Effect of Lichen Metabolites on Thylakoid Electron Transport and Photophosphorylation in Isolated Spinach Chloroplasts[1]

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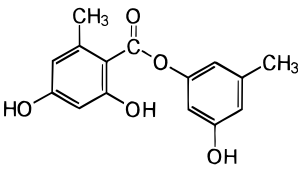
Investigation of the lichen *Parmotrema tinctorum* led to the isolation of several known compounds. Among them, lecanorin (**1**), methyl-β-orcinol carboxylate, methyl orsellinate, orcinol, and methyl haematommate (**3**) caused significant inhibition of the radicle growth and germination of seedlings of *Amaranthus hypochondriacus* and *Echinochloa crusgalli*. In addition, lecanorin (**1**) and gyrophoric acid (**2**) significantly inhibited the light-dependent synthesis of ATP and uncoupled electron transfer on the reducing side of photosystem II in freshly lysed, illuminated spinach chloroplasts. The targets of **1** and **2** were located at the water-splitting enzyme level and in one of the redox enzymes in the range of electron transport from P_{680} to Q_A, respectively.

Parmotrema tinctorum Nyl. (Hale) (Parmeliaceae) is a foliose lichen widely distributed in East Mexico. Previous chemical investigation on *P. tinctorum* resulted in the isolation of orsellinic, lecanoric, usnic, diffractic, and gyrophoric acids as well as methyl orsenillate and atranorin.[2−4] Some of these metabolites demonstrated antiproliferative and cytotoxic activities.[3,4] Lichen compounds have clear ecological significance because of vital roles as antimicrobial and allelopathic agents. Several investigations have demonstrated that a number of metabolites and aqueous extracts of various lichens are capable of inhibiting seed germination and growth of both wild and agricultural races of vascular plants, thus representing potential leads for the developing of new herbicidal agents.[5] The present investigation was to evaluate the effect of the major phytoxins isolated from *P. tinctorum* on several photosynthetic reactions in isolated spinach chloroplasts.



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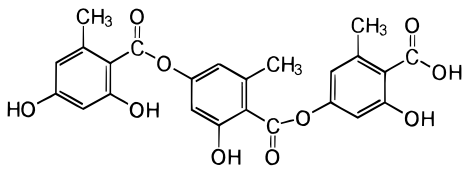

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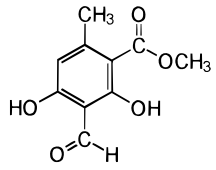

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Results and Discussion

P. tinctorum was extracted with $CHCl_3$−MeOH (1:1). The resulting extract inhibited germination and growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli* seeds using a Petri-dish bioassay.[6] Bioactivity-directed fractionation of the extract led to the isolation of the known compounds lecanorin (**1**),[7] gyrophoric acid (**2**),[8] methyl haematommate (**3**),[9] methyl-β-orcinol carboxylate,[9] lichexanthone,[10] methyl orsellinate,[11] and orcinol.[11] The compounds were identified by IR, NMR, and mass spectral analyses and by comparison with the spectroscopic data previously described.[7−11]

The isolated compounds were tested for their ability to inhibit the seed germination and radicle growth of *A. hypochondriacus* and *E. crusgalli*. Compounds **1**, methyl-β-orcinol carboxylate, methyl orsellinate, orcinol, and **3** significantly reduced both processes in a concentration-dependent manner (Table 1). In general, *A. hypochondriacus* was more sensitive to the treatments, with **3** being the most potent compound.

The effect of these compounds was also tested for inhibition of photosynthetic activities in freshly lysed, illuminated spinach chloroplasts. The activities investigated included ATP-synthesis, H^+-uptake, electron transport rate, and partial reactions of the photosystems I (PSI) and II (PSII).[6] Photosynthetic phosphorylation from water to methyl viologen (MV) in spinach thylakoids was significantly inhibited by **1** ($I_{50} = 1.36 \times 10^{-4}$ M) and **2** ($I_{50} = 8.71 \times 10^{-5}$ M) and partially affected by **3**, lichexanthone, and methyl orsellinate at 300 μM. However, **1** and **2** were less potent than 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a synthetic inhibitor employed as a positive control ($I_{50} = 1.40 \times 10^{-7}$ M). On the other hand, the light-dependent proton-uptake was also inhibited by **1** and **2** ($I_{50} = 1.40 \times 10^{-4}$ M and 5.97×10^{-5} M, respectively).

The light-dependent synthesis of ATP and H^+-uptake by illuminated thylakoids might be inhibited in a numbers of ways: (a) by uncoupling ATP synthesis from the electron transport, (b) by inhibiting the electron transport, and/or (c) by blocking the phosphorylation reaction itself. To understand the mechanism by which the most active compounds, **1** and **2**, inhibited photophosphorylation, their effects on the electron transport chain were investigated. Figures 1 and 2 show that the noncyclic electron transport

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Table 1. Phytogrowth-Inhibitory Activity of Compounds from *P. tinctorum*

compound	seedling growth IC_{50} M		seedling germination IC_{50} M	
	A. hypochondriacus	*E. crusgalli*	*A. hypochondriacus*	*E. crusgalli*
1	5.8×10^{-4}	1.8×10^{-4}	7.6×10^{-4}	7.1×10^{-4}
2	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}
3	2.1×10^{-5}	2.9×10^{-5}	6.8×10^{-6}	1.7×10^{-5}
methyl-β-orcinol carboxylate	3.2×10^{-4}	6.1×10^{-4}	7.6×10^{-4}	1.1×10^{-3}
lichexanthone	$> 2.0 \times 10^{-3}$	$> 2.0 \times 10^{-3}$	$> 2.0 \times 10^{-3}$	$> 2.0 \times 10^{-3}$
methyl orsellinate	2.9×10^{-4}	3.1×10^{-4}	3.6×10^{-4}	6.8×10^{-4}
orcinol	1.0×10^{-3}	1.1×10^{-3}	1.7×10^{-3}	$> 8.1 \times 10^{-3}$
2,4-D[a]	1.8×10^{-4}	$> 2.3 \times 10^{-4}$	8.8×10^{-4}	$> 2.3 \times 10^{-4}$

[a] Positive control.

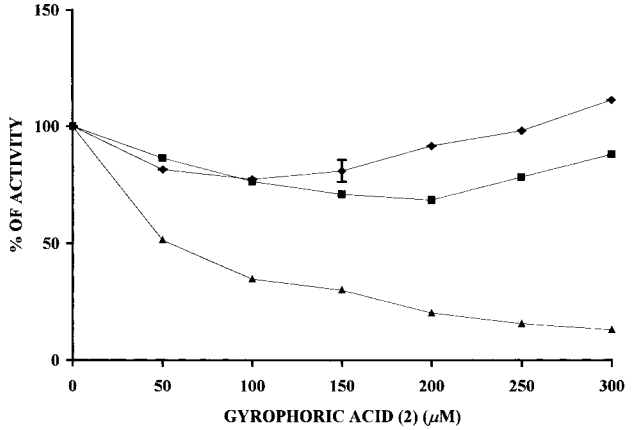

Figure 1. Noncyclic electron transport from water to MV as a function of gyrophoric acid (**2**) concentration in chloroplast thylakoids isolated from spinach leaves (*S. oleracea* L.). Each cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions are described under Experimental Section. Control value rates for basal (◆), phosphorylating (■), and uncoupled (▲) electron transport were 400.0, 554.1, and 1025.0 μequiv·e^{-}·h^{-1}·mg of Chl^{-1}, respectively. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts. Vertical bars represent maximum standard deviation.

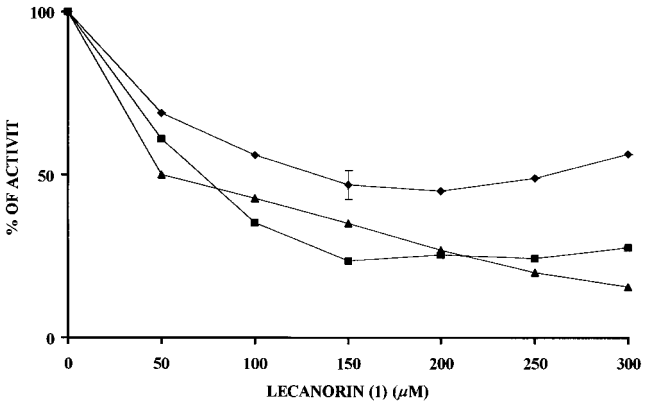

Figure 2. Noncyclic electron transport from water to MV as a function of lecanorin (**1**) concentration in chloroplast thylakoids isolated from spinach leaves (*S. oleracea* L.). Each cuvette contained chlorophyll 20 μg/mL in the reaction medium. Other conditions were described under Experimental Section. Control value rates for basal (◆), phosphorylating (■), and uncoupled (▲) electron transport were 331.4, 457.1, and 1117.8 μequive^{-}·h^{-1}·mg of Chl^{-1}, respectively. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts. Vertical bars represent maximum standard deviation.

from water to MV in uncoupled conditions was drastically inhibited by compounds **1** and **2**. Therefore, these lichen metabolites behave as Hill reaction inhibitors.

To localize the inhibition site of **1** and **2**, their effect on PSI and PSII electron transport was evaluated using appropriate inhibitors and artificial electron donors and acceptors.[12,13] Uncoupled PSI measured from dichlorophenol−indophenol (DCPIP)/ascorbate to MV was not significantly affected by compounds **1** and **2** (data not shown). However, uncoupled PSII electron flow was drastically inhibited by both compounds (Tables 2 and 3). Uncoupled PSII from water to DCPIP/K_3[Fe(CN)$_6$], from water to silicomolybdate (SiMo), and from diphenilcarbazide (DPC) to DCPIP were inhibited as the concentrations of **2** increased (Table 2). Therefore, the mode of action of **2** involves the inhibition of electron transfer between P_{680} and Q_A on the reducing side of PSII. On the other hand, the site of inhibition of **1** was at the water-splitting enzyme level, because it inhibited uncoupled electron transport from water to DCPIP/K_3[Fe(CN)$_6$] and from water to SiMo, without affecting uncoupled electron transport from DPC to DCPIP and uncoupled photosystem electron transport from DCPIP/ascorbate to MV (Table 3).

The level activity of compound **3** on radicle growth and germination and the effect of **1** and **2** on photosynthesis

Table 2. Effect of Gyrophoric Acid (**2**) on Uncoupled Photosystem II Electron Transport Rate[a]

concentration, μM	reactions		
	H_2O to DCPIP (H_2O to cyt b_6/f)	H_2O to SiMo (H_2O to PheO)	DPC to DCPIP (P_{680} to cyt b_6/f)
0	100.0	100.0	100.0
50	72.1	93.4	84.3
100	49.7	79.9	58.5
150	31.3	62.5	40.3
200	16.8	41.0	33.8
250	4.1	0.0	29.9
300	0.0	0.0	17.9

[a] Control value rates μequiv e^{-}·h^{-1}·mg of Chl^{-1} from photosystem II (from H_2O to DCPIP, H_2O to SiMo, DPC to DCPIP) were 324.7, 86.6, and 106.0, respectively. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts. The corresponding range of the electron transport chain is given in parentheses.

Table 3. Effect of Lecanorin (**1**) on Uncoupled Photosystem II Electron Transport Rate[a]

concentration, μM	reactions		
	H_2O to DCPIP (H_2O to cyt b_6/f)	H_2O to SiMo (H_2O to PheO)	DPC to DCPIP (P_{680} to cyt b_6/f)
0	100.0	100.0	100.0
50	64.3	59.1	93.6
100	37.5	25.0	86.9
150	15.7	8.4	88.9
200	0.0	0.0	96.0
250	0.0	0.0	111.8
300	0.0	0.0	135.2

[a] Control value rates μequiv e^{-}·h^{-1}·mg of Chl^{-1} from photosystem II (from H_2O to DCPIP, H_2O to SiMo, DPC to DCPIP) were 200.0, 25.0, and 98.0, respectively. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts. The corresponding range of the electron transport chain is given in parentheses.

clearly reveal the potential of these metabolites as leads for the development of new herbicidal agents. The concentrations of metabolite **1** required for inhibition of photophosphorylation, electron transfer, and radicle growth inhibition are comparable. However, in the case of **2** the inhibitory effect on photosynthesis was higher than the preemergent phytotoxic activity. Finally, it is important to point out that the phytotoxins isolated from *P. tinctorum* represent the most common classes of lichen metabolites.

Experimental Section

General Experimental Procedures. Melting point determinations were performed on a Fisher-Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks on a Perkin-Elmer 599B spectrophotometer. UV spectra were obtained on a Shimadzu 160 UV spectrometer in MeOH solution. NMR spectra including COSY, NOESY, HMBC, and HMQC experiments were recorded on a Varian Unity Plus 500 spectrometer or on a Bruker DMX500 at 500 MHz (^{1}H) or 125 MHz (^{13}C) NMR. EIMS were obtained on a JEOL JMS-AX505HA mass spectrometer. Open column chromatography: Si gel 60 (70−230 mesh, Merck). TLC: Si gel 60 F_{254} (Merck).

Plant Material. The plant material of *P. tinctorum* was collected in Los Tuxtlas, Veracruz, Mexico. A voucher specimen (G.C.-1996) has been deposited in the Herbarium of Instituto de Ecología (XAL), Jalapa, Veracruz.

Extraction and Bioassay-Directed Fractionation. The air-dried lichen material (2.6 kg) was ground into powder and extracted by maceration with $CHCl_3$−MeOH (1:1) at room temperature. After filtration, the extract was evaporated under reduced pressure to yield 260 g of residue, which was subjected to column chromatography over Si gel (2.6 kg) and eluted with a gradient of hexane−CH_2Cl_2 (10:0→0:10) and CH_2Cl_2−MeOH (9:1→5:5). Fractions of 400 mL each were collected and pooled based on TLC profiles to yield nine major fractions. Fractions 2, 4, and 8 were phytotoxic according to the bioautographic phytogrowth-inhibitory bioassay (BPIB).[6] Compound **1** (46 mg) crystallized from active fraction 2. Preparative TLC of fraction 4 (150 mg) using CH_2Cl_2−MeOH (99:1) led to the isolation of the compound orcinol carboxylate (55 mg). Successive preparative TLC of fraction 3 (3.5 g) using hexane−CH_2Cl_2 (2:8), three developments, yielded the compound lichexanthone (80 mg). Methyl orsellinate (9.3 g) crystallized from fraction 5 (12 g). Further column chromatography of fraction 8 (55 g) on Si gel (750 g), eluting with a concentration gradient of hexane−CH_2Cl_2−MeOH, starting with hexane−CH_2Cl_2 (1:1), afforded eight secondary fractions (A−H). According to the BPIB, the phytotoxic activity was concentrated in secondary fractions F and H. Preparative TLC of secondary fraction F using CH_2Cl_2−MeOH (93:7) yielded orcinol (45 mg) and **3** (20 mg). Finally, from secondary fraction H precipitated **2** (18.6 g) as an amorphous solid.

Lecanorin (1): ^{13}C NMR (C_5D_5N, 125 MHz) δ 170.1 (C-7), 165.3 (C-4), 164.2 (C-2), 159.7 (C-2′), 151.7 (C-4′), 143.3 (C-6), 140.4 (C-6′), 114.5 (C-1′), 113.6 (C-5′), 112.4 (C-5), 107.3 (C-3′), 105.6 (C-1), 101.7 (C-3), 23.6 (C-8), 21.1 (C-7′).

Lichexanthone: ^{13}C NMR ($CDCl_3$, 125 MHz) δ 182.4 (C-9), 165.8 (C-6), 163.8 (C-1), 159.4 (C-5a,-4a), 157.0 (C-3), 143.5 (C-8), 115.4 (C-7), 113.0 (C-8a), 104.2 (C-9a), 98.5 (C-5), 96.8 (C-2), 92.1 (C-4), 55.7 (OCH_3-3,-6), 23.4 (CH_3-8).

Methyl orsellinate: ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.8 (C-7), 164.4 (C-2), 161.5 (C-4), 143.3 (C-6), 111.4 (C-5), 104.2 (C-3), 100.4 (C-1), 51.3 (OCH_3-7), 23.8 (C-8).

Orcinol: ^{13}C NMR ($CDCl_3$, 125 MHz) δ 157.7 (C-2,-4), 139.7 (C-6), 107.5 (C-1,-5), 99.9 (C-3), 21.1 (C-7).

Phytogrowth-inhibitory Bioassays. The phytogrowth-inhibitory activity of the extract and of pure compounds was evaluated on seeds of *A. hypochondriacus* and *E. crusgalli* using a Petri-dish bioassay.[6] A BPIB system was employed to guide secondary fractionation and to speed up the isolation of active compounds.[6] The results were analyzed by ANOVA ($p < 0.05$), and IC_{50} (50% inhibitory concentration) values, expressed in molar concentrations, were calculated by Probit analysis based on percent of radicle growth inhibition. The extract and pure compounds were evaluated at 1, 10, 100, and 1000 μg mL^{-1}. 2,4-Dichlorophenoxy acetic acid (2,4-D) was used as the positive control. The bioassays were all performed at 28 °C.

Chloroplast Isolation and Chlorophyll Determination. Intact chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L.) obtained from a local market as previously described.[13] Chloroplasts were suspended in the following medium: 400 mM sucrose, 5 mM $MgCl_2$, 20 mM KCl, and buffered with 30 mM Na^+-tricine at pH 8.0. They were stored as a concentrated suspension in the dark for 1 h at 0 °C. Chlorophyll concentration was measured spectrophotometrically as reported.[14]

Measurement of Proton Uptake, ATP Synthesis, and Electron Transport. Proton uptake was measured as pH value increase between 8.0 and 8.1, using a combination microelectrode Orion model 8103 Ross connected to a Corning potentiometer model 12, with expanded scale.[15] The pH changes were registered using a Gilson recorder. The reaction medium used contained 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 1 mM Na^+-tricine, at pH 8.0. ATP synthesis was determined titrametrically by the procedure of Dilley.[15] The 0.05 mM MV was employed as electron acceptor for the Hill reaction. Noncyclic electron transport activity from water to MV was monitored with a Yellow Springs Instrument model 5300 oxygen monitor and a Clark electrode. The reaction medium was the same as in the proton uptake assay, except that the tricine concentration was 15 mM, and 0.5 mM KCN was added (basal electron transport). Phosphorylating noncyclic electron transport was measured as basal noncyclic electron transport, except that 1 mM ADP and 3 mM KH_2PO_4 were added to the reaction medium. Uncoupled electron transport was tested in the basal noncyclic electron medium, and 6 mM NH_4Cl was added. All reaction mixture was illuminated with actinic light of a projector lamp (Gaf 2669) passed through a 5-cm filter of a 1% $CuSO_4$ solution for 2 min.[16] Uncoupled PSII was measured by the reduction of DCPIP-supported O_2 evolutions monitored polarographically.[16] The reaction medium was the same as in electron transport assay, but 300 μM $K_3[Fe(CN)_6]$ was used instead of MV and the following reagents were added: 1 μM 2,5-dibromo-3-methyl-6-isopropyl-1,4-*p*-benzoquinone (DBMIB), 100 μM DCPIP, and 6 mM NH_4Cl. Uncoupled electron transport from water to SiMo, was determined with the same reaction mixture as in PSII, except that 200 μM SiMo and 10 μM DCMU were added.[17] Uncoupled electron transport from DPC to DCPIP was measured spectrophotometrically as reported previously;[18] 200 μM DPC, 100 μM DCPIP, 1 μM DBMIB, and 6 mM NH_4Cl were added to the medium, and chloroplasts previously treated with Tris 0.8 M, pH 8.0 were used. PSI electron transport was determined also in a similar form to noncyclic electron transport but the following reagents were added: 10 μM DCMU, 100 μM DCPIP, 300 μM ascorbate, 6 mM NH_4Cl, and 0.05 mM MV, without $K_3[Fe(CN)_6]$.

In all cases the compounds were tested at 50, 100, 150, 200, 250, and 300 μM. DCMU, employed as positive control for ATP synthesis assay, was tested at 0.01, 0.1, 1.0, and 10.0 μM. The I_{50} value for each activity was extrapolated using the graph of percent activity versus concentration of compounds. I_{50} is the concentration producing 50% inhibition. The concentration threshold required for photosynthesis inhibitors in similar experiments is often $\leq 10^{-4}$ M.[19]

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References and Notes

(1) Taken in part from the Ph.D. Thesis of I. S. Rojas entitled "Phytotoxic Compounds from Selected Lichens Species." Facultad de Química, Universidad Nacional Autónoma de México, México D. F., 2000.
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