzoni et al. 2001). Thus, the potential among ascomycetes to form both symbiotic associations and diverse secondary metabolites suggests a possible evolutionary correlation between these characteristics. Molecular phylogenetic analyses suggest that major groups of non-lichenized fungi, such as the Eurotiomycota, are derived from lichenized ancestors. It has been hypothesized that members of the Eurotiomycota, which

include many well known producers of natural compounds, such as the mold genera *Aspergillus* and *Penicillium*, acquired their propensities for secondary metabolite production as a consequence of their lichen-mutualistic ancestry (Lutzoni et al. 2001). However, lichenized fungi produce biologically active metabolites only in association with suitable algal partners.

In nature, lichen metabolites have multiple functions (Lawrey 1986, 1995; Rikkinen 1995). They may be light filters to shelter the photobiont from excessive radiation (Gauslaa and Solhaug 2001) or they may be deterrents to damage from grazing by herbivorous invertebrates, or they may have antibiotic properties to protect against microbial degradation (Emmerich et al. 1993); other secondary metabolites may be involved in maintaining the symbiotic equilibrium (Kinraide and Ahmadjian 1970, Huneck 2003) or the weathering of rocks for better attach-

ment of lichens to their substrate (Rundel 1978).

Humans have exploited lichens for diverse purposes. Their most important use has been for dyeing textiles. Dye-stuffs made from lichens reached considerable economic importance during the 18th century, particularly in the Canary Islands. Long before then, however, the Romans dyed their togas with orchil, a purple pigment from *Roccella* spp. found around the Mediterranean Sea, whereas the brown pigment crottal was extracted from *Parmelia*, *Ochrolechia* and *Evernia* spp. Chemists still use paper strips impregnated with litmus, a water-soluble mixture of dyes extracted from lichens, especially the genus *Roccella*, to estimate pH values. Besides dyeing, lichens were used extensively in traditional medicines and for cosmetic purposes (Agelet and Vallès 2001, González-Tejero et al. 1995, Hawksworth 2003, Huneck 1999, Proksa

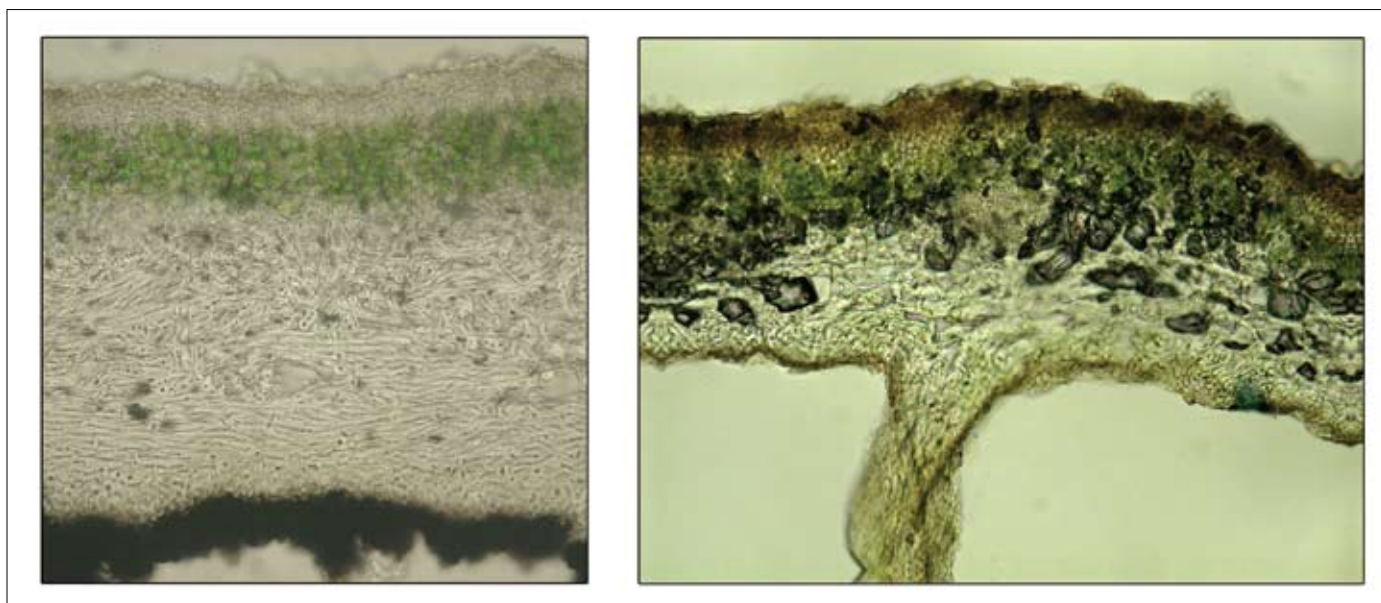


Figure 2. Cross-section through a stratified lichen thallus. **Left figure:** An *Umbilicaria* sp. Four layers can be discerned (from top to bottom): upper cortex (fungal hyphae), algal layer, medullary layer (fungal hyphae), lower cortex (fungal hyphae) (photo: Robert Lücking, reproduced with permission). **Right figure:** The bark-inhabiting species *Physcia stellaris*. The brownish tinged upper surface contains crystals of atranorin (a parapepside) (photo: Walter Obermayer, reproduced with permission).

et al. 1994, Rancan et al. 2002; Richardson 1988, Schindler 1988). Lichen substances exhibit a great diversity of biological effects, including antimicrobial, anti-inflammatory, analgesic, antipyretic, and antiproliferative and cytotoxic activities, and there has been a growing interest in the pharmaceutical properties of compounds derived from lichens (Boustie and Grube 2005). However, relatively few lichen substances have been screened in detail for biological activity and therapeutic potential, due principally to difficulties in obtaining them in quantities and purities sufficient for structural elucidation and pharmacological testing.

A brief history of lichen chemistry

Early reports on chemical peculiarities in lichens were presented in the middle of the 19th century by the Finnish botanist and entomologist William Nylander (1822-1899) (Nylander 1866; also see Vitikainen 2001). However, the first systematic treatment of lichen chemistry came from the laboratory of the German biologist Friedrich Wilhelm Zopf (1846-1909) (Zopf 1895; also see www.

catalogus-professorum-halensis.de/zopf-wilhelm.html). Japanese researchers subsequently succeeded in characterizing the chemical structures of numerous lichen compounds. In particular Yasuhiko Asahina (1880-1975; see Shibata 2000), Shōji Shibata (b. 1915), and co-workers studied the biosynthesis of lichen substances and introduced micro-recrystallization as a simple method for determining lichen substances (Asahina 1936; Shibata 1958, 2000; and citations therein), based on the fact that in the presence of certain reagents some lichen substances assume characteristic crystal forms which can be identified microscopically. Subsequent application of thin layer chromatography (TLC) and high performance LC (HPLC) by lichen systematists led to a clearer understanding of the distribution of secondary metabolites in lichens, which had a profound impact on lichen chemotaxonomy and which revealed interesting evolutionary relationships. Some secondary metabolites occur over broad evolutionary lineages, whereas others are highly variable even within a given species. Data for about 430 lichen substances, including their distribution

in different species, was published by C. F. Culberson in three influential books (Culberson 1969, 1970; Culberson et al. 1977). The number of structurally defined lichen substances has increased considerably since 1977 and more than 800 compounds were included in the last major compilations (Huneck 2001, Huneck and Yoshimura 1996). Structures for more than 1000 lichen substances are available and a backlog of a similar number of lichen substances remains to be chemically characterized. However, even if all of the easily-detected extrolites were to be identified, a large number of additional secondary metabolites, occurring mainly at low concentrations, certainly would remain to be characterized.

Structures of lichen substances

Most known lichen substances are phenolic compounds (e.g., orcinol and β -orcinol), anthraquinones (e.g., parietin), dibenzofurans (e.g., usnic acid), depsides (e.g., barbatic acid), depsidones (e.g., salazinic acid), depsones (e.g., picrolichenic acid), γ -lactones (e.g., protolichesterinic acid, nephrosterinic acid), and pulvinic acid

derivatives (e.g., vulpinic acid) (Fig. 3). Lichens had to evolve diverse biosynthetic pathways to produce such complex arrays of secondary metabolites. The polyketide biosynthetic pathway appears to be responsible (in whole or in part) for most of the classes of compounds mentioned before, whereas vulpinic acids are shikimate derivatives, and the abundance of di- and triterpenoids found in lichens (Abdullah et al. 2007) are formed via the mevalonate pathway (Dayan and Romagni 2001). Large numbers of different lichen substances can be generated from a limited set of monoaromatic units. Metabolites, which arise via phenolic coupling (Geissman and Crout 1969) and form a substantial fraction of the chemical diversity of lichens, including *p*- or *m*-depsides and depsidones, consist of multiple phenolic units. Products of phenolic coupling can be modified by decarboxylation, halogenation, methylation, or esterification with short- and long-chain fatty acids.

Beside compounds common to all or most lichen groups, some species contain unique products. Arthogalin, for example, is a cyclic depsipeptide known only from a species endemic to the Galapagos Islands (Huneck and Himmelreich 1995). The cytotoxic scabrosin esters (epidithiodioxopiperazines) (Ernst-Russell et al. 1999), which probably derive from phenylalanine (Gardiner et al. 2005), occur in only a few, unrelated lichen species (Elvebakk et al. 2007). Uncommon structures include structural modifications arising from intramolecular arrangements (e.g., *m*-scrobiculin [Elix et al. 1986]) or conjugation with carbohydrate moieties (Řezanka and Guschina 2000). Řezanka and coworkers described brominated depsidones (Řezanka and Guschina 1999) and brominated acetylenic fatty acids (Řezanka and Dembitsky 1999) recovered from lichens native to central Asia, and monotetrahydrofuranic acetogenins from the lichenized ascomycete *Torn-*

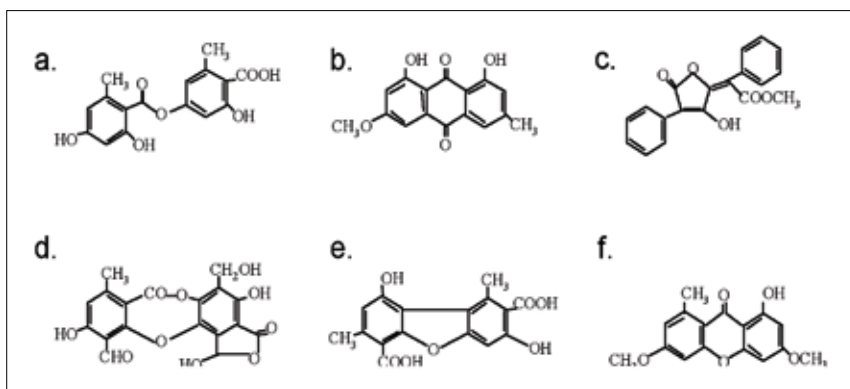


Figure 3. Structures of some common lichen compounds: lecanoric acid (a, depside), parietin (b, quinone), vulpinic acid (c, pulvinic acid derivative), salazinic acid (d, depsidone), pannaric acid (e, dibenzofuran), lichexanthone (f, xanthone).

abea scutellifera (Řezanka et al. 2004). γ -Lactones containing glycosylated long-chain fatty acids (Řezanka and Guschina 2000, 2001a,b) and macrolactone glycosides (gobienines, Řezanka and Guschina 2001c) were identified in lichens from central Asia. Mono- and diprenylated xanthone glucosides (umbilicaxanthosides, Řezanka et al., 2003) have been obtained from the lichen *Umbilicaria proboscidea* collected from the Ural Mountains; and Torres et al. (2004) reported that the lichenized ascomycete *Collema cristatum* contains a glucosylated mycosporine which they called collemin A.

Additional unique or uncommon compounds (Fig. 4) undoubtedly will be detected as the search for such substances proceeds and analytical methodologies become more refined.

Biological effects and potential uses

Recent reviews have discussed the pharmaceutical potential of lichen substances (Huneck 1999, Müller 2001, Yamamoto 2000, Boustie and Grube 2005). Products containing usnic acid as a topical antiseptic are available in some countries (e.g., Gessato™ shaving treatment from Italy, Camillen 60 Fudes spray and nail oil from Germany), though in fact many pharmacological properties have been attributed to this compound (Ingólfssdóttir 2002). However, usnic acid can provoke allergic reactions, and the compound is toxic at high doses (acute oral toxicity [LD₅₀] in the mouse, 0.84 g/kg). Fatal hepatotoxicity has been attributed to ingestion of ~500 mg/day of usnic acid in nutraceuticals marketed as weight-loss aids (Durazo

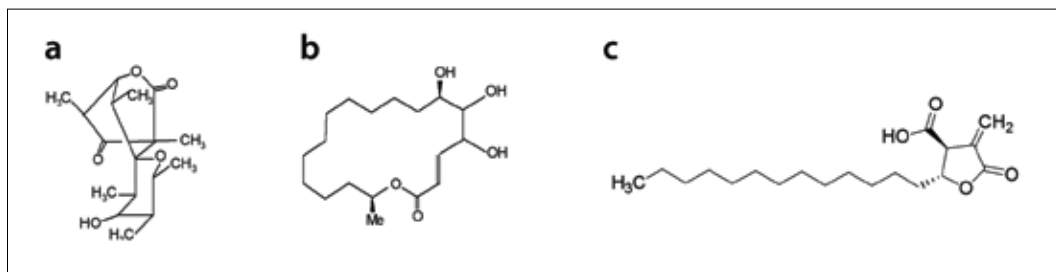


Figure 4. Unusual structures of lichen compounds: (a) portentol, (b) aspicilin, (c) nephrosterinic acid. Portentol, a polypropionate produced by *Rocella portentosa*, derives from acetate and methionine (Aberhart et al. 1970). Aspicilin is undoubtedly the product of a PKS, whereas the biosynthesis of nephrosterinic acid may be related to that of A-factor (Gräfe 1989, Kato et al. 2007).

et al. 2004, Neff et al. 2004).

Cold remedies formulated from Icelandic lichen are sold under the trade name Broncholind[®] by MCM Klosterfrau Vertriebsgesellschaft mbH (Cologne, Germany) and Isla-Moos[®] by Engelhard Arzneimittel GmbH & Co. KG (Niederdorfelden, Germany). A small number of other cosmetic, nutraceutical, or pharmaceutical firms (e.g., Ichimaru Pharcos Co., Ltd., Gifu, Japan) also manufacture products containing lichen substances or extracts.

Antibiotic and antifungal activities

The antibacterial properties of lichen extracts have been known for many years (Burkholder et al. 1944), and incorporation of usnic acid into medical devices inhibits bacterial biofilm formation on polymer surfaces (Francolini et al. 2004).

Minimal inhibitory concentrations (MICs) for acetone and methanol extracts of the lichens *Cladonia furcata*, *Parmelia caperata*, *P. pertusa*, *Hypogymnia physodes*, and *Umbilicaria polyphylla* were determined towards six species of bacteria and ten species of fungi (Ranković et al. 2007a, cf. Ranković et al. 2007b). Strongest antimicrobial activities were recorded for extracts of *P. pertusa*, whereas weakest activities were observed for *P. caperata*. *Bacillus mycoides* was the most sensitive bacterial species tested, whereas *Candida albicans* was the most sensitive fungal species examined. Anti-Gram-positive activities have been reported for evernic acid, vulpinic acid and hirtusneanoside (Lawrey 1986, Řezanka and Sigler 2007). Antifungal activities been reported for the anthraquinone parietin isolated from *Caloplaca cerina* (Manojlovic et al. 2005), as well as for extracts of the Andean lichens *Protousnea poeppigii* and *Usnea rigida*, which contain divaricatinic acid, isodivaricatic acid, usnic acid, and 5-propylresorcinol (Schmeda-Hirschmann et al. 2007).

The riminophenazine antibiotics, exemplified by clofazimine (Lamprene[®]),

were developed as antimycobacterial drugs (Reddy et al. 1999). Their synthesis followed from identification of the antituberculous activity of derivatives of diploicin, a depsidone produced by the Irish lichen *Buellia canescens* (Barry 1946, Barry and Twomey 1950, Nolan et al. 1948).

Antiviral activity

Antiviral properties have been attributed to various lichen substances. Usnic acid extracted from *Ramalina celastri* displayed antiviral activity, whereas parietin extracted from *Teloschistes chrysophthalmus* was virucidal towards the Junín and Tacaribe (South American) arenaviruses (Fazio et al. 2007). Lichenan, a linear {1→3, 1→4} β-D glucan (Tvaroska et al. 1983) widely distributed among lichens (Common 1991) and believed to be a structural component of the mycosymbiont cells wall (Honegger and Haish 2001), inhibited symptom development and virus accumulation in four greenhouse-grown *Nicotiana* spp. infected by tobacco mosaic virus, probably by interfering with an early event in virus replication. The antiviral activity of partially hydrolyzed lichenan was comparable to that of intact lichenan (Stübler and Buchenauer 1996).

Antitumor and antimutagenic activities

Colleflaccinosides, bisanthraquinone glycosides isolated from *Collema flaccidum*, showed significant antitumor activity in the crown gall tumor inhibition test (Řezanka and Dembitsky 2006). Likewise, the depsidone pannarin reportedly inhibits cell growth and induces apoptosis in human prostate carcinoma DU-145 (Maier et al. 1999) and human melanoma M14 cells (Russo et al. 2006, 2008). The antiproliferative activities of tenuiorin (a tridepside) and methyl orsellinate extracted from *Peltigera leucophaeobia* were tested on human breast (T-47D), pancreatic (PANC-1) and colon (WIDR) cancer cell lines; the former compound caused a weak-to-moderate reduction in [³H]thymidine uptake by the pancreatic and colon

cells, whereas methyl orsellinate was without effect (Ingólfssdóttir et al. 2002).

Enzyme inhibitory and antioxidant activities

Extracts of the lichen *Umbilicaria esculenta* strongly inhibited disaccharide hydrolytic enzymes of mold and mammalian origin. The purified inhibitor was identified as 1-deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol), already known as a product of *Streptomyces* spp. and plants of the genus *Morus*. Extracts of *Parmelia austrosinensis* and *Parmelia praesorediosa* had similar glucosidase inhibitory activities (Lee and Kim 2000). Inhibition of glycosylation is believed to affect melanin biosynthesis in human melanoma cells. Kim and Cho (2007) concluded that methanol extracts of lichens *Usnea longissima* and *U. esculenta* affect the activity of tyrosinase via inhibition of tyrosinase glycosylation. Inhibition of lipoxygenase (Ingólfssdóttir et al. 2002) and of prostaglandin (Sankawa et al. 1982) and leukotriene B₄ biosyntheses (Kumar and Müller 1999) might correlate with the anti-inflammatory, analgesic and antipyretic activities of some lichen substances (Okuyama et al. 1995).

Many lichen extracts have antioxidant properties, probably due to their phenolic content. Jayaprakasha and Rao (2000) examined the antioxidant properties of methyl orsellinate, atranorin, orsellinic acid, and lecanoric acid. Bhattarai et al. (2008) reported stronger antioxidant activities in extracts from Antarctic lichens than from lichens native to temperate or tropical regions.

Products of axenically cultured mycobionts

Lichen mycobionts synthesize significant quantities of secondary metabolites only under permissive conditions, as has been demonstrated repeatedly in cultivation experiments (Hamada et al. 1996, Hamada and Ueno 1987, Kinoshita 1993, Leuckert et al. 1990, Yamamoto et al. 1995). Permissive conditions appear to be those mimicking the conditions to which lichens are exposed “in the wild,” since stable culture conditions do not promote production

*PKSs are classified as type I, II, or III. For a review of PKS nomenclature, see Moss et al. (2004).

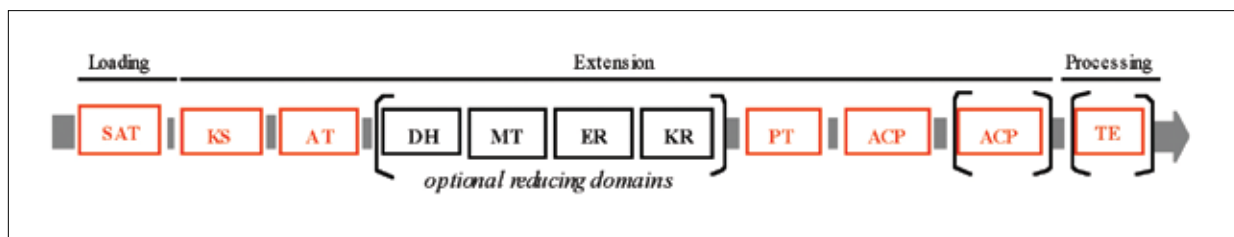


Figure 5. Gene architecture of fungal type I PKS: SAT, starter unit acyl transacylase; KS, ketosynthase; AT, acyltransferase; DH, dehydratase; MT, methyl transferase; ER, enoyl reductase; KR, ketoreductase; PT, product template; ACP, acyl-carrier protein; TE, thioesterase (or CYC, Claisen cyclase). Non-reducing domains in red, optional reducing domain in black. The ACP domain may occur in pairs, whereas the TE domain may be absent.

of lichen-specific secondary metabolites (Stocker-Wörgötter 2008). This may, in part, be attributed to elevated osmotic conditions in vitro, since high levels of sugars, for example, mannitol, often are needed to initiate secondary metabolite production in axenic cultures. Alteration of secondary metabolite production was reported for the mycobiont of *Xanthoria elegans* grown on different media (Brunauer et al. 2007), whereas in resynthesis and relichenization experiments with *Cladia retipora* and *Dactylina artica* complete thalli containing typical lichen substances were formed (Stocker-Wörgötter and Elix 2006).

However, cultured mycobionts often produce new, interesting metabolites not encountered in nature and synthesis of these novel products may be enhanced by varying the type and concentration of nutrients in the growth medium, or by simulating stress situations (ultraviolet irradiation, changes in thermal or osmotic conditions). Axenic cultures of *Bunodophoron patagonicum* isolated either from spores or from thallus fragments formed two chemosyndromes (suites of chemotypes) of depsides and dibenzofurans synchronically (Stocker-Wörgötter 2004). Alternatively, different carbon sources in the culture medium can switch secondary metabolite biosynthesis from the polyketide pathway to the fatty acid biosynthetic pathway. Thus, *Physconia distorta* grown on nutrient-rich media produced mainly oleic acid, linoleic acid, stearic acid, and their triglyceride derivatives, which were excreted as oil drops on the mycelia sur-

face, rather than phenolics (Molina et al. 2003).

Biosynthesis of polyketides

Fatty acid and polyketide biosynthesis both proceed by sequential additions of C_2 or related chemical units; however, whereas fatty acid synthetases (FASs) make use predominantly of acetyl~SCoA (and, less commonly, malonyl~SCoA) moieties, polyketide synthetases (PKSs) can utilize a greater variety of activated organic acids, including propionyl~SCoA, butyryl~SCoA, methylmalonyl~SCoA, and ethylmalonyl~SCoA (Hopwood 1997, Shen 2000). Moreover, whereas successive chain elongation steps during fatty acid biosynthesis typically proceed via a fixed sequence of keto reduction, dehydration, and enoyl reduction, certain reactions typical of fatty acid biosynthesis can be suppressed by PKSs at specific biosynthetic steps; that is, chain elongation intermediates during polyketide biosynthesis may undergo all, some, or none of the aforementioned biosynthetic reactions, depending upon the particular catalytic domains in a given PKS. The result is a remarkable diversity of polyketide structural motifs much more diverse than the products of FAS biosynthesis, which can be enhanced further by post-PKS modifications (“tailoring reactions”) (O’Hagen 1991, Rawlings 1999). Tailoring enzymes, such as oxidoreductases or methylases, may obscure the biosynthetic origin of PKS products (Pfeifer and Khosla 2001, Rix et al. 2002).

Diversity of PKS genes in lichen-forming fungi

Fungal type I PKSs are multifunctional proteins containing a single reiteratively-used ketoacyl synthase (KS) domain targetable by PCR primers (Bingle et al. 1999, Boustie and Grube 2005, Grube and Blaha 2003, Lee et al. 2001, Miao et al. 2001, Nicholson et al. 2001, Sauer et al. 2002, Schmitt et al. 2005). Different polyketides are produced depending upon which other domains are present (Fig. 5). Besides the KS domain, type I PKSs also contain acyltransferase (AT) and acyl-carrier protein (ACP) (or phosphopantethein, PP) domains. At the N-terminus of the PKS, anterior to the KS domain, there resides a starter unit, the ACP transacylase (SAT) domain which mediates loading of a starter unit, whereas the product template (PT) domain, presumably responsible (at least in part) for polyketide chain length and cyclization, lies downstream of the AT domain (Cox 2007). Using a deconstruction approach, Crawford et al. (2008) demonstrated that the PT domain of PksA, which initiates biosynthesis of aflatoxin B_1 in *Aspergillus parasiticus*, is involved in maintaining the correct aldol addition intermediate and promoting its dehydration, thereby facilitating non-enzymic release of the shunt product naphthopyrone or of norsolorinic acid anthrone. Other domains, such as ones with a thioesterase/Claisen cyclase (TE/CYC) motif or which function as ketoreductases (KRs), dehydratases (DHs), enoyl reductases (ERs), or methyltransferase (MeTs) are optional, and

are found in PKSs generating polyketides with different degrees of reduction and/or substitution.

The composition of domains correlated with phylogenetic lineages in a phylogenomic study of the PKS family (Kroken et al. 2003). Not all fungal aromatic polyketides, however, are necessarily produced by type I PKSs; for example, a type III PKS is responsible for production of the pentaketide alkylresorcinol acid in *Neurospora crassa* (Funa et al. 2007). Preliminary analyses suggest that these synthases are also present in lichens. Partially degenerate primers targeting fungal type III-like PKS detected corresponding genes so far in six lichen species (Muggia et al. unpublished data).

Phylogenetic analyses in conjunction with bioinformatic approaches to fungal genomes which have been sequenced completely (Kroken et al. 2003, Varga et al. 2003) have revealed a large diversity of paralogous PKSs. Likewise, phylogenetic analyses of mycobionts suggest high numbers of paralogous genes (Grube and Blaha 2003, Opanowicz et al. 2005, Schmitt et al. 2005). The clades of these paralogs can now be tested for functional divergence using statistical methods (Gu 1999, Grube and Blaha 2003), but two crucial questions remain:

- How did the diversity of paralogs evolve?
- What is the function of the paralogs?

Sequence analyses of PKS genes from lichenized fungi

Paralogous PKS genes in non-lichenized fungi likely evolved via gene duplication (Kroken et al. 2003). Muggia et al. (2008) analyzed all available sequence data for KS domains of mycobiont PKS genes and generated a phylogenetic framework for studying the evolutionary forces acting on nucleotide sequences. According to this work, strong purifying selection shapes the evolution of PKS genes in lichens, while no clear evidence was found for neutral evolution or positive selection.

As with non-lichenized fungi, the wide distribution of certain paralogs indicates that gene duplication led to the diversity of polyketide synthases found in lichens. It is postulated that multiple paralogs were lost in different lineages during evolution, and that different paralogs might be involved in production of similar products in different lineages.

~~Schmitt et al. (2008) showed that lichen fungi also contain 6-methylsalicylic acid synthase (6-MSAS)-like polyketide synthase genes, and speculated that these genes could have been acquired by horizontal transfer from bacterial genomes.~~ Such horizontal transfer might be facilitated by high abundances of bacterial communities, as observed in some lichens (Cardinale et al. 2008). In the absence of complete genome data from lichen mycobionts it is currently impossible to know whether acquisition of novel genes by means of horizontal transfer is a common or rare event.

Functional characterization and heterologous expression of PKSs

Despite much progress in the elucidation of the diversity of lichen PKS genes, little is known about the roles of individual paralogs. Phylogenetic analyses of the KS domain revealed large monophyletic clusters of reducing and non-reducing PKSs from various fungi, suggesting considerable degrees of paralogy within these main lineages (Boustie and Grube 2005, Kroken et al. 2003, Opanowicz et al. 2005, Schmitt et al. 2005). Such analyses applied to the secondary metabolites found in a mycobiont are insufficient to predict the function of any of its paralogs (Schmitt et al. 2005). Routine analytical methods in lichenology (typically TLC or HPLC) probably do not detect the full spectrum of secondary metabolites produced by lichens. As studies of non-lichenized fungi show, similar products may be synthesized by unrelated PKSs in different fungi: the biosynthesis of dihydroxynaphthalene as common precursor of melanin formation in ascomycetes can proceed via penta- or heptaketides in fungi (Tsai et al. 2001). Preliminary evidence from inhibition as-

says with tricyclazole, a fungicide that acts as a non-competitive inhibitor of trihydroxynaphthalene reductase (Viviani et al. 1993), suggests that lichen melanins are comprised of dihydroxynaphthalene precursors (G. Brunauer, personal communication), but it cannot be excluded that some lichens might produce melanins from tyrosine (Laufer et al. 2006).

Functional assignment of a particular PKS to a particular polyketide product remains a complicated task, particularly since the technique of reverse genetics (determining how a change in gene sequence affects phenotype), which has been used for other fungi (Gold and Alic 1993, Magee et al. 2003), is not feasible for mycobionts because no transformation system has been devised yet for these microorganisms. Moreover, heterologous expression of lichen PKSs in surrogate hosts may not be as straight-forward as expected, since the complex metabolism of potential surrogate hosts might influence or interfere with mycobiont PKS biosynthesis or function and lead to different polyketide products, analogous to the “hybrid antibiotics” observed for actinomycetes by Schlegel and Fleck (1980) and by Hopwood et al. (1985). Thus, ectopic expression of *Colletotrichum lagenarium* PKS1 in *A. oryzae* led to unexpected coproduction of the tetraketide orsellinic acid and the pentaketide 6,8-diacetoxy-3-methylisocoumarin (Fujii et al. 1999). Even though fungal PKS genes have already been expressed in heterologous hosts such as bacteria, yeast, other filamentous fungi, and plants (Pfeifer and Khosla 2001, Schumann and Hertweck 2006), attempted expression of a PKS gene from the mycobiont of the lichen *Xanthoparmelia semiviridis* (Chooi et al. 2008) and the mycobiont of the lichen *Solorina crocea* (Gagunashvili et al. 2009) in *A. nidulans* did not result in a product. Possible reasons for failure include inefficient priming of polyketide biosynthesis, absence of required post-PKS enzymes, inappropriate expression systems, lack of auxiliary enzymic activities, codon usage differences, incorrect protein folding, even toxicity of *X. semiviridis* and/or *S. crocea* polyketide products towards the

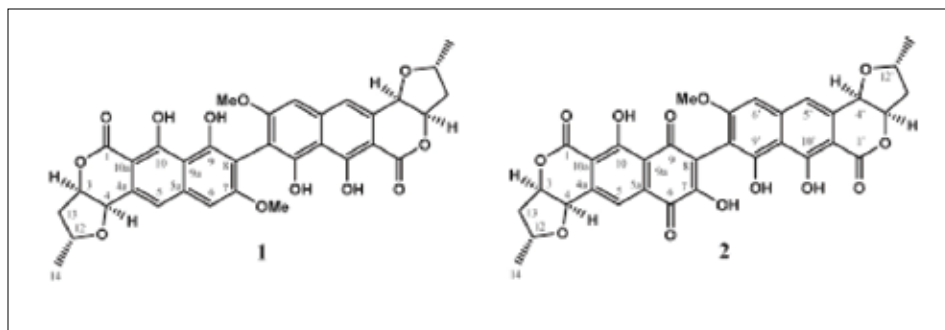


Figure 6. Structures of lichenicolins A (1) and B (2).

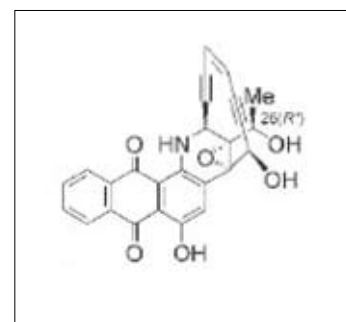


Figure 7. Structure of unciamycin.

heterologous host.

Present data about diversity of PKS genes in lichen fungi suggest that their genomes contain more functional paralogs than can be accounted for in terms of identified secondary metabolites. This parallels the results from genomic exploration in non-lichenized fungi (Kroken et al. 2003) and suggests a considerable number of silent pathways. Recently, Williams et al. (2008) awakened silent pathways in fungi and obtained new secondary metabolites by disrupting heterochromatin formation with DNA methyltransferase and histone deacetylase inhibitors. Thus, epigenetic remodeling of the metabolome may lead to the discovery of many new compounds in lichenized fungi.

Genome sequence information is certainly required for further investigations of PKS diversity in lichens. This will enable creation of specific markers for PKS genes not yet detectable with current primers, and will shed further light on the genealogy and evolution of lichen PKSs in foreseeable future. Although current methods for genome sequencing, such as 454 technology, are much more rapid than earlier techniques (Rothberg and Leamon 2008, Shendure and Ji 2008), isolation of novel genes for cloning and sequencing, as is done for easy-to-cultivate fungi, relies predominantly on genomic libraries, which are still rare for lichen fungi (Chooi et al. 2008; Gagunashvili et al. 2009; Miao et al. 2001; D. Armaleo, personal communication). Establishment of representative genomic libraries requires sufficient amounts of axenic lichen mycobionts,

since genomic material extracted directly from whole lichens will, by definition, contain DNA from at least two species.

Secondary compounds from microbial associates of lichens

While lichens are generally considered symbioses between only a fungus and one or more species of green algae or cyanobacteria, recent studies report that many lichens contain a stable consortium of other microorganisms, including parasitic fungi (Lawrey and Diederich 2003), endophytic fungi (Arnold et al. 2008), and bacteria (Cardinale et al. 2006). The diversity of co-inhabiting fungi is well-studied: more than a thousand species parasitic for lichens having been documented (Lawrey and Diederich 2003), and they can be producers of interesting metabolites in their own right. For example, two new bisnaphthopyrone compounds, lichenicolins A and B, were isolated from an unidentified lichen-inhabiting fungus (He et al. 2005) (Fig. 6). Lichenicolin A was active against Gram-positive bacteria, including strains of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*, whereas it showed variable activity against strains of *Escherichia coli*. In contrast, lichenicolin B was devoid of antibacterial and anticandidal activity.

The bacterial constituents of lichens are still largely underexplored. Cardinale et al. (2008) used fluorescence in situ hybridisation to show that bacterial associates colonize lichens in a biofilm-like manner. They suggested that lichens might constitute a rich pool of bacterial diversity, in-

cluding as-yet undescribed species which may serve as sources of biotechnologically interesting compounds, enzymes, and/or strains. Gonzales et al. (2005) highlighted the diversity of actinomycetes in lichens by DNA fingerprinting, and used specific primers to screen 337 isolates for genes involved in secondary metabolite production. High frequencies of positive PCR amplification were obtained for PKS I (62.6%), PKS II (64.7%) and non-ribosomal peptide synthetases (58.5%). Their results suggest that lichens constitute an extremely diverse reservoir not only of actinomycetes but of secondary metabolites generally, which may prove important in the search for new antibiotics. For example, unciamycin (Davies et al. 2005) (Fig. 7), a new enediyne produced by a streptomycete isolate occurring on the surface of a lichen from British Columbia (*Cladonia uncialis*), had strong antibacterial activity against both Gram-positive and Gram-negative human pathogens, including *Burkholderia cepacia* (MIC, 0.001 µg/mL) and *S. aureus* (MIC, 0.0064 µg/mL). However, enediyne seem to be generally cytotoxic due to their reactivity towards nucleic acids (Battigello et al. 1995, Cosgrove and Dendon 2003).

Secondary metabolite production by lichen photobionts has not been investigated in much detail. Among the very few such compounds reported are the nostocliides (Yang et al. 1993), γ -butyrolactones from a *Nostoc* sp. recovered from the lichen *Peltigera canina*, with moderate anticytotoxic activities; microcystins, hepatotoxic cyclic heptapeptides produced by a *Nostoc*

sp. recovered from the lichen *Pannaria pezizoides* (Oksanen et al. 2004), β -*N*-methylamino-L-alanine, a neurotoxic amino acid produced by a *Nostoc* sp. recovered from a *Peltigera* sp. of lichen (Cox et al. 2005), and cryptophycins, peptolides with promising antineoplastic activities, from *Nostoc* sp. ATCC53789 (isolated from a lichen found on Aaron Island, Scotland) (Margarvey et al. 2006).

Future prospects

Lichens encompass not just the traditionally recognized algal and fungal symbionts but also diverse lichen-inhabiting (lichenicolous and endophytic) fungi as well as a plethora of bacteria and harbor an enormous diversity of secondary metabolites. Investigations on the natural product composition of whole lichens or lichen constituents and their biosynthetic machineries lag behind those of non-lichenous bacteria and fungi, but the range of bioactivities identified to date portend well for further research that eventually may result in the commercial production and exploitation of pharmaceutically interesting lichen substances. However, until the establishment of appropriate expression systems for lichen-derived DNA, genomics-driven discovery (Bergmann et al. 2007) and metagenomic mining will be the only means of obtaining new insights into the evolution and diversity of the biosynthetic genes of these organisms. It is expected that at least two mycobiont genome sequences, from the lichens *Xanthoria parietina* and *Cladonia grayi*, will become available in near future, and provide baseline information about total PKS gene diversity in lichen fungi.

Microorganisms rapidly acquire resistance to antibiotics, so there is a continuous demand for new antimicrobial agents. Lichens could be valuable source of new lead structures. Moreover, it is anticipated that the genetic engineering and combinatorial biosynthesis approaches currently used to expand the repertoire of polyketides from “conventional” bacteria and fungi will become applicable to lichen PKS genes. A noteworthy (non-lichenous) precedent is the work of Watanabe and

Ebizuka (2002), who succeeded in producing in *A. oryzae* a novel hexaketide, 2-acetyl-1,3,6,8-tetrahydroxynaphthalene, synthesized from a chimeric gene derived from a pentaketide synthase of *Colletotrichum lagenarium* and a heptaketide synthase of *A. nidulans*. Lichenized fungi, with their diversity of metabolites, could provide very interesting sets of PKS genes for creation of novel and pharmacologically interesting secondary metabolites

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