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Inhibition of Photophosphorylation and Electron Transport Chain in Thylakoids by Lasiodiplodin, a Natural Product from *Botryosphaeria rhodina*

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Four natural products were isolated from the fungus *Botryosphaeria rhodina*, and their effects on photosynthesis were tested. Only lasiodiplodin (**1**) inhibited ATP synthesis and electron flow from water to methylviologen; therefore, it acts as a Hill reaction inhibitor in freshly lysed spinach thylakoids. Photosystem I and II and partial reactions as well as ATPase were measured in the presence of **1**. Three new different sites of **1** interaction and inhibition were found: one at CF_1, the second in the water-splitting enzyme, and the third at the electron-transfer path between P_{680} and Q_A; these targets are different from that of the synthetic herbicides present. Electron transport chain inhibition by **1** was corroborated by fluorescence induction kinetics studies.

KEYWORDS: ***Botryosphaeria rhodina*; lasiodiplodin; Hill reaction inhibitor; photosystem II inhibitor**

INTRODUCTION

Microorganisms have long served mankind by virtue of the myriad enzymes and secondary metabolites they produce. Furthermore, only a relatively small number of microbes are used directly in industrial applications (e.g., cheese, wine, and beer production), in environmental cleanup operations, and in the biological control of pests and pathogens. It seems that we have by no means exhausted the world of its hidden microbes, and a much more comprehensive search of the Earth's various niches might yet reveal novel microbes which have direct usefulness to human societies. These uses could be either of the microbes themselves or of one or more of their natural products (*1*).

In recent years it was evident that many species of fungus such as *Botryosphaeria* had reached a great number of mushrooms and geographic distribution; even so, it is distribution worldwide is limited mainly to an area 40° south and 40° north of the equator (*2*). These fungi attack plants with weaknesses and insect damage, during dry conditions or severe winters, etc.; more than 100 sorts of symptoms become visible due to the stress induced by the fungus. These can vary depending on the type of plant and the extent of infection.

The chemical profile of *Botryosphaeria rhodina* had been found to include compounds such as jasmonic acid and its derivatives; also frequently found were some polyketides, such as lasiodiplodin and isocoumarin among other composite classes (*3*, *4*). Here, we are interested in studying if one of these natural products affects photosynthesis as a mechanism for phytotoxicity in vitro.

MATERIALS AND METHODS

Tested Material. Four natural products [lasiodiplodin (**1**), *p*-hydroxyphenylethanol (**2**), inosin (**3**), and ergosterol (**4**)] (**Figure 1**) were isolated from the ethanolic extract from the fungus *B. rhodina* as previously described (*4*). Stock solutions for compounds **1**−**4** were prepared using dimethyl sulfoxide (DMSO), and the maximum concentration of solvent mixture in the media was <1%.

Isolation of Lasiodiplodin (1). The dichloromethanic extract (1.71 g) of the fungus *B. rhodina* was subjected to column chromatography over silica gel (35 mm, 50 g), and the elution was carried out with hexane, ethyl acetate, and methanol gradients to give a group of 25 fractions. Fraction 8 was subjected to thin layer chromatography with hexane/ethyl acetate (15%) to produce compound **1**, which was identified with spectroscopic and spectrometric techniques: ^{1}H and ^{13}C NMR, including 2D experiments (HSQC, HMBC, and NOESY) as previously reported (*4*).

Isolation of *p*-Hydroxyphenylethanol (2), Inosin (3), and Ergosterol (4). The ethanolic extract (2.5 g) of *B. rhodina* was subjected to column chromatography over silica gel (45 mm, 100 g), and the elution was carried out with hexane, dichloromethane, and methanol gradients

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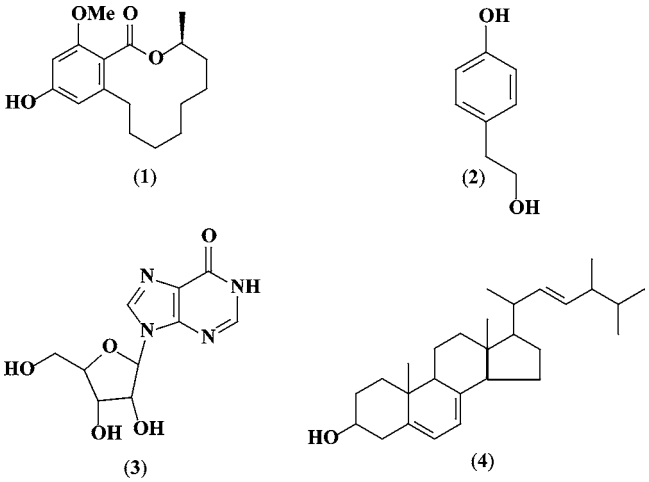

Figure 1. Structures of compounds **1**–**4** isolated from *Botryosphaeria rhodina*.

to give nine fractions; from fraction 7 were obtained compounds **2** and **4** (*5*, *6*), and from fraction 6 was obtained compound **3** (*7*). Compounds **2**−**4** were identified with the same techniques used to identify compound **1**.

Chloroplast Isolation and Chlorophyll Determination. Intact chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L.) obtained from a local market as previously described (*8*, *9*). Chloroplasts were suspended in the following medium: 400 mM sucrose, 5 mM $MgCl_2$, and 10 mM KCl, buffered with 0.03 M Na^+ tricine at pH 8.0. They were stored as a concentrated suspension in the dark for 1 h at 0 °C. Intact chloroplasts were efficiently lysed to yield free thylakoids prior to each experiment by incubating them in the following electron transport medium: 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, and 30 mM tricine [(*N*-tris[hydroxymethyl]methylglycine; *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine) buffer (pH 8 with the addition of KOH)]. Chlorophyll concentration was measured spectrophotometrically as reported (*10*).

Measurement of ATP Synthesis. ATP synthesis was determined titrametrically using a microelectrode Orion model 8103 Ross connected to a Corning potentiometer model 12, with expanded scale as reported (*11*). The ATP synthesis reaction medium contained 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, 50 μM methylviologen (MV) used as electron acceptor, and 1 mM Na^+-tricine (pH 8.0) in addition of 20 μg/mL of chlorophyll when the intact chloroplasts were freshly lysed.

Measurement of Noncyclic Electron Transport Rate. Light-induced noncyclic electron transport activity from water to MV was determined by using a Clark-type electrode, as published, in the presence of 50 μM MV as electron acceptor (*11*). Basal electron transport was determined by illuminating chloroplasts during 1 min (equivalent of 20 μg/mL of chlorophyll) lysed in 3.0 mL of the reacting medium: 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM KCN, 30 mM Na^+-tricine, and 50 μM MV at pH 8.0. The sample was illuminated in the presence or absence of 6 mM NH_4Cl (*12*). 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 from water to MV was tested in the basal noncyclic electron transport medium, and 6 mM NH_4Cl was added. All reaction mixtures were illuminated with the actinic light of a projector lamp (GAF 2660) passed through a 5 cm filter of a 1% $CuSO_4$ solution for 1 min.

Uncoupled Photosystem II (PSII) and Photosystem I (PSI). Electron Flow Determination. Electron transport activity was monitored with a YSI (Yellow Springs Instrument) model 5300 oxygen monitor using a Clark electrode. The reaction medium was the same as in the electron transport assay. Uncoupled PSII from $H_2O \rightarrow$ DCPIP was measured by the reduction of 2,6-dichlorophenol indophenol (DCPIP) supported O_2 evolutions monitored polarographically. The reaction medium for assaying PSII activity contained the same basal electron transport medium in the presence of 1 μM 2,5-dibromo-3-methyl-6-isopropyl-1,4-*p*-benzoquinone (DBMIB), 100 μM DCPIP/300 μM $K_3[Fe(CN)_6]$, and 6 mM NH_4Cl. Uncoupled PSI electron transport from $DCPIP_{red}$ to MV was determined in a similar form to basal noncyclic electron transport medium. The following reagents were added: 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 100 μM DCPIP, 50 μM MV, 300 μM ascorbate, and 6 mM NH_4Cl. Uncoupled PSI electron transport from reduced phenylmetasulfate (PMS) to MV was determined using KCN-poisoned chloroplasts. The reaction medium was the same as in PSI except that 500 μM PMS/100 μM ascorbate was used as electron donor to P700 (PSI reaction center), MV as PSI electron acceptor, 10 μM DCMU as inhibitor to Q_B (secondary quinone electron acceptor of PS II), and 6 mM NH_4Cl used as uncoupler to PSI. Cyanide-treated chloroplasts were prepared by incubating chloroplasts for 30 min at 4 °C in 30 mM KCN and then centrifuged at 8000*g* (Sorvall super T21) for 1 min and resuspended in the reaction medium (*12*). Moroever, electron paramagnetic resonance (EPR) spectroscopy confirmed the ability of reduced PMS to interact directly with P700 (*13*). 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.

Mg^{2+}-ATPase Assay. Chloroplasts were isolated from 30−40 g of spinach leaves, which were ground in 160 mL of medium containing 350 mM sorbitol, 5 mM ascorbic acid, and 20 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 6.5. Chloroplasts were centrifuged at 3000*g* for 60 s, washed once in 40 mL of grinding medium, and resuspended in 35 mM HEPES, pH 7.6. Light-triggered Mg^{2+}-ATPase activity bound to thylakoid membranes was measured as described previously (*9*). Released inorganic P was measured as reported (*14*).

Mg^{2+}- and Ca^{2+}-ATPase Activities from Isolated CF_1. Light-triggered Mg^{2+}-ATPase activity bound to thylakoid membranes was done as in ref *9*. To obtain CF_1-depleted chloroplasts and solubilized CF_1, an aliquot of fresh chloroplasts was diluted with 0.75 M ethylenediaminetetraacetic acid (EDTA), pH 7.6, and incubated for 10 min at 20 °C. CF_1-depleted membranes were removed by centrifugation. Of this EDTA supernatant (containing CF_1 complex), 0.5 mL was added to 0.5 mL of 20 mM tricine, pH 8.0, 2 mM EDTA, 10 mM DTT, and 40 mM ATP and heated at 60 °C for 4 min. Of the resulting ATPase-activated mixture, a 0.1 mL aliquot was incubated for 20 min at 37 °C with 0.9 mL of a medium containing 50 mM Tris, pH 8.4, 5 mM $CaCl_2$, and 5 mM ATP (*15*, *16*). Released inorganic phosphate was measured as previously described (*14*). Protein was determined according to the Lowry method (*17*).

Chlorophyll *a* (Chl *a*) Fluorescence Determination. Chl *a* fluorescence was measured with a Hansatech Fluorescence Handy PEA (plant efficiency analyzer) in 5 min dark-adapted chloroplasts (20 μg/mL) at room temperature (*18*), using red light intensity (broad band 650 nm) of 3000 μmol m^{-2} s^{-1}, provided by an array of three light-emitting diodes. The pulse duration was 2 s. The reaction medium used was the one employed in basal noncyclic electron transport measurements. To monitor Chl *a* fluorescence transients, aliquots of dark-adapted thylakoids were placed by gravity on filter paper with a dot-blot apparatus (Bio-Rad) to ensure a homogeneous and reproducible distribution of thylakoids in the filter paper and then dipped immediately in 3 mL of electron transport medium with a 300 μM concentration of the test compound.

RESULTS AND DISCUSSION

ATP Synthesis. Figure 2 shows typical results from experiments measuring the effects of purified compounds **1**−**4** on the rates of ATP synthesis by freshly lysed spinach chloroplasts with MV as electron acceptor. An increasing concentration of **1** resulted in an increasing inhibition of ATP synthesis (open squares). The I_{50} value was 35.6 μM. **Figure 2** also shows that compounds **2**−**4** have no effect on ATP formation; therefore, they were not further studied.

Elucidation of the Mechanism of Action. The light-dependent synthesis of ATP by illuminated thylakoid may be inhibited in a number of ways: (a) by blocking the electron transport, (b) by uncoupling ATP synthesis from the electron

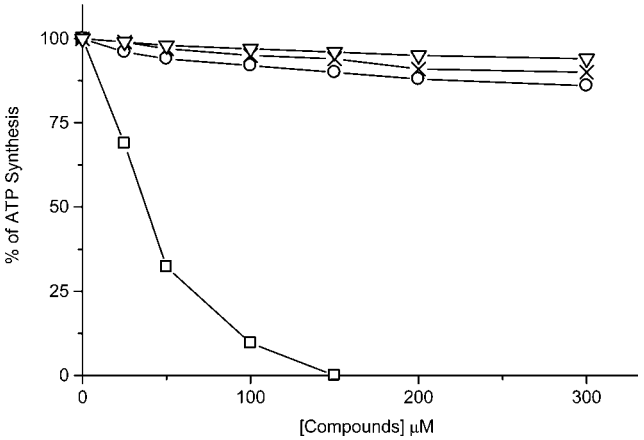

Figure 2. Effect of compounds **1** (□), **2** (×), **3** (○), and **4** (▽) on ATP synthesis. Control rate value for **1** was 1276 μM ATP·h^{-1}·mg of Chl^{-1}.

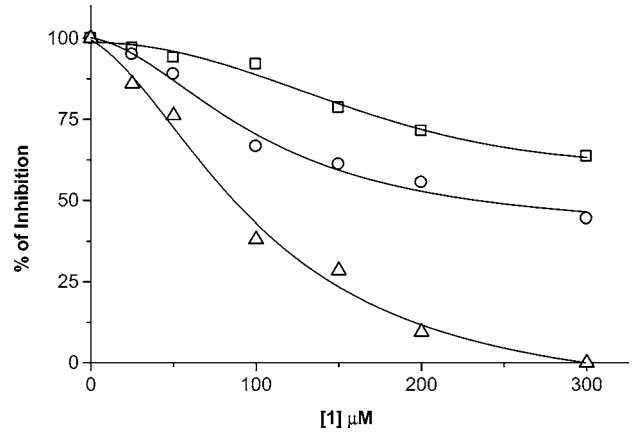

Figure 3. Effect of compound **1** on electron flow (basal, phosphorylating, and uncoupled) from water to MV in spinach chloroplasts. Control rate values for electron transport from basal (□), phosphorylating (○), and uncoupled (△) conditions were 933, 1200, and 1400 μequiv·e$^-$·h^{-1}·mg of Chl^{-1}, respectively.

transport, and (c) by blocking the phosphorylation reaction itself. Reagents that block electron transport avoid ATP synthesis because the generation of the transmembrane electrochemical gradient is not formed; the driving force for ATP synthesis is dependent upon electron flow. Chemicals that increased the proton permeability of thylakoid membranes uncouple phosphorylation from electron flow. Uncoupling agents inhibit ATP synthesis by decreasing the proton gradient but allow electron transport to occur at high rates. In contrast, direct inhibitors of photophosphorylation block both phosphorylation and that portion of electron transport that is a consequence of proton efflux linked to phosphorylation (*19*).

Thus, the described inhibition of photophosphorylation produced by **1** can be explained by an effect of lasiodiplodin on either the electron transport flow or the energy-transfer reactions.

It was decided to analyze the mechanism of action of **1** to determine its effect on the rates of electron transport in different conditions (basal, phosphorylating, and uncoupled). It was measured in the absence or presence of ADP, P_i, or NH_4Cl and using MV as electron acceptor; compound **1** inhibited oxygen uptake by illuminated chloroplasts. **Figure 3** shows that **1** inhibited all conditions of electron flow. It was concluded that **1** acts as a Hill reaction inhibitor. The uncoupled electron transport rate was the most inhibited (100% at 300 μM); however, compared with ATP synthesis inhibition (100% at 150 μM), a 2 times greater concentration of **1** is needed to inhibit uncoupled electron flow, and basal electron flow is less affected. Therefore, **1** has more than one mechanism of action and may be inhibiting H^+-ATPase.

Table 1. Effect of Lasiodiplodin (**1**) on Uncoupled PSII Electron Transport from Water to DCPIP and the Partial Reactions of PSII from Water to SiMo and from DPC to DCPIP

	PSII					
	H_2O to DCPIP		H_2O to SiMo		DPC to DCPIP	
concn (μM)	μequiv·e$^-$ mg^{-1} Chl	%	μequiv·e$^-$ mg^{-1} Chl	%	μM $DCPIP_{red}$ mg^{-1} Ch h^{-1}	%
0	433	100	400	100	449	100
50						
100	266	62	320	80	389	87
200	200	46	280	70	336	75
300	133	31	160	40	120	27
400	0	0	0	0		

Table 2. Effect of Lasiodiplodin (**1**) on the Bound to Thylakoid Membranes H^+-ATPase and the Light-Activated Membrane-Bound Mg^{2+}-ATPase and the Heat Ca^{2+}-ATPase Activity of Purified Coupling Factor 1 (CF_1) of Chloroplasts[a]

concn (μM)	H^+-ATPase	Ca^{2+}-ATPase (%)	Mg^{2+}-ATPase (%)
0	100	100	100
25		95.6	62.3
50	95.0	87.5	54.5
150	98.5	84.1	49.4
200	97.7		

[a] Control values for Mg^{2+}- and Ca^{2+}-dependent ATPases were 47.3 μmol of P_i released/mg of Chl·h and 55.4 mmol of Pi/mg of protein, respectively.

Localization of Lasiodiplodin (1) Site(s) of Interaction on PSI and PSII and Partial Reactions. To determine the site of inhibition on the thylakoid electron transport chain, the effect of **1** on uncoupled PSII, PSI, and partial reactions was determined using appropriate artificial electron donors, acceptors, and inhibitors (*20*). Lasiodiplodin (**1**) inhibited PSII uncoupled electron flow from water to DCPIP (**Table 1**), from water to SiMo, and from DPC to DCPIP (**Table 1**). **1** inhibited by 100% all activities of PSII electron transport rate and partial PSII reactions at 400 μM (**Table 1**); the polarographic measurement indicated that the PSII electron transport chain contains two inhibition sites for **1**: one, the water-splitting enzyme, and the other, the electron-transfer path between P_{680} and Q_A.

Effect of Lasiodiplodin (1) on Membrane-Bound Mg^{2+}-ATPase and Mg^{2+}- and Ca^{2+}- Dependent ATPase Activities from Isolated CF_1. To determine whether **1** interacts with the catalytic unit of the H^+-ATPase complex when it inhibits the photophosphorylation, its effects on Mg^{2+}-ATPase and Mg^{2+}- and Ca^{2+}-dependent ATPase activities of CF_1 were investigated. **Table 2** shows that increasing concentrations of **1** partially inhibit the three activities. Mg^{2+}-ATPase from isolated CF_1 was the most inhibited (approximately 51% at 150 μM). This last result indicates that **1** has another site of interaction and inhibition at CF_1; thus, it acts as an energy-transfer inhibitor, too.

Chl *a* Fluorescence. To further characterize the mode of action of **1** in thylakoid, the Chl *a* fluorescence induction curves were measured. A polyphasic curve exhibiting an OJIP sequence of fluorescence transients was observed with thylakoid used as control (**Figure 4**). These transients were similar to those previously published (*21*). **Figure 4** also shows the effect of **1** at 300 μM on the fluorescence induction curves on freshly lysed

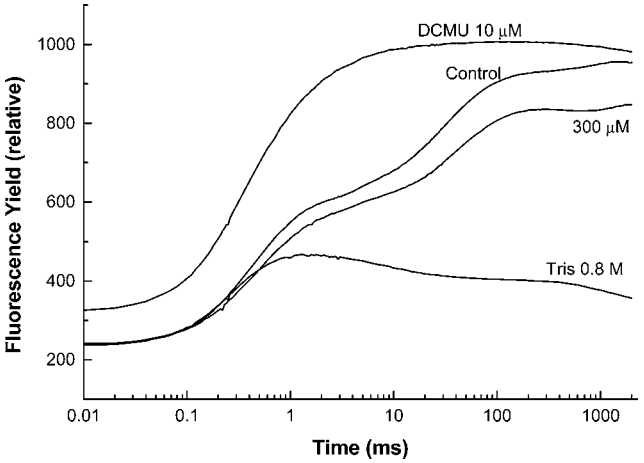

Figure 4. Fluorescence rise kinetics of freshly lysed broken chloroplasts infiltrated with lasiodiplodin (**1**) at 300 μM, DCMU, and Tris-treated thylakoid. Control chloroplasts are shown for comparison. Chl *a* fluorescence induction curves were measured at room temperature. Details are under Materials and Methods. Data are of three replicates.

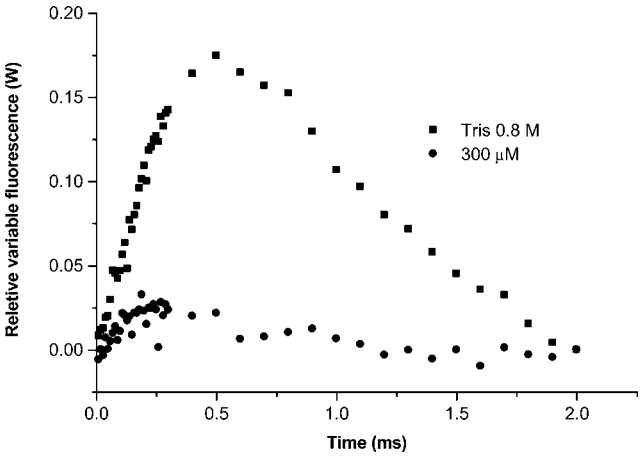

Figure 5. Appearance of the K-band at about 300 μs. Difference of *W* values of each curve as $W_{inhibited} - W_{control}$ from the normalized relative variable fluorescence on the amplitude $F_j - F_0$, $W = (F_t - F_0)/(F_j - F_0)$. Lasiodiplodin 300 μM (●) and broken chloroplasts were incubated with 0.8 M Tris (■).

intact chloroplasts. Curves in the presence of either 10 μM DCMU and 0.8 M Tris were used as positive controls. Addition of 10 μM DCMU resulted in a fast rise of the fluorescence yield during the first 2 ms of illumination, transforming the OJIP transient into an OJ sequence, indicating that DCMU displaces the secondary quinone acceptor, Q_B, from its binding site at the D_1 protein of PSII (*22*, *23*). Addition of 0.8 M Tris, pH 8.0, a well-known donor site inhibitor of PSII (*24*), resulted in the formation of a K phase followed by a dip; the K step arises when the electron flow to the acceptor side exceeds the electron flow from the donor side (*25*). The K step appears clearly and consists of a rapid rise to a maximum (at 300 μs) followed by a decrease to a level close to F_0 (**Figure 5**). All other steps, J and I, are absent from the transient, as is shown by heat-treated samples (*26*), conditions in which the electron flow from P_{680} to Q_A results in the formation of the K step. The fast initial fluorescence rise is due to the reduction of Q_A followed by the reduction of P^+_{680} by Z without the direct participation of the OEC. The subsequent reduction in fluorescence yield apparently results from the opening of the reaction center by reoxidation of Q_A and/or accumulation of P^+_{680}, both of which are effective fluorescence quenchers (*25*).

Table 3. Effect of Lasiodiplodin (**1**) on Fluorescence Parameters of Thylakoids Previously Incubated for 5 min in the Dark and with 0.8 M Tris, pH 8.0

compound	F_0	F_M	F_V/F_M	area
control	226	956	0.764	29200
10 μM DCMU	307	1006	0.695	1000
0.8 M Tris	227	468	0.515	0
300 μM	232	918	0.747	23800

However, the analysis of the Chl *a* fluorescence transient in this work indicates that the water-splitting enzyme was mildly blocked by **1** (**Figures 4** and **5**). In this work, the F_0 and F_M values and the area above the curve between F_0 and F_M (**Table 3**) decreased slightly in the presence of a 300 μM concentration **1**; these decreased values indicate that the electron transfer to the quinone pool size is partially blocked by **1**.

Conclusion. From the fungus *B. rhodina* were isolated compounds **1**−**4**. Compound **1** acts as a Hill reaction inhibitor in a similar way as other natural products such as trachyloban-19-oic acid (*26*), xanthorrhizol (*27*), tricolorin A (*28*), and 6-(3,3-dimethylallyloxy)-4-methoxy-5-methylphthalide (*29*). Polarographic measurements and chlorophyll *a* fluorescence measurements indicate that **1** inhibits partially the water-splitting enzyme. In this work, we found for the first time that the lasiodiplodin (**1**) behaves as a Hill reaction inhibitor of the oxygen-evolving complex on chloroplasts and also that **1** interacts at CF_1 by inhibiting CF_1 Mg^{2+}-ATPase activity.

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