Production and Bioactivity of Common Lichen Metabolites as Exemplified by *Heterodea muelleri* (Hampe) Nyl.

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Abstract Based on results of a former study in 2005, this investigation aimed at quantifying UV- and cold temperature stress-induced changes within the secondary metabolite production of the cultured mycobiont of the lichen *Heterodea muelleri* (Hampe) Nyl. The chemical profiles of the mycobiont cultures and the lichen thallus were determined by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) analyses. The voucher specimen of *H. muelleri* produced diffractaic acid as a major polyketide and barbatic acid as a satellite compound, whereas the untreated mycobiont did not contain any detectable secondary metabolites. While UV-C stress caused a general increase in substance formation, cold temperature stress resulted in a strong activation of barbatic acid biosynthesis. A further series of experiments focused on the effect of diffractaic and barbatic acids on the growth of the symbiotic photobiont *Trebouxia jamesii*; usnic acid was similarly tested. Pure substances were obtained from mycobiont cultures by performing preparative TLC. A determined quantity of algae was incubated on BBM plates that contained three different concentrations of the pure lichen metabolites. The growth of the photobionts was monitored over a period of 1 mo to evaluate the impact of each substance on the cultured algae. While diffractaic and usnic acid had no noticeable effect, barbatic acid strongly inhibited algal growth and resulted in cell death.

Introduction

Lichen secondary metabolites, especially polymalonyl-derived polyketides, have been found to exhibit manifold biological activities in various screenings (Huneck 1999, 2001; Dayan and Romagni 2001; Müller 2001). Although the biological activities of lichen substances are well known, the biological and ecological functions of these compounds for the lichen symbiosis and the lichen itself are poorly understood. Although more than 800 lichen substances are known and their structures have been elucidated (Huneck 2001), many others remain to be characterized. In general, lichen secondary metabolites can be grouped into three classes according to their biosynthetic origin; polymalonate, mevalonate, and shikimate derivatives (Asahina and Shibata 1954; Culberson 1969, 1970). Lichens, like other ascomycetous fungi, form chemically heterogenous polyketides that comprise aliphatic and aromatic structural motifs. The orcinol and β-orcinol derivatives (depsides, depsidones, dibenzofurans) are especially interesting as they presumably play a role in the establishment of the lichen symbiosis and probably also in the interaction between the symbionts and with their environment (Armaleo 1995). A few of the structurally simple depsides have been found in non-lichenized fungi, such as lecanoric acid in the genus *Pyricularia* (Umezawa et al. 1974), a plant pathogenic fungus. In former studies, this group of organisms has been considered as the possible origin of the establishment of fungal symbioses that result in the lichen symbiosis (Ahmadjian 1970; Gargas et al. 1995). More

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recent and extensive studies by Lutzoni et al. (2004) and James et al. (2006) have superseded the former speculations, indicating that many pathogens are derived from lichen-forming fungi.

The over-riding interest in these compounds has been in respect to their potential source as prospective pharmacophores (González-Tejero et al. 1995; Huneck 1999), but it is difficult to produce and isolate large quantities. To obtain rare compounds in large quantities without endangering the natural populations of the source organisms, optimized cultures of lichen fungi are desirable. It is, therefore, relevant to investigate what the ecological implications are in producing particular substances and what factors trigger the fungus to express a certain chemical profile (Culberson and Armaleo 1992; Armaleo 1995; Hamada and Miyagawa 1995).

The secondary metabolites of *Heterodea muelleri* (Hampe) Nyl. were previously analyzed by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) and described by Hager and Stocker-Wörgötter (2005). Under particular stress conditions (e.g., exposure to UV light), cultured mycobionts produced the typical depsides, diffractaic and barbatic acids. In another screening experiment, Nishitoba et al. (1987) showed that diffractaic and barbatic acids strongly inhibited the growth of lettuce seedlings, and Takahagi et al. (2006) reported an inhibition of photosystem II in tobacco cells by barbatic acid.

Inspired by these results, we investigated whether these metabolites have any effect on the photobionts of *H. muelleri*. For this reason, we tested whether lichen substances protect the lichen photobiont against environmental stress and control its photosynthesis and growth. The major aim was to test the effects of typical lichen metabolites, diffractaic and barbatic acids, formed both by natural thalli and by cultured mycobionts on the growth of the cultured algal symbionts. A further experiment studied the effects of usnic acid extracted from *Heterodea beaugleholei*, the closest relative of *H. muelleri*.

Methods and Materials

Voucher Specimens The voucher specimens, fruticose thalli of *H. muelleri*, were collected from the hills of Canberra (Mt. Ainslie, S 35°20′57″, E 149°07′32″) in the Australian Capital Territory (Fig. 1a). The collected material is kept and preserved in the Herbarium of the University of Salzburg (Herbarium SZU L 23035).

Culture Method Single spore isolation was performed according to Ahmadjian (1973). Apothecia were cut off the thallus and transferred to 10 ml of water in a beaker (for 10 min.). After removal of the water film with filter paper, the apothecia were fixed to the top cover of a petri dish with petroleum jelly. Spores were discharged onto a sterile S2% medium in a petri dish [Sabouraud-2%–Glucose–Agar (Fluka 84086)]. After 2 d, several groups of spores were present on the surface of the agar plate. The cover that contained the apothecia was then removed to avoid further contamination. Germinated, single spore isolates were transferred to tubes containing S2%. Mycelia that developed from these spores (incubated for 4–6 mo) were gently homogenized to obtain subcultures.

Culture Media for Photobionts Algal symbionts were isolated and subcultured on Bold's basal medium(BBM; Deason and Bold 1960). For the growth experiment, petri dishes with BBM+b (BBM, as a solid medium impregnated by barbatic acid), BBM+d (BBM with diffractaic acid), and BBM+u (BBM with usnic acid) were used.

Culture Conditions Cultures were maintained in a culture chamber under a changing 12 hr/27°C:12 hr/24°C L:D regime, and a light intensity of 50–100 μmol m^{-2} s^{-1} (=standard conditions).

Stress Parameters (1) Well-developed subcultures (spore isolates, 4 mo old) of *H. muelleri* were subjected to a low temperature stress of 4°C for 6 wk. A control was kept

Fig. 1 a *Heterodea muelleri* thallus from Mt. Ainslie (Canberra, Australia). **b** Nutrient media growth experiment; mycobiont cultures on three different media after 5 mo (showing strong pigmentation on MS); *bar*=5 mm
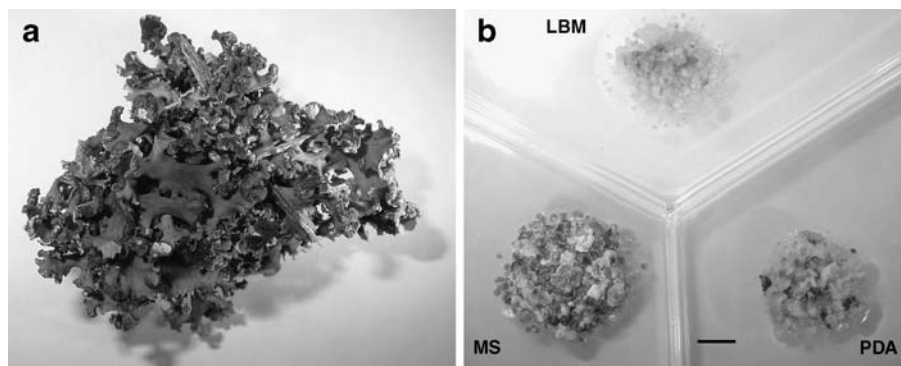
under standard conditions. Methanol extracts of the freeze–dried cultures were analyzed by HPLC. (2) Four-month-old subcultures of the lichen fungus were exposed to UV-C (254 nm; 300 $\mu W\ cm^{-2}$) for 1 hr each day at room temperature (under a sterile hood with deactivated airflow to avoid dryness) over a period of 2 wk. Cultures were maintained under standard conditions (as described above) for the remaining time. A control culture was kept as a reference. Methanol extracts of freeze–dried cultures were analyzed by HPLC.

Preparation of Specimens, DNA and Chemical Analyses

Preparation Circular plugs (ca 1 cm in diameter) were cut from each agar plate overgrown by the mycobiont, and the origin of each subculture was recorded. To prevent contamination, the plugs for chemical testing were prepared in a hood under sterile conditions. Samples were vacuum freeze–dried at −42°C for 24 hr by using an ice condenser connected to a vacuum pump. The dried discs were transferred to glass tubes and extracted with methanol for 4 hr. Extracts were then transferred to HPLC vials, and an aliquot of 20 µl from every sample was injected.

DNA Extraction Total DNA was extracted from cultured mycobionts and for the comparisons from the voucher specimens (Armaleo and Clerc 1995).

DNA Amplification and Purification The ITS-regions, the 5.8 region, and the flanking parts of the small and large subunits (SSU 18S and LSU 28S) of the rDNA were amplified by using a GeneAmp polymerase chain reaction (PCR) system thermal cycler. Primers for the PCR were ITS1F (Gardes and Bruns 1993), ITS2, and ITS4 (White et al. 1990) designed for the lichen fungus. The PCR mix contained 1.25 U of Dynazyme Taq polymerase (Finnzymes), 0.2 mM of each of the 4 dNTPs, 0.5 µM of each primer, and 10–50 ng genomic DNA. The PCR products were cleaned by using a Quiaquick PCR product purification kit. Sequences were run on an ABI 310 automated sequencer. For the alignments of sequences, a Pile-up Programme was used, and the adjustments were done manually. For the photobionts, special *Trebouxia* primers (ITS1T and ITS4T, Kroken and Taylor 2000) were used.

Chromatographic Analysis Lichen thalli (100 mg dry weight) and vacuum-dried mycobionts (removed from the medium; 100 mg dry weight) were extracted for 4 hr with methanol at room temperature for HPLC. HPLC analysis was performed following Stocker-Wörgötter and Elix (2002) by using a Hitachi/Merck HPLC system that included two pumps, a Photodiode array detector (190–

800 nm wavelength range), a Beckman 5C18 column (4.6× 250 mm, 5 µm), and a computer system with an integration package based on Windows NT.

Extraction of Pure Metabolites To obtain pure barbatic and diffractaic acids (Fig. 2a,b), a preparative TLC was performed: the yield of ten mycobiont subcultures was collected and vacuum freeze–dried for 12 hr. Dried cultured material (ca 5–10 g) was extracted with acetone for 4 hr at room temperature. The mixture was transferred to a silica plate (as one continuous line, 160×5 mm). As the two substances are closely related, the separation was achieved by using solvent B (120 ml hexane, 90 ml methyl tertiary-butyl ether, 230 ml formic acid) and on TLC plates (20× 20 cm). The substance bands were scraped from the silica plate and redissolved in acetone. The supernatant that contained the pure lichen compound was carefully separated from the silica gel, and the remaining acetone was evaporated in a hood. Finally, the purity of the substance was checked by HPLC.

Algal Growth Experiments and Effect of Biologically Active Metabolites Growth experiments with *Trebouxia jamesii* (photobiont) were done on BBM agar plates over a period
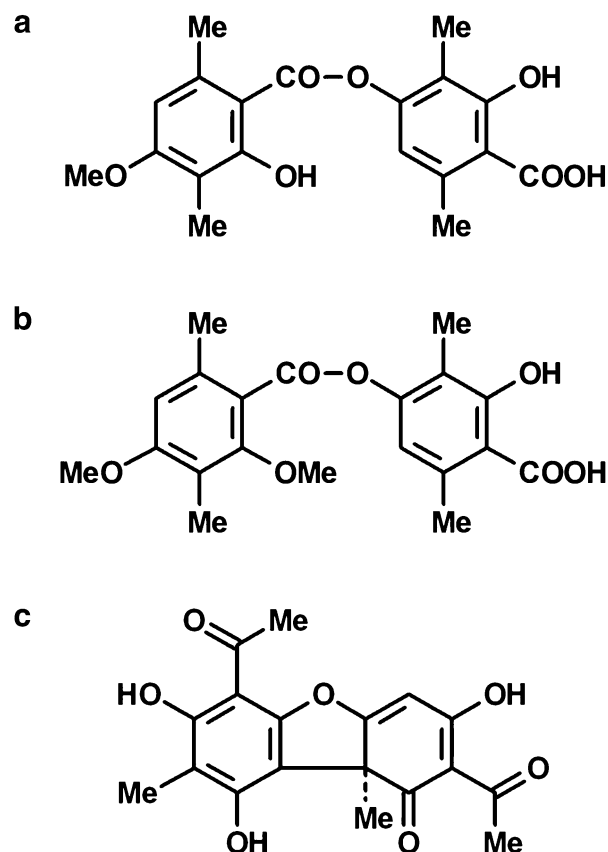
Fig. 2 Molecular structures of barbatic acid (**a**), diffractaic acid (**b**), and usnic acid (**c**)

of 1 mo in three different, independent experiments. In each, a layer of pure diffractaic or barbatic acid—extracted from metabolite-forming *Heterodea* mycobiont—was super-imposed onto the nutrient media by using three different concentrations (0.5, 2, and 4 mg cm^{-2}; the quantity was chosen considering the overall thallus contents). The effect on the growth of the algae (starting with algal colonies, ca 3 mg fresh weight) was documented by a weekly analysis of images taken by a digital camera fixed to a light microscope. In another study, the effect of usnic acid (extracted from *H. beaugleholei*, Fig. 2c) on photobiont division rates was tested in a similar way. After 1 mo, the algal cells were harvested, weighed, and the obtained values were statistically evaluated by considering mean values and SDs.

Statistical Analyses Statistics were calculated by using ANOVA procedure of SigmaStat 3.11 for Windows; SigmaPlot 9.01 for Windows was used for figure prepara-tion (Systat Software, Inc. 2004).

Results

HPLC analyses demonstrated that the voucher samples produce diffractaic acid as a major substance, whereas barbatic acid was detected as a minor satellite compound (Fig. 2a,b). Mycobiont cultures showed the highest growth rates on Murashige and Skoog (MS) medium (Stocker-Wörgötter 2001); development was accompanied by a more intense pigmentation than was found in cultures from other tested media (Fig. 1b).

Mycobiont cultures only began secondary metabolite production when they had been exposed to cold tempera-ture stress (4°C for 6 wk) or UV-C light (254 nm, 1 hr per d over 2 wk). As a consequence of this treatment, cultures produced diffractaic acid, barbatic acid, and probably their precursors and intermediates. UV-C treatment doubled the proportion of produced substances and provoked a strong pigmentation.

In the UV-C experiment, diffractaic acid was the major substance, but cold temperature exposure resulted in large amounts of barbatic acid; overall values increased more than 60 times compared with voucher specimens (Fig. 3). In both experiments, the overall secondary metabolite contents were significantly higher than those of the voucher specimen. Based on the quantities of lichen acids in the thallus (4.80 mg cm^{-2} diffractaic acid and 0.36 mg cm^{-2} barbatic acid), the concentrations used for the algal test series were calculated as 0.5, 2, and 4 mg cm^{-2} on the surface of the agar plate.

After 3 d of incubation, algal growth was reduced considerably on the plates with barbatic acid. However, the
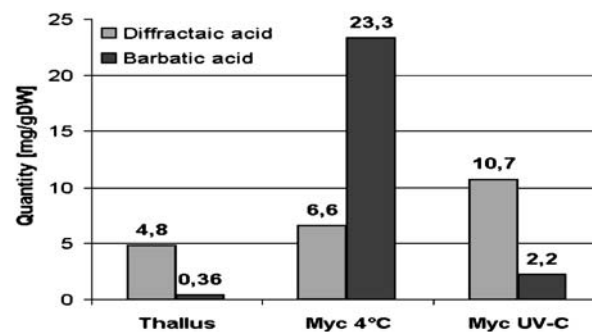
Fig. 3 Changes in lichen acid production of the cultures mycobionts (myc) due to temperature and UV-C stress in comparison with the thallus content

algal biomass in the dishes that contained diffractaic and usnic acids and in the negative control, algal growth increased from 550 to 650% after 1 mo in culture (Table 1, Fig. 4). No significant differences were recognized between the diffractaic and usnic acid treatment and the control culture with no lichen metabolites. *Trebouxia* colonies were able to double their biomass at low barbatic acid concen-trations (0.5 mg cm^{-2}), whereas the algal cells were seriously damaged at higher concentrations (2 mg cm^{-2}). Most of the cells died at the highest test concentration of 4 mg cm^{-2} (Figs. 5, and 6).

Discussion

As former studies have shown (Stocker-Wörgötter 2001; Hager and Stocker-Wörgötter 2005, Brunauer et al. 2006, 2007), the culturing of lichen mycobionts requires long-term experiments; numerous "case" studies have demon-strated that every "new" lichen fungus prefers a different composition of nutrient medium and specially adapted culture conditions. Many culture experiments are necessary to trigger the aposymbiotically grown mycobiont to produce specific secondary metabolites.

Table 1 Biomass increase of algal colonies on control and test-media after 1 mo (starting from 3 mg algal cells, $N=3$)

| Medium (BBM)	Concentration (mg cm^{-2})	Mean (mg)	Increase (%)	SD
Control		19.13	537.66	18.23
+Barbatic acid	0.5	6.60	120.00	7.39
	2.0	2.58	−14.00	5.01
	4.0	1.95	−35.00	7.83
+Diffractaic acid	0.5	19.66	555.33	23.36
	2.0	20.65	588.33	28.47
	4.0	21.39	613.00	33.69
+Usnic acid	0.5	20.21	573.66	12.45
	2.0	20.44	581.33	37.51
	4.0	21.33	611.00	28.22
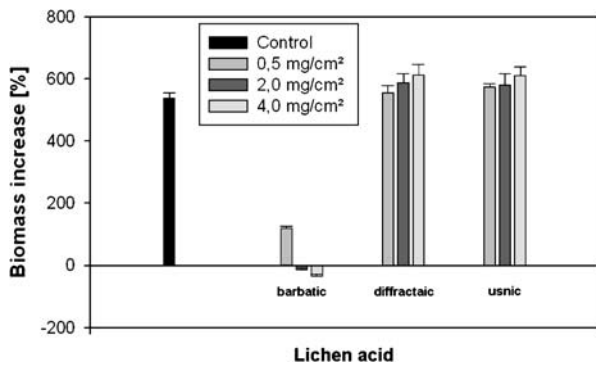
Fig. 4 Increase of algal colony biomass on the different lichen acid layers after 1 mo. *Error bars* represent SE

In some cases, lichen culturing is further complicated by the isolation of epi- and endolichenic fungi and algae. To identify the lichen mycobiont and to distinguish it from occasionally isolated, lichenicolous fungi, DNA canalyses of the ITS region have been performed. If the mycobiont produces lichen substances in culture, the fungus can be identified by its chemical profile, and the produced lichen substances can be used as chemical markers. Chemical analyses by HPLC, TLC, and microcrystallization tests cannot screen contaminations by other fungi.

Culture of *H. muelleri* mycobionts on MS medium enriched with sugars resulted in increased and optimized growth rates. The characteristic brown pigmentation found in cultures grown on MS is probably correlated with the brown pigment that is normally formed by the apothecia of the voucher specimens (Miyagawa et al. 1994).

Stress experiments with *H. muelleri* mycobiont cultures yielded surprising results, especially the more than 60-fold increase of barbatic acid that resulted from cold temperature treatment. Low temperatures can set off an increased production of secondary metabolites, as reported by Stocker-Wörgötter (2001) for *Umbilicaria mammulata*. The question as to why stress due to UV-C exposure resulted in an increased biosynthesis of medullary lichen acids (in the intact lichen formed by medullary hyphae) remains open. Further experiments, using different types of UV irradiations (A, B, and C) have to be performed, as different wavelengths may play a crucial role in secondary compound synthesis. According to Swanson and Fahselt (1997) and BeGora and Fahselt (2001), UV-A irradiation alone caused the production of higher contents of phenolic compounds in lichens than exposure to both UV-A and UV-B. A definite correlation between production of lichen acids and the effects of UV-light was recently reported by

Fig. 5 Algal growth experiment example: growth over 1 mo of algal colonies on different lichen acid layers, concentration 2 mg cm^{-2}; *top*: algae on barbatic (**a**), diffractaic (**b**), and usnic acid (**c**) layer after transfer; *middle*: colonies on barbatic (**d**), diffractaic (**e**), and usnic acid (**f**) layer after 2 weeks; *bottom*: colonies on barbatic (**g**), diffractaic (**h**), and usnic acid (**i**) layer after 1 mo; *bar*=3 mm
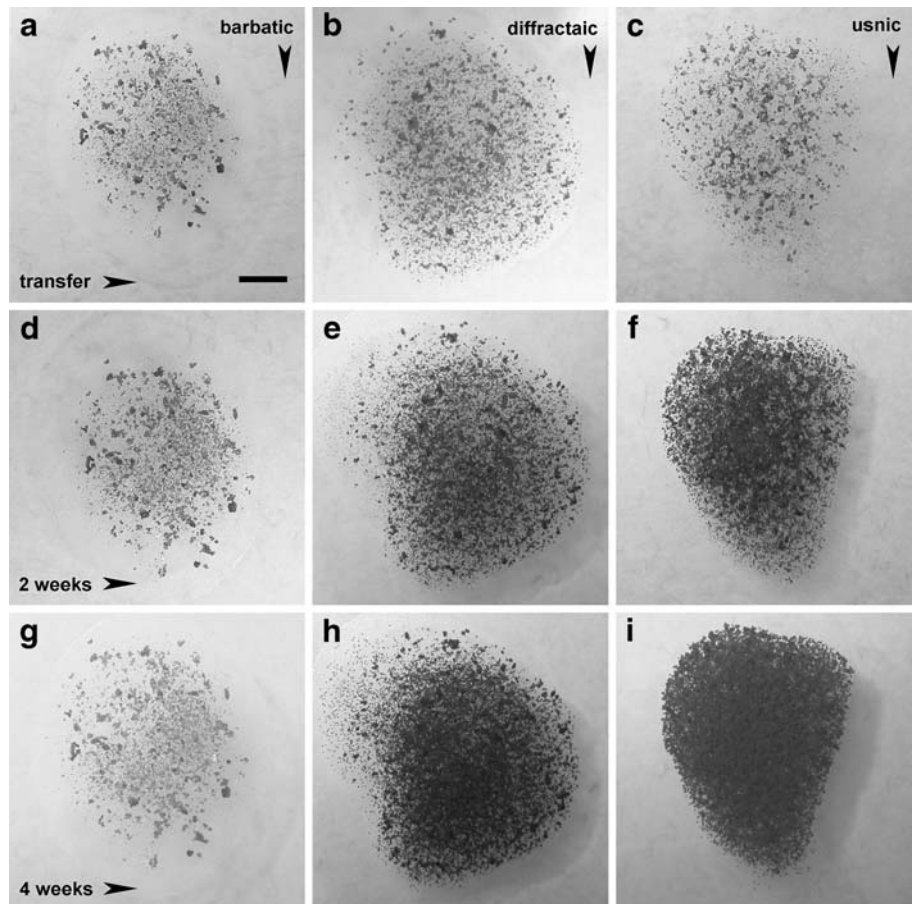
Fig. 6 Control colony after 1 mo (**a**); *bar*=3 mm. Algal cells of control colony (**b**), and of colony on 2 mg/cm² barbatic acid (**c**) after 1 mo; *bar*=10 μm
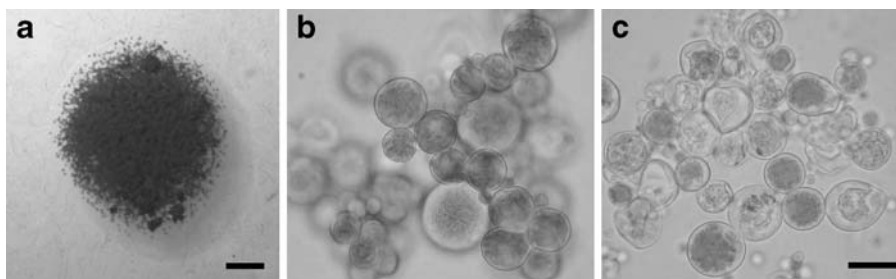
Solhaug et al. (2003) for parietin, an anthraquinone that is known to act as an UV-protecting agent in the cortex of *Xanthoria parietina*.

While barbatic and diffractaic acids are known to be biologically active metabolites that act as inhibitors of specific functions such as cell division and growth, usnic acid is a molecule with manifold activities (Table 2; Ingólfsdóttir 2002). Although the molecules are closely related, our experiments and those of others indicate that barbatic and diffractaic acids exhibit diverging biological activities. Barbatic acid, for example, impairs the function of photosystem II in higher plants, whereas diffractaic acid has fungicidal properties (Nishitoba et al. 1987; Dayan and Romagni 2001, 2002; Takahagi et al. 2006). In agreement with these experiments, our test series showed that the growth of photobionts exposed to barbatic acid concentrations higher than 0.5 mg.cm^{-2} was inhibited, and a significant loss of vitality and cell-bleaching was observed.

At such high concentrations, algae were killed depending on the lengths of exposure times. Whether cell death of the algae was caused by barbatic acid inhibiting photosystem II or due to another mode of action remains to be determined. The concentration of 0.5 mg.cm^{-2}, which corresponds to the quantity present in the *H. muelleri* thallus, slowed down cell division rates of the *Trebouxia* photobionts. This result clearly shows that barbatic acid acts as a regulating agent for algal growth and mitosis in the lichen.

Photobionts incubated on BBM+d and BBM+u were not affected in comparison to the negative control. Although Schimmer and Lehner (1973) reported that usnic acid inhibited the growth of the free-living unicellular green alga *Chlamydomonas reinhardii*, it had no impact on the growth of *Trebouxia jamesii* in our experiments. The increase in algal biomass on BBM+u was similar to that of the negative control. As usnic acid is accumulated solely in the thallus cortex, this outcome was expected. Effects of usnic acid on

Table 2 Overview of known activity of the lichen acids tested in this study

Metabolite	Activity	Publication
Barbatic acid	Inhibition of EBV activation	Yamamoto et al. 1995
	Non-redox inhibitors of Leukotriene B4 biosynthesis	Kumar and Müller 1999b,c
	PS II inhibitor	Dayan and Romagni 2001
	Inhibition of PSII in atrazine-tolerant tobacco cells	Takahagi et al. 2006
Diffractaic acid	Inhibition of EBV activation	Yamamoto et al. 1995
	Analgesic and antipyretic agent	Okuyama et al. 1995
	Moderate inhibitor of arachidonate 5-lipoxygenase	Kumar and Müller 1999a,b,c
	Fungitoxic	Dayan and Romagni 2001
	Antiulcerogenic	Bayir et al. 2006
Usnic acid	Most potent antibiotic agent	Klosa 1953
	Growth inhibition of *Chlamydomonas reinhardii*	Schimmer and Lehner 1973
	Antitumor activities (Lewis lung-carcinoma test system)	Kupchan and Kopperman 1975
	Inhibits mitosis in *Allium*	Huovinen and Lampero 1989
	Antitranspiratory	Carbonnier et al. 1993
	Inhibition of EBV activation	Yamamoto et al. 1995
	Antipyretic and analgesic component	Okuyama et al. 1995
	In vitro activity against *Staphylococcus* and *Enterococcus*	Lauterwein et al. 1995
	Antimitotic effects	Cardarelli et al. 1997
	Antiproliferative activity, antisporiatic properties	Kumar and Müller 1999c
	Antimicrobial, fungicidal, herbicidal activity	Dayan and Romagni 2001
	Antifungal activity	Halama and Van Haluwin 2004
	anticancer properties	Mayer et al. 2005

epibiontic algae that grow on the lichen have to be tested. Usnic acid has various biological activities in several plants and even humans. According to Swanson and Fahselt (1997) it also has important functions in the lichen itself, e.g., as UV protection. While usnic acid located in the cortex could act against uncontrolled propagation of epibiontic algae without having any impact on the photobiont, compounds such as diffractaic acid, deposited and accumulated by medullary hyphae, could effectively restrict the growth of endolichenic fungi (Dayan and Romagni 2001).

It has to be emphasized that the two depsides tested, diffractaic and barbatic acids, exhibit two fundamentally different biological activities, although the molecules differ merely in one functional unit (diffractaic acid has a CH_3-group at the side chain of C2, barbatic acid has a hydrogen), a phenomenon often observed by closely related biologically active substances. Further investigations are planned to identify the effects of barbatic acid on functional aspects of the lichen symbioses The investigated substances and their derivatives will be tested also on the photobionts of other lichens and on free-living algae, and their influence on the photosynthesis will be evaluated.

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