The phytotoxic lichen metabolite, usnic acid, is a potent inhibitor of plant *p*-hydroxyphenylpyruvate dioxygenase

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Abstract The lichen secondary metabolite usnic acid exists as a (−) and a (+) enantiomer, indicating a α or β projection of the methyl group at position 9b, respectively. (−)-Usnic caused a dose-dependent bleaching of the cotyledonary tissues associated with a decrease of both chlorophylls and carotenoids in treated plants whereas no bleaching was observed with the (+) enantiomer. (−)-Usnic acid inhibited protophorphyrinogen oxidase activity (I_{50} = 3 μM), but did not lead to protoporphyrin IX accumulation. Bleaching appears to be caused by irreversible inhibition of the enzyme 4-hydroxyphenylpyruvate dioxygenase by (−)-usnic acid (apparent IC_{50} = 50 nM).

Key words: Bleaching herbicide; Lichen; *p*-Hydroxyphenylpyruvate dioxygenase; Phytotoxin; *Alectoria sarmentosa*

1. Introduction

Lichens, the quintessential symbiosis between a fungal (mycobiont) and algal and/or cyanobacterial (photobiont) partner, produce a variety of secondary compounds. These compounds typically arise from the secondary metabolism of the fungal component and are deposited on the surface of the hyphae rather than compartmentalized in the cells. Most of these compounds are unique to lichens, with only a small minority (ca. 60) occurring in other fungi or higher plants [1]. While the secondary chemistry of lichen compounds is better documented than in any other phylogenetic group due to a long history of chemotaxonomic study, the bioactivity associated with these compounds has been generally ignored.

Of the more than 20 000 known species of lichens, only a few have been analyzed and identified as containing biologically active secondary compounds. A prime example is the antimicrobial compound, usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H9βH)-dibenzofurandione), commonly found in the genus *Usnea* [1] (Fig. 1). Usnic acid has been documented to have antihistamine, spasmolytic, antiviral, and antibacterial activities. Two biologically active natural enantiomers of usnic acid, differing in the orientation of the methyl group at 9b on the otherwise rigid molecule, have been identified as showing different biological activities and mechanisms of action. Proska et al. [2] reported that (−)-usnic acid inhibited urease and arginase activity. There are several reports [3,4] that the (+)-enantiomer is a more effective antimicrobial agent, although no specific mode of action was determined.

Limited studies have documented phytotoxic effects of usnic acid including inhibition of transpiration and oxygen evolving processes in maize and sunflower seedlings [5]. Studies of mouse mitochondria have suggested that (+)-usnic acid uncouples oxidative phosphorylation at levels of 1 μM [6]. However, a definitive explanation of the phytotoxicity of usnic acid has, to our knowledge, never been reported.

The objective of this study was to determine the primary mechanism of action of usnic acid on plants using a systematic approach developed by our laboratory [7]. A second objective was to compare the effect of the two enantiomers to ascertain whether they possessed different mechanisms of action.

2. Materials and methods

2.1. Materials

(−)-Usnic acid was isolated from *Alectoria sarmentosa* (Ach.) collected in Coeur d'Aléne, ID in August, 1999. Lichen samples were collected and identified by J. Romagni and voucher specimens are located in the University of Mississippi herbarium. All other chemicals, including (+)-usnic, were purchased from Sigma-Aldrich (Sigma-Aldrich, Milwaukee, WI, USA).

2.2. Isolation of (−)-usnic acid

(−)-Usnic acid was extracted with acetone (3×500 ml) from *A. sarmentosa* at ambient temperature. The acetone extract was evaporated and the residue was sonicated with methylene chloride and filtered. The filtrate was evaporated and the residue was crystallized from ether to give (−)-usnic acid (1.8 g) that was recrystallized from chloroform/ethanol to give yellow crystals (melting point 200–201°C; $[\alpha]_D$ −486°C, (c = 0.34, $CHCl_3$)); Spectral data were in agreement with those reported [8].

2.3. Dose-response assays

Twenty-five lettuce seeds (*Lactuca sativa* cv. iceberg) or onion (*Allium cepa* L. cv. Evergreen Bunching) were placed on sterile 55-mm diameter filter paper fitted to 60×15-mm petri dishes. Filter paper was premoistened with 3 ml of the test solutions. Usnic acid stock solutions were prepared in acetone to obtain final assay concentrations of 100, 33, 10, 3, 1, 0.3, 0.1, and 0.03 μM with the volume of carrier solvent being $<1\%$ in the assay. Controls received similar amounts of acetone. The plates were maintained in a growth chamber (25°C, 200 μmol m^{-2} s^{-1}, 16/8 h light cycle) for 6 days. Growth was monitored by measuring root length in mm. Each treatment was triplicated and the experiment was repeated.

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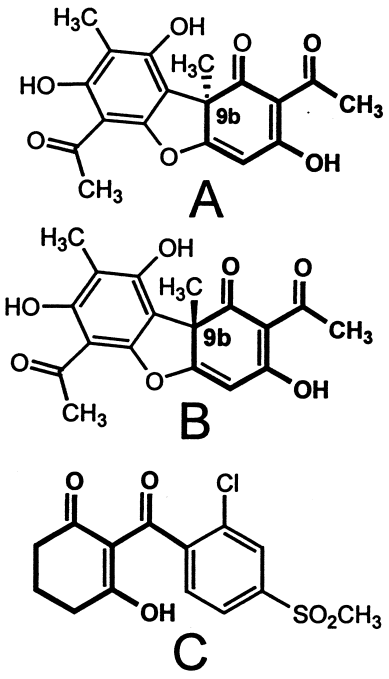

Fig. 1. Structures of (A) (−)- and (B) (+)-usnic acids showing the triketone reactive centers in bold and the stereogenic center at 9b. The structure of sulcotrione (C), a synthetic triketone herbicide targeting HPPD.

2.4. Effect of usnic acid on chlorophyll and carotenoid content

Chlorophyll was extracted from 40 mg of leaf tissue per treatment in 3 ml of dimethyl sulfoxide [9]. Chlorophyll concentration was determined spectrophotometrically according to Arnon [10]. Carotenoid analysis was determined spectrophotometrically using methods from Sandmann [11].

2.5. Determination of chlorophylll/carotenoids spectra by HPLC

Treated and control cucumber cotyledons were collected in dim green light and homogenized in 2 ml of basic methanol (methanol: 0.1 N NH_4OH; 9:1 vol.:vol.). The samples were centrifuged at $9000\times g$ for 20 min and the supernatant was collected. The pellet was re-suspended in an additional 1 ml basic methanol and spun at $9000\times g$ for an additional 20 min. The resultant supernatant was collected and added to the original 2 ml, and filtered through a 0.2-μm nylon syringe filter before analysis by HPLC.

2.6. HPLC conditions

HPLC conditions were modified from Dayan et al. [12]. The HPLC system was composed of Waters Associates (Milford, MA, USA) components, which included a Model 510 pump, a Model 712 autosampler, a Millennium 2010 controller and Models 470 fluorescence and 996 photodiode spectrophotometric detectors. The column was a 4.6×250-mm (ID) Spherisorb 5 μm ODS-1 reversed phase preceded by a Bio-Rad ODS-5S guard column. The solvent system consisted of a gradient beginning at 60% HPLC-grade methanol and 40% ddH_2O. At 10 min, the gradient was 100% methanol and at 30 min, it returned to the original settings. The injection volume was 100 μl. Pigment detection was performed with a fluorescence detector with excitation and emission wavelength settings at 440 and 630 nm, respectively, and the peaks were confirmed by scanning them from 300 to 700 nm with the photodiode array detector.

2.7. Effect of usnic acids on membrane integrity

Cucumber seedlings (*Cucumis sativus* 'Long Green Improved') were grown in a growth chamber maintained at a temperature of 25°C and a photon flux density (PFPD) of 200 μmol s^{-1} m^{-2} continuous illumination. Electrolyte leakage induced by usnic acid was determined using 4-mm cotyledon discs as described by Kenyon et al. [13]. The Petri dishes were placed in the dark at 25°C for 22 h and then exposed to 325 μmol m^{-2} s^{-1} of light for the remainder of the experiment.

2.8. Protoporphyrinogen oxidase (Protox) assay and binding study

Crude etioplast preparations were obtained from 10-day-old dark-grown barley seedlings according to the method of Sherman et al. [14]. Preparation of the enzyme substrate protoporphyrinogen and the Protox assay were performed as described by Dayan et al. [12]. The ability of usnic to displace the diphenyl ether herbicide acifluorfen from its binding site on Protox was done according Tischer and Strotmann [15] as modified by Dayan et al. [12].

2.9. Determination of p-hydroxyphenylpyruvate dioxygenase (HPPD) activity in vitro

Recombinant HPPD from *Arabidopsis thaliana* was overexpressed in *Escherichia coli* JM105 with pTrc 99A-AT4-HPPD plasmid grown as described by Maniatis et al. [16]. Expression of the vector was induced by IPTG (1 mM) when bacterial growth was equivalent to an A_{600} of 0.6. The cells were incubated for another 17 h at 30°C and harvested by centrifugation ($6000\times g$). The pellet was resuspended in buffer (20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM DTT, 1 mM 6-aminohexanoic acid, 1 mM benzamidine) and lysed by sonication (Branson Sonifier 450, Danbury, CT, USA). A cell-free supernatant was obtained by centrifugation at $35\,000\times g$ for 30 min.

The reaction mixture, consisting of 185 μl of assay buffer (50 mM sodium ascorbate in 100 mM Tris–HCl, pH 7.5) and 50 μg protein, was incubated for 15 min on ice with various concentrations of inhibitors. Compounds were tested at final concentrations ranging from 0.01 to 100 μM in half-log increments. Controls received the same volume (4 μl) of solvent used to deliver the inhibitors. The reaction was initiated by adding 5 μl of 4-hydroxyphenylpyruvate (10 mM in methanol) for a total volume of 200 μl.

The reaction was stopped after 15 min incubation at 30°C by addition of 70 μl of 20% perchloric acid (vol./vol.). The supernatant obtained after centrifugation ($20\,000\times g$ for 5 min) was subjected to HPLC analysis for the determination of homogentisic acid produced. The HPLC system was identical to the one described above, except chromatographic separation was obtained with a 3.9-mm×15-cm Pico Tag reversed phase column preceded by a Bio-Rad ODS-5S guard column. The solvent system consisted of a linear gradient beginning at 0% (100% A) to 70% B from 0 to 17 min, 70 to 100% B from 17 to 20 min, 100% B from 20 to 24 min, 100 to 0% B from 24 to 28 min and 0% B from 28 to 35 min. The flow rate was 1 ml min^{-1} and the injection volume was 100 μl. Solvent A was 0.1% (vol./vol.) trifluoroacetic acid in ddH_2O and solvent B was 0.07% (vol./vol.) trifluoroacetic acid in 80% (vol./vol.) HPLC-grade acetonitrile/ddH_2O.

2.10. Binding study of HPPD

The nature of the interaction between the usnic acid enantiomers and their binding site on HPPD was determined according to Ellis et al. [17] by incubating various concentrations of HPPD 15 min with either 0.03 μM (−)-usnic acid or 0.1 μM (+)-usnic acid. HPPD activity was then measured as described above.

2.11. Computer modeling of usnic acids

The (+) and (−) usnic enantiomers were built using fragments from the structure library provided in Sybyl 6.3 (Tripos associates, St Louis, MO, USA) on a Silicon Graphics O_2 250 MHz R10 000. Each molecule was assigned appropriate stereochemistry and minimized according to Dayan and Allen [18]. The structures were then subjected to full geometry optimization via MOPAC using AM1 parameterization. Potential intramolecular hydrogen bonding was determined using the default settings.

3. Results

(−)-Usnic caused a dose-dependent bleaching of the cotyledonary tissues (Fig. 2) that ultimately led to the death of the seedlings, whereas (+)-usnic did not cause any significant changes in chlorophyll content. Early growth of lettuce seedlings as measured by root growth was not inhibited by usnic acid (data not shown).

Loss of chlorophylls in response to phytotoxins can be associated with light-dependent destabilization of cellular and sub-cellular membranes, but usnic acid apparently acts differ-

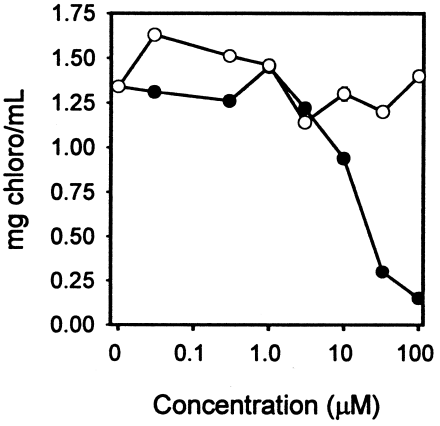

Fig. 2. Effect of (−)-usnic acid (-●-) and (+)-usnic acid (-○-) (0.03–100 μM; no data for 0.1 μM) on chlorophyll concentration in lettuce cotyledons after 6 days of growth.

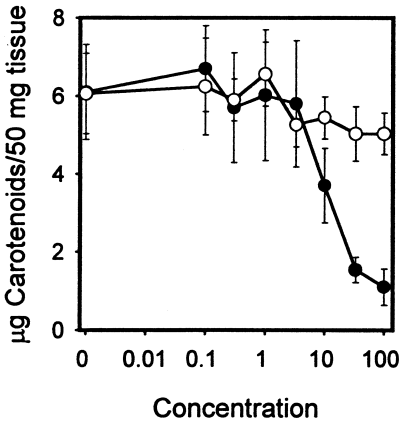

Fig. 4. Effect of (−)-usnic acid (-●-) and (+)-usnic acid (-○-) (0.1–100 μM) on carotenoid concentration in lettuce cotyledons after 6 days of growth.

ently since both enantiomers caused membrane leakage in the absence of light (Fig. 3). Nonetheless, the rate of electrolyte leakage was enhanced when the samples were exposed to 325 μE of light (Fig. 3), suggesting that the physiological or biochemical processes affected by usnic acid are connected with light. Although both enantiomers cause membrane leakage, (−)-usnic has a greater effect.

Many photobleaching herbicides act by inhibiting the enzyme Protox, which catalyzes the last step in common between chlorophylls and hemes. Usnic acid shares some structural features in common with these herbicides, such as the diphenyl ether scaffolding. The inhibitory activity of (−)-usnic acid on Protox was similar to that of the herbicide acifluorfen (I_{50} ca. 3 μM), whereas the non-bleaching (+)-usnic was approximately 20× less active with an I_{50} of ca. 60 μM. However, these compounds did not displace acifluorfen from its binding site on Protox (data not shown), indicating that this natural product interacts with Protox differently than other inhibitors. The presence of the bridge between the *ortho*-carbons of diphenyl ethers preventing the rotation that is normally possible in the diphenyl ether herbicides may be involved. Furthermore, the primary mechanism of action of usnic acid could not be attributed to inhibition of Protox because treated cucumber cotyledons did not accumulate protoporphyrin IX (Proto IX) (data not shown), as occurs with herbicides that act by inhibition of Protox.

Inhibitors of carotenoid synthesis also led to chlorophyll destruction by destabilizing the photosynthetic apparatus. HPLC analyses showed a strong decrease in β-carotene content in samples treated with (−)-usnic acid and total carotenoids decreased with increased usnic concentration (Fig. 4). The non-bleaching (+)-usnic acid did not cause a similar decrease in carotenoid levels.

Carotenoid biosynthesis can be interrupted by inhibiting the enzyme phytoene desaturase that converts phytoene to carotenes or by inhibiting the enzyme HPPD responsible for plastoquinone (PQ) (required for phytoene desaturase activity) synthesis [17]. Usnic acid possesses some of the structural features of the triketone HPPD inhibitors, such as sulcotrione (Fig. 1) [18]. (−)-Usnic acid had a strong inhibitory activity on HPPD, with an apparent IC_{50} of 70 nM, surpassing the activity obtained with the commercial herbicide sulcotrione (Fig. 5). The non-bleaching (+)-usnic was about 10-fold less active than its (−)-enantiomer.

We also compared the activity of both enantiomers with sulcotrione, an irreversible inhibitor of HPPD. We determined that (−)-usnic acid was an irreversible inhibitor (Fig. 6A).

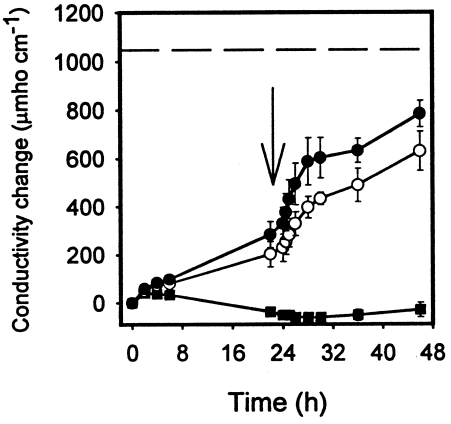

Fig. 3. Effect of (−)-usnic acid (-●-) and (+)-usnic acid (-○-) on electrolyte leakage from cucumber cotyledons. The arrow represents the time when the samples were exposed to 325 μmol m^{-2} s^{-1} light. Dashed line represents maximum leakage obtained from boiled samples and leakage from untreated samples are shown (-■-).

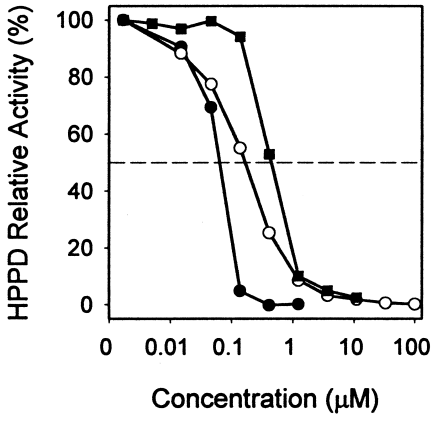

Fig. 5. Effect of (−)-usnic acid (-●-) and (+)-usnic acid (-○-) (0.01–100 μM) on activity of HPPD. The activity of the herbicide sulcotrione was added for comparison (-■-).

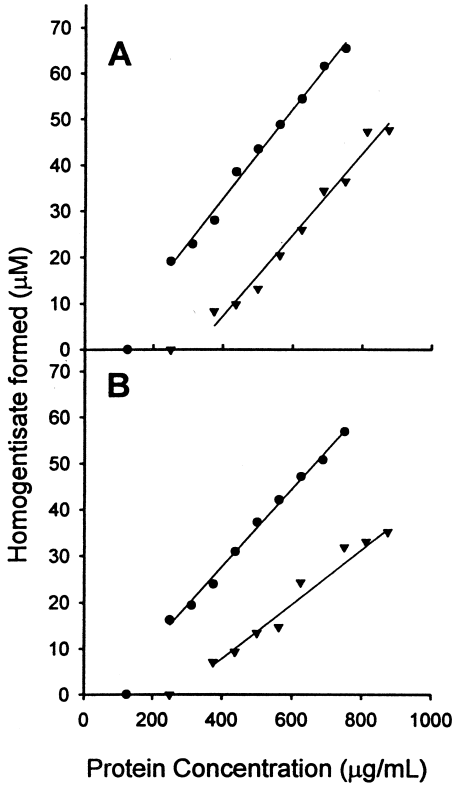

Fig. 6. Representative plot of the effect of (A) (−)- and (B) (+)-usnic acid upon the formation of HGA from HPPA (control (-●-) and inhibitor (▼). Concentrations of inhibitor were 0.03 and 0.1 μM ((−)-and (+)-usnic acid, respectively).

Results also suggest that HPPD was irreversibly inhibited by (+)-usnic acid, however they were less conclusive (Fig. 6B).

4. Discussion

(−)-Usnic acid had the greatest phytotoxic activity of the two enantiomers, causing severe bleaching of cotyledonary tissues of developing seedlings. The chromatographic profiles of light-grown plants exposed to (−)-usnic acid clearly illustrated that the decrease in chlorophyll concentration was due to degradative processes rather than due to inhibition of the chlorophyll biosynthetic pathway.

Although the inhibitory activity of (−)-usnic acid on Protox was similar to the commercial herbicide acifluorfen, this mechanism of action could not be attributed to usnic acid since there was no discernible buildup of Proto IX normally associated with inhibition of this enzyme [21]. In addition, binding studies with radiolabelled acifluorfen showed no inhibition of the enzyme. Furthermore, unlike most Protox inhibitors, membrane leakage was not strictly light-dependent, with some level of leakage occurring in the dark. Evidence suggested that bleaching caused by (−)-usnic acid was due to destabilization of the photosynthetic apparatus in a manner similar to that observed with inhibition of carotenoid synthesis [11].

Inhibition of phytoene desaturase has been well established as a cause of bleaching. Many different classes of compounds inhibit this enzyme, however they are normally highly lipophilic compounds that are unionized at neutral pH [22]. Usnic acid does not resemble inhibitors of phytoene desaturase and did not lead to phytoene accumulation normally associated with this mechanism of action. Rather, usnic acid possesses a 2-keto-cyclohexane-1,3-dione common to many triketone herbicides such as sulcotrione. Inhibition of HPPD leads to bleaching symptoms through the blocking of PQ, an essential cofactor of the enzyme phytoene desaturase [19–22]. Further investigation supported the hypothesis that (−)-usnic acid, due to its configuration, behaved as an irreversible inhibitor of the enzyme [17].

Carotenoids play an important role in quenching excess excitation energy in the photosynthetic apparatus. Under normal conditions, chlorophyll reaches singlet excitation state following absorption of a photon. The singlet chlorophyll transfers an electron to PQ and returns to ground state rapidly by receiving an electron from the splitting of water. Under excessive light intensity, chlorophyll can reach the more stable triplet state. This excess energy is transferred to carotenoids through intersystem crossing and is consequently released in a non-radiating way, allowing the chlorophyll molecules to return to ground state and preventing photodestruction. In the absence of carotenoids, the photosynthetic apparatus is destabilized [23]. The excess energy from the chlorophyll in their triplet state is transferred to oxygen, causing formation of singlet oxygen. Singlet oxygen is highly reactive and causes bleaching of pigments and lipid peroxidation of membranes. These symptoms (e.g. chlorophyll degradation and electrolyte leakage) were observed in vivo in seedlings treated with (−)-usnic acid, suggesting that the primary mechanism of action of this natural product is associated with inhibition of HPPD.

(−)-Usnic acid was a strong HPPD inhibitor, with an apparent IC_{50} of 70 nM. (+)-Usnic acid was an order of magnitude less active than its enantiomer. The basis for this difference in activity may be associated with the effect that the methyl group at 9b has on the configuration of the structures. There are no differences between the two enantiomers except that the space occupied by the other ring is either above or below the plane of the triketone center. This steric difference may affect the binding of usnic acid on HPPD. Computer modeling analysis illustrated how the triketone portion of usnic acid overlays closely with portion of the herbicide sulcotrione (Fig. 7). It has been inferred that triketone herbicides were derived from the natural product leptospermone isolated from bottlebrush (*Calistermon* spp.) [18]. However, this is the first report demonstrating that natural products can be potent irreversible inhibitors of HPPD.

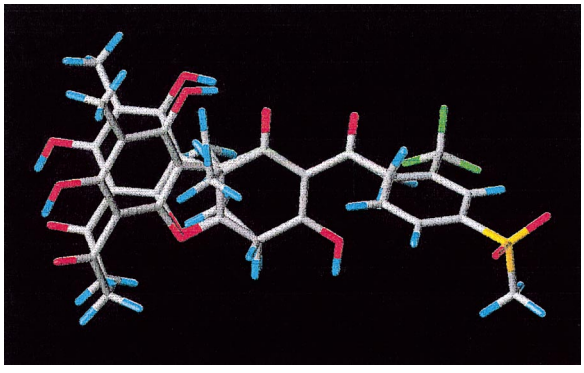

Fig. 7. Overlay of (−)-usnic acid, (+)-usnic acid, and sulcotrione on the triketone center common to the three molecules.

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