Lichen Secondary Metabolites from the Cultured Lichen Mycobionts of *Teloschistes chrysophthalmus* and *Ramalina celastri* and their Antiviral Activities

Alejandra T. Fazio[a], Mónica T. Adler[a], María D. Bertoni[a], Claudia S. Sepúlveda[b], Elsa B. Damonte[b], and Marta S. Maier[c,*]

[a] Laboratorio de Liquenología, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, (1428) Buenos Aires, Argentina
[b] Laboratorio de Virología, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, (1428) Buenos Aires, Argentina
[c] Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, (1428) Buenos Aires, Argentina. Fax: 54 11 45 76 33 85. E-mail: maier@qo.fcen.uba.ar

* Author for correspondence and reprint requests

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Lichens and spore-derived cultured mycobionts of *Teloschistes chrysophthalmus* and *Ramalina celastri* were studied chemically, and results indicated that they produced, respectively, parietin and usnic acid as major secondary metabolites, which were purified and identified. Identification of the compounds was performed by high performance liquid chromatography and structural elucidation by nuclear magnetic resonance (^{1}H) and electron impact mass spectrometry. Usnic acid exhibited antiviral activity whereas parietin had a virucidal effect against the arenaviruses Junín and Tacaribe.

Key words: Lichen Mycobiont, Parietin, Usnic Acid

Introduction

Since the pioneer works of Ahmadjian, culture investigations of separated lichen symbionts were developed to study their behaviour and to understand the symbiosis. Some of these studies, particularly on cultured mycobionts, tried to improve the culture conditions to trigger and enhance the synthesis of secondary metabolites, to reveal the factors involved in this process, to understand the role of each of the partners in the synthesis of the lichen substances, or to find sources of therapeutic agents. Chemical studies of isolated cultured mycobionts reported different types of results. In some cases the aposymbiotic fungal strains synthesized the same secondary metabolites as the natural lichen, all (Stocker-Wörgötter and Elix, 2004) or only some of them (Ahmadjian, 1993; Huneck and Yoshimura, 1996). Other examples showed that some mycobionts produced secondary metabolites different from those present as major compounds in the symbiotic state (Ahmadjian, 1993; Huneck and Yoshimura, 1996), including novel molecules such as the graphislactones, graphenone and xanthones (Hamada *et al.*, 2001).

Many lichen secondary metabolites exhibit antibiotic, antitumour, antimutagenic, allergenic, antifungal, antiviral, enzyme inhibitory and plant growth inhibitory properties (Huneck and Yoshimura, 1996; Elix, 1996). Lichen mycobionts, as other fungi, could therefore be a potential source in the search for pharmaceutical useful chemicals (Crittenden and Porter, 1991).

The primary goal of the present work was to study in axenic culture the aposymbiotic mycobionts isolated from the lichens *Teloschistes chrysophthalmus* (L.) Fr. and *Ramalina celastri* (Spreng.) Krog & Swinscow, and establish conditions where secondary metabolites are synthesized successfully. Finally, the purified substances from these sources were assayed for antiviral and virucidal activities.

Results and Discussion

Polyspore cultures of mycobionts were obtained after ascospore discharge from apothecia onto agarized BBM (Bold's basal medium) (Ahmadjian, 1993). The aposymbiotic mycobionts of both lichen species were transferred to different media.

Mycobiont growth on Lilly and Barnett medium with glucose (LBG) (Ahmadjian, 1993) and MME (modified malt extract; 2% malt extract, 0.1% peptone, 1% glucose, 2% agar) medium (Culberson *et al.*, 1992) of both species was poor whereas the development on MEYE (2% malt extract, 0.2% yeast extract, 2% agar) (Ahmadjian, 1993) medium was considered satisfactory (about 1 mm in diameter per month, or more). After 8 months of culture, colonies of *T. chrysophthalmus* were conical to cerebroid, pinkish orange to brownish orange and about 0.5–0.8 cm in diameter whereas those of *R. celastri* attained 1.0–1.5 cm in diameter and were also conical to cerebroid, pink at first, turning light greenish yellow with age, and slightly tan at the apex when harvested.

The acetone extracts of the lichen *T. chrysophthalmus* and its mycobionts were purified by chromatographic procedures to afford 6.3 mg (0.60%) and 8.0 mg (0.48%) of orange crystals of parietin (**1**), respectively. Compound **1** was identified by comparison of its ^{1}H NMR and EI-MS spectra with published data (Huneck and Yoshimura, 1996; Manojlovic *et al.*, 2000). Analysis by HPLC-DAD of the extracts and comparison of the retention time of compound **1** (34.74 min) and its UV spectrum with those of a standard allowed to identify parietin (Fig. 1) in the lichen and the mycobiont. The acetone extract of *R. celastri* mycobionts was purified by preparative TLC to afford usnic acid (**2**) (1.6 mg, 0.58%) as yellow crystals. Its structure was characterized by ^{1}H NMR and EI-MS and comparison with reported data (Huneck and Yoshimura, 1996; König and Wright, 1999) (Fig. 1). The rotatory power of **2** suggested the presence of a mixture of both enantiomers with predominance of (+)-usnic acid (Huneck and Yoshimura, 1996).

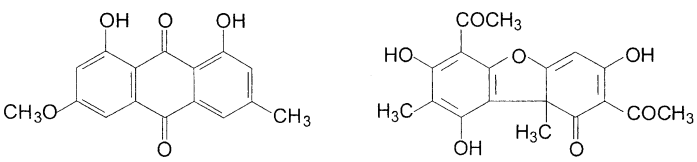
The concentration of usnic acid (**2**) in the voucher specimen of *R. celastri* was very low. Its content in the lichen extract was quantified by reversed phase HPLC-DAD. Analysis of the chromatographic peak at a retention time of 31.81 min and comparison of its UV spectrum with that of a standard allowed the identification of usnic acid and the determination of its content in the lichen (0.08%).

Previous publications reported the synthesis of parietin and usnic acid by some *in vitro* cultured mycobionts (Nakano *et al.*, 1972; Honegger and Kutasi, 1989; Takenaka *et al.*, 2002; Rosso *et al.*, 2003; Komiya and Shibata, 1969; Hamada, 1991; Hamada *et al.*, 1996, 1997). Our results are the first reports of biosynthesis of these secondary metabolites by the isolated mycobionts of *T. chrysophthalmus* and *R. celastri*. As both metabolites were produced by isolated axenic mycobionts, it can be concluded that in these two cases, the photobionts in the lichens do not play a decisive role in their synthesis.

Present yields of parietin were 0.48% in mycobionts of *T. chrysophthalmus* and 0.60% in lichen thalli, whereas those of usnic acid in *R. celastri* were 0.58% for the mycobionts but only 0.08% in thallus material. In the first case the mycobionts produced similar levels of the major secondary metabolite as the lichen, but in the second case the isolated mycobiont yielded much higher contents of usnic acid. Consequently in these two species lichen materials could be satisfactorily substituted by their respective mycobionts as secondary metabolites sources. The present data are promising with regard to the use of cultured mycobionts for the production of secondary metabolites *in vitro*.

To evaluate the biological activities of the two mycobiont-isolated compounds, parietin (**1**) and usnic acid (**2**), their cytotoxicity to Vero cell cultures was analyzed. After incubation with serial two-fold dilutions of each compound, the concentrations required for 50% reduction in Vero cell viability (CC_{50}) were determined by the MTT method. Parietin (**1**) was the less cytotoxic compound to Vero cells, with a CC_{50} value of 347.0 μM, whereas the CC_{50} for usnic acid (**2**) was 65.1 μM (Table I).

The antiviral activity of both compounds was studied at concentrations below the CC_{50} by a virus yield inhibition assay. The arenavirus JUNV (Junin virus), the agent of Argentine hemorrhagic



1 **2**

Fig. 1. Chemical structures of parietin (**1**) and usnic acid (**2**).

Table I. Cytotoxicity, antiviral activity and selectivity indices.

Compound	CC_{50}[a] [μM]	EC_{50}[b] [μM]		IS[c]	
		JUNV	TCRV	JUNV	TCRV
Parietin (**1**)	347.0	> 200	> 200	Inactive	Inactive
Usnic acid (**2**)	65.1	9.9	20.6	6.8	3.2

[a] Cytotoxic concentration 50%: compound concentration required to reduce cell viability by 50%.
[b] Effective concentration 50%: compound concentration required to reduce virus yield by 50%.
[c] Selectivity index: ratio CC_{50}/EC_{50}.

fever in humans (Damonte and Coto, 2002) and included in the Category A Pathogen List of the Centers for Disease Control and Prevention (CDC, USA) as potential agent of bioterrorism (Rotz *et al.*, 2002), was used as model system. Usnic acid (**2**) specifically reduced JUNV production from infected Vero cells in a dose-dependent manner and the 50% inhibition in JUNV yield was obtained at a concentration of 9.9 μM (Table I). By contrast, no inhibition of JUNV yield was observed after treatment with parietin (**1**) at concentrations up to 200 μM (Table I). The antiviral properties of usnic acid were also evaluated against TCRV (Tacaribe virus), a non-pathogenic member of the family Arenaviridae which antigenically closely related to JUNV (Damonte and Coto, 2002). The EC_{50} value for compound **2** against TCRV was 20.6 μM (Table I). Thus, the selectivity indices (ratio CC_{50}/EC_{50}) of usnic acid for JUNV and TCRV were 6.8 and 3.2, respectively (Table I), indicating that the antiviral activity of this metabolite against these viruses is specific and not a consequence of its action on cell toxicity. Again, parietin did not exhibit antiviral activity against TCRV (Table I).

To test the possibility if these compounds had virus-inactivating properties by a direct effect on the virion particles, a virucidal assay was performed. When suspensions of JUNV or TCRV particles were incubated with usnic acid before cell infection, no differences in remaining infectivity titres between compound-treated and untreated virus suspensions were detected (Fig. 2). Thus, the virus inhibitory effect observed in the yield inhibition assay was due to a real antiviral activity exerted during virus multiplication in the host cell. Surprisingly, **1** exerted a direct virucidal effect producing a dose-dependent inactivation of JUNV and TCRV suspensions after incubation of both viruses with the compound (Fig. 2). The IC_{50} values of parietin (**1**) (concentrations required to inactivate 50% of virions) were 9.7 μM and 20 μM for JUNV and TCRV, respectively.

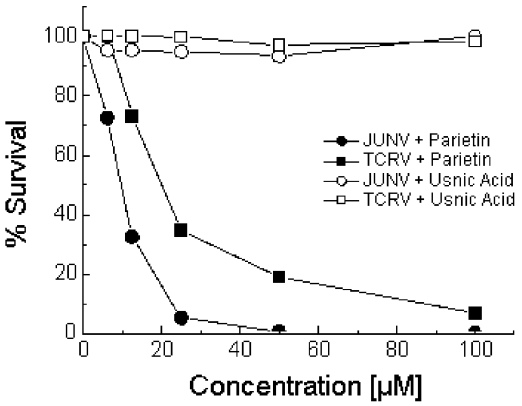


Fig. 2. Virucidal activity of parietin (**1**) and usnic acid (**2**). Suspensions of JUNV or TCRV particles were incubated with different concentrations of usnic acid or parietin for 1.5 h at 37 °C, and then remaining infectivity was determined by a plaque formation assay. Each value is the mean of two independent experiments.

Only a few studies on antiviral effects of usnic acid (**2**) have shown that it has *in vitro* inhibitory action on proliferation or cytopathic effects of mouse polyomavirus (Campanella *et al.*, 2002), herpes simplex type 1 (HSV-1) and polio type 1 viruses (Perry *et al.*, 1999). The results presented in this study have extended the spectrum of antiviral properties of **2**, demonstrating a selective inhibition of the multiplication of the arenaviruses JUNV and TCRV in Vero cells at concentrations not affecting the cell viability. Concerning parietin (**1**), the present study is the first report on its virus inhibitory effects. Other naturally occurring anthraquinones were found active against HSV-1, but only in the presence of light (Cohen *et al.*, 1996). Here, parietin has been shown to contain a specific virucidal action against both arenaviruses, JUNV and TCRV, inactivating virus infectivity.

Studies cited earlier and others by *e.g.* Ahmadjian (1993) and Huneck and Yoshimura (1996) showed that so far, no universal culture conditions have been found, that, combined, will induce the synthesis of natural lichen substances in all or most axenically grown mycobionts. On the contrary, each mycobiont strain usually has its own particular conditions for optimum growth and substance production. Indeed, important differences of behaviour in culture have been observed in mycobionts isolated from related species [*e.g.* of *Xanthoria* (Honegger and Kutasi, 1989)]. Improvement of mycobiont culture for the production of secondary metabolites has a theoretic value with respect to the lichen symbiosis. It also has potential for practical application. If substances with significant biological activity can be produced *in vitro* with high yields, they could be of pharmaceutical interest. The results described in this paper, suggest that there are promising prospects for using lichen mycobiont cultures as a source of useful bioactive compounds. They also demonstrate the need for further studies on lichen mycobionts, whose behaviour is still poorly understood.

Experimental

General procedures

^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker AM 500 spectrometer. Electron impact mass spectra were collected on a Shimadzu QP-5000 mass spectrometer. Optical rotation was determined on a Perkin-Elmer 343 polarimeter. Analytical HPLC was carried out on a Gilson 506C HPLC system using a Phenomenex Hypersil 5 μm column (25 cm × 4.6 mm i.d.). Compounds were detected using a 170 photodiode array detector set at 245 nm, operated in series with a Unipoint System software, recording the absorption spectrum in the range 200–400 nm.

Lichen materials

Lichens on tree bark were collected at two different localities in Argentina: *T. chrysophthalmus* at Suipacha (Buenos Aires Province) by N. Venedikian, and *R. celastri* in the surroundings of El Palmar National Park (Entre Ríos Province) by B. Lechner. After collection lichens were air-dried at room temperature. Voucher specimens of both lichens are kept at BAFC (Buenos Aires Facultad de Ciencias Exactas y Naturales Herbarium) (Holmgren *et al.*, 2001), under numbers 39167 and 39217, respectively.

Culture procedures

Fresh collected apothecia were selected and washed for 30 min in tap water with drops of Tween 20, soaked and rinsed in sterile water for 1 h and blotted to remove excess water. Apothecia were then fixed with petroleum jelly to the cover of Petri dishes that were turned upside down to let the spores discharge upwards onto the medium (Hamada, 1989). After several days of incubation at 23 °C in a culture chamber in continuous light, the germinated spores were transferred to MEYE medium (2% malt extract, 0.2% yeast extract, 2% agar) (Ahmadjian, 1993). The colonies were successively subcultured on the same medium to obtain enough colonies for chemical studies, and finally incubated for 8 months in the same medium and light conditions. BAFC-culture registration numbers of mycobiont strains are 3119 (*T. chrysophthalmus*) and 3120 (*R. celastri*).

Extraction and isolation of lichen thalli secondary metabolites

Dried lichen material of *T. chrysophthalmus* (1.0 g dry weight) was extracted with acetone at room temperature for one week. After evaporation to dryness, the extract (36.3 mg) was purified by preparative TLC (silica gel) eluting with CH_2Cl_2/AcOEt/AcOH (90:8:2) to afford compound **1**.

Dried lichen material of *R. celastri* (192 mg dry weight) was extracted with acetone at room temperature for one week and evaporated to dryness to obtain 2.2 mg of extract. Due to the scanty amount of extract the identification and quantification of compound **2** was performed by HPLC-DAD.

Extraction and isolation of mycobiont secondary metabolites

Freshly harvested colonies of *T. chrysophthalmus* (1.66 g dry weight at 50 °C) and *R. celastri* (0.28 g dry weight at 50 °C) were extracted with acetone at room temperature during one week. After evaporation to dryness, the extract of *T. chrysophthalmus* mycobionts (187.7 mg) was purified by silica gel column chromatography using cyclohexane and cyclohexane/acetone mixtures with increasing amounts of acetone. Compound **1**

(2.6 mg) was eluted with cyclohexane/acetone (96:4). Fractions eluted with cyclohexane/acetone (80:20), (70:30) and (50:50) were submitted to preparative TLC [silica gel F_{254}, 2 mm, Merck, CH_2Cl_2/AcOEt/AcOH (90:8:2)] to give 5.4 mg of **1**. Therefore, 8.0 mg of compound **1** were obtained.

The acetone extract of *R. celastri* mycobiont was evaporated to dryness (17.4 mg) and purified by preparative TLC [silica gel F_{254}, 2 mm, Merck, toluene/AcOH (170:30)] to give 1.6 mg of compound **2**.

*Parietin (**1**)*: Orange amorphous powder; m.p. 207–208 °C (after crystallization in acetone). – ^{1}H NMR (500 MHz): δ = 2.46 (s, 3H, CH_3), 3.94 (s, 3H, OCH_3), 6.69 (d, J = 2.5 Hz, 1H, H-2), 7.09 (m, 1H, H-7), 7.38 (d, J = 2.7 Hz, 1H, H-4), 7.64 (m, 1H, H-5), 12.11 (s, 1H, OH), 12.25 (s, 1H, OH). – EI-MS: *m/z* (rel. int.) = 284 [M^+] (100), 255 (15), 241 (9), 226 (6), 213 (6).

*Usnic acid (**2**)*: Yellow amorphous powder; m.p. 203–204 °C (after crystallization in $CHCl_3$/EtOH). – $[\alpha]_D^{25}$ + 18.3° ($CHCl_3$, *c* 1.00). – ^{1}H NMR (500 MHz): δ = 1.77 (s, 3H, CH_3-13), 2.12 (s, 3H, CH_3-16), 2.67 (s, 3H, CH_3-15), 2.68 (s, 3H, CH_3-18), 5.98 (s, 1H, H-4), 11.03 (s, 1H, OH). – EI-MS: *m/z* (rel. int.) = 344 [M^+] (4), 284 (5), 260 (4), 241 (2), 233 (7).

Analysis of extracts by HPLC

10 μl aliquots of the lichen and mycobiont extracts were analyzed by reversed-phase HPLC-DAD based on a protocol reported previously (Feige *et al.*, 1993). The samples were eluted with a two solvent system at a rate of 1 ml min^{-1}. Solvent A was 1% aqueous orthophosphoric acid/MeOH (7:3) and solvent B was MeOH. The gradient started with 0% B and raised to 58% B within 15 min, then to 100% B within 15 min, followed by 100% B for 10 min. In order to quantify the content of compound **2** in the acetone extract of *R. celastri*, a standard of usnic acid was diluted to concentrations of 380, 190, 95 and 45 μg ml^{-1} to prepare the calibration curve under the same experimental conditions as described above.

Cells and virus

Vero cells were grown in Eagle's minimal essential medium (MEM, GIBCO, Rockville, MD) containing 5% inactivated fetal bovine serum and 50 μg/ml gentamycin. Maintenance medium (MM), pH 7.5, consisted of MEM supplemented with 1.5% fetal bovine serum and gentamycin. The naturally attenuated IV4454 strain of Junin virus (JUNV) obtained from a mild human case (Contigiani and Sabattini, 1977) and the TRLV 11573 strain of Tacaribe virus (TCRV) (Downs *et al.*, 1963) were used. Virus stocks were prepared in Vero cell cultures and titrated by plaque formation.

Cytotoxicity assay

Vero cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich] method (Mosmann, 1983) as described elsewhere (García *et al.*, 2002). Parietin and usnic acid were solubilized in DMSO to a final concentration of 100 mM, stored at 4 °C and serially diluted (100 μM – 6.25 μM). Cytotoxicity was calculated as the compound concentration required to reduce the MTT signal by 50% compared to controls (CC_{50}).

Antiviral and virucidal activities

For the antiviral assay, Vero cell monolayers grown in 24-well microplates were infected with JUNV at a MOI of 0.1 for 1 h at 37 °C in 5% CO_2. Virus inocula were removed and refed with MM containing different concentrations of the compounds (2 wells per concentration). After 24 h of incubation at 37 °C in 5% CO_2, supernatant cultures were harvested and extracellular virus yields were determined by a plaque assay. The 50% effective concentration (EC_{50}) was calculated as the concentration required to reduce the virus yield by 50% in the compound-treated cultures compared with untreated ones.

To measure the virucidal activity, equal volumes of a virus suspension containing approximately 1×10^6 PFU of either JUNV or TCRV and various concentrations of compounds in MM were mixed, incubated for 1.5 h at 37 °C, and then remaining infectivity was evaluated as described previously (García *et al.*, 2002). The 50% inactivating concentration (IC_{50}), the concentration required to inactivate virions by 50%, was calculated.

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