Interference of 1,2,3,4-Tetramethoxy-5-(2-propenyl)benzene with Photosynthetic Electron Transport*

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The effect of 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene, the major phytogrowth-inhibitory agent isolated from the leaves, stem bark and wood of *Malmea depressa* (Annonaceae), on several photosynthetic activities has been investigated using freshly lysed spinach chloroplasts. The results indicate that this compound inhibits proton-uptake, ATP synthesis and electron flow (basal, phosphorylating and uncoupled) in a concentration dependent manner, therefore acting as a Hill reaction inhibitor. Uncoupled electron transport through photosystem I from reduced dichlorophenol-indophenol to methylviologen is unaffected by this compound. On the other hand, uncoupled electron transport through photosystem II from water to dichlorophenol-indophenol, from water to silicomolibdate and from diphenylcarbazide to dichlorophenol-indophenol is inhibited by this phenylpropanoid, suggesting that the site of inhibition is located in the span from P_{680} to Q_A.

Introduction

As a part of our search for biologically active compounds of agrochemical interest it was previously demonstrated that the aqueous lixiviates, organic extracts and the essential oil, prepared from the stem bark of *Malmea depressa* (Baill.) R. E. Fries (Annonaceae), inhibited seedling growth of *Amaranthus hypochondriacus* (L.) and *Echinochloa crusgalli* (L.) Beauv. Furthermore, bioactivity directed fractionation of the $CHCl_3$ extract and the essential oil led to the isolation of several phytotoxic principles. The most active compound was the phenylpropanoid 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene (Jiménez *et al.*, 1996).

The process of photosynthesis is a target of a wide range of compounds which destroy or inhibit plant growth (Einhelling, 1995). However, the effect of phenylpropanoids on energetic metabolism (i.e. respiration or photosynthesis) remains largely unexplored (Einhelling, 1995). Therefore, in this paper we describe the effect of 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene (**1**), the major and most active phytogrowth-inhibitory principle of *M. depressa,* on several photosynthetic activities including proton uptake, ATP synthesis and electron flow (basal, phosphorylating and uncoupled).

Materials and Methods

General experimental procedures

GC was performed on a Hewlett-Packard Model 5890 gas chromatograph, equipped with PAS-1701-tested 1701 silicone column (25 m×0.32 mm i.d.) programmed from 1–150 °C at the rate of 7 °C×min; the carrier gas was He (7 psi, 1 ml/min). Analytical and preparative TLC were performed on Si gel 60 F_{254} E. Merck plates, and the spots were visualized by spraying with a 10% solution of H_2SO_4, followed by heating at 110 °C.

Abbreviations: MV, methylviologen; DCMU, 3(3,4-dichlorophenyl-1,1-dimethylurea; DCPIP, dichlorophenol-indophenol; $K_3[Fe(CN)_6]$, potassium ferricyanide; SiMo, silicomolybdate; DPC, diphenylcarbazide

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Plant material

The leaves of *M. depressa* were collected in Catemaco, Veracruz, Mexico in September 1995. The wood was collected in Carrillo Puerto Quintana Roo, Mexico in March 1993. Voucher specimens were deposited in the Instituto de Ecología Herbarium (XAL), Jalapa and National Herbarium (MEXU), México D. F.

Extraction and identification

The essential oils were prepared by distillation from 200 g of plant material to yield 4.8 g from leaves and 3.4 g from wood. Preparative TLC of the essential oils on silica gel plates, using benzene–EtOAc 9:1 as the eluent, allowed the isolation of compound **1** (4.3 g from the leaves and 2.9 g from the wood). The spectroscopic and spectrometric properties of **1** were identical to those of an authentic sample previously isolated from the stem bark of *M. depressa* (Jiménez *et al.*, 1996).

Chloroplasts, isolation and chlorophyll determination

Chloroplast thylakoids were isolated from market spinach (*Spinacea oleracea* L.) as described earlier (Saha *et al.*, 1971; Mills *et al.*, 1980; Calera *et al.*, 1996) and suspended, unless otherwise indicated, in three ml of a medium composed of 400 mM sucrose, 5 mM $MgCl_2$, and 10 mM KCl and buffered with 0.030 M Na^+-tricine at pH 8.0 (KOH, 1 M). KCN (0.1 mM) was added to inhibit any catalase activity. Chlorophyll concentration was measured according to Strain *et al.* (1971).

Measurement of proton uptake, ATP synthesis and electron transport

Proton uptake was measured as the pH value increase between 8.0 and 8.1 (Dilley, 1972), using a combination microelectrode connected to a Corning Potentiometer with expanded scale. The pH changes were registered using a Gilson recorder. The reaction medium was 100 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, 1 mM Na^+-tricine, pH 8.0 (KOH, 1M). ATP-synthesis was determined titrametrically by the procedure of Dilley (Dilley, 1972). Methylviologen (MV) (0.05 mM) was employed as electron acceptor for the Hill Reaction.

Light-induced noncyclic electron transport in the presence of MV was monitored with a YSI (Yellow Spring Instrument C) model 5300 oxygen monitor using a Clark electrode in a temperature regulated flask at 20 °C. The reaction medium was the same as in the proton uptake assay except for the tricine concentration (15 mM) and the presence or absence of 6 mM ammonium chloride (NH_4Cl) (Saha, *et al.*, 1971; Mills *et al.*, 1980; Calera *et al.*, 1996).

Photosystem I was determined in a similar way as noncyclic electron transport (Calera *et al.*, 1995; Calera *et al.*, 1996). The following reagents were added: 6 mM NH_4Cl, 10 μM 3(3,4-dichlorophenyl-1,1-dimethylurea (DCMU), 100 μM dichlorophenol-indophenol (DCPIP), 50 μM MV and 500 μM ascorbic acid. Photosystem II electron transport was measured in the presence of 100 μM DCPIP, 1 μM 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB), 500 μM potassium ferricyanide ($K_3[Fe(CN)_6]$), and 6 mM NH_4Cl (Calera *et al.*, 1995; Calera *et al.*, 1996).

Uncoupled electron transport from water to silicomolibdate (SiMo) was determined as in photosystem II except that 200 μM SiMo and 10 μM DCMU were added to the reaction medium (Giaquinta *et al.*, 1984). Uncoupled electron transport from diphenylcarbazide (DPC) to DCPIP was measured spectrophotometrically as reported (Vernon and Shaw, 1969) but in this medium MV was omitted, and 200 μM DPC were added. All reaction mixtures were illuminated with actinic light from a projector lamp (GAF 2660) and were passed through a 5 cm filter of a 1% $CuSO_4$ solution. The temperature was 20 °C. For each reaction a blank experiment was performed with the chloroplasts alone in the reaction medium. All the experiments were done in triplicate and the data analyzed by ANOVA. The I_{50} value for each activity was extrapolated using the graph of percent activity vs concentration of phenylpropanoid. I_{50} is the concentration producing 50% inhibition.

Results and Discussion

Isolation of 1,2,3,4-Tetramethoxy-5-(2-propenyl)-benzene

GC analysis of the essential oils revealed the presence of compound **1** in a proportion of 95% in the case of the leaves and 90% in the case of

the wood. Identification was made by comparison with an authentic sample via coinjection during GC analysis) (Jiménez *et al.*, 1996). These results indicated that this phenylpropanoid is the major component not only of the essential oil from the stem bark, but also from those of the leaves and wood of *M. depressa*.

Preparative TLC of the essential oils from the leaves and of wood of *M. depressa* allowed the isolation of 1,2,3,4-tetramethoxy-5-(2-propenyl)-benzene (Fig. 1). The spectroscopic and spectrometric properties were identical to those of an authentic sample (Jiménez *et al.*, 1996).

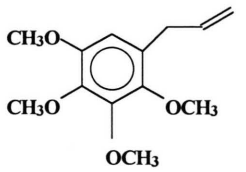

Fig. 1. Structure of 1,2,3,4-tetramethoxy-5-(2-propenyl)-benzene.

Biological activity of 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene

The effect of this phenylpropanoid on several photosynthetic processes, including ATP-synthesis, H^+-uptake, electron transport rate (basal, phosphorylating and uncoupled) and partial reactions of the photosystems I and II, was investigated using freshly lysed spinach chloroplasts (Calera *et al.*, 1995; Calera *et al.*, 1996).

Effect of 1,2,3,4-tetramethoxy-5-(2-propenyl)-benzene on basal, phosphorylating and uncoupled electron transport

1,2,3,4-Tetramethoxy-5-(2-propenyl)benzene inhibited basal, phosphorylating and uncoupled electron transport from water (electron donor) to MV (electron acceptor) in a concentration-dependent manner (Fig. 2). The uncoupled electron flow was most drastically inhibited. The I_{50} values for each type of electron transport (basal, phosphorylating and uncoupled) were 3.6, 5.0 and 2.7 μM, respectively. These data clearly indicate that this compound behaves as a Hill reaction inhibitor. These results also show that the target of **1** in the thylakoid membranes (are) is exposed in the non-energized state (uncoupled by 6 mM NH_4Cl), as indicated by the lowest I_{50} value being obtained at this state.

Localization of the site of inhibition of 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene

In order to determine the site of inhibition, the effect of phenylpropanoid **1** on partial photosynthesis reactions (photosystems I and II) was measured using artificial electron donors and acceptors (Lotina-Hennsen *et al.*, 1991; Calera *et al.*, 1995). Compound **1** inhibited electron flow in photosystem II from water to DCPIP, from water to SiMo and from DPC to DCPIP (this last activity was explored in Tris-treated chloroplasts [Vernon and Shaw, 1969]). At the concentration of 6.05 μM this compound completely inhibited both electron transport from water to SiMo and from water to DCPIP by 96.1%. The I_{50} for both activities was

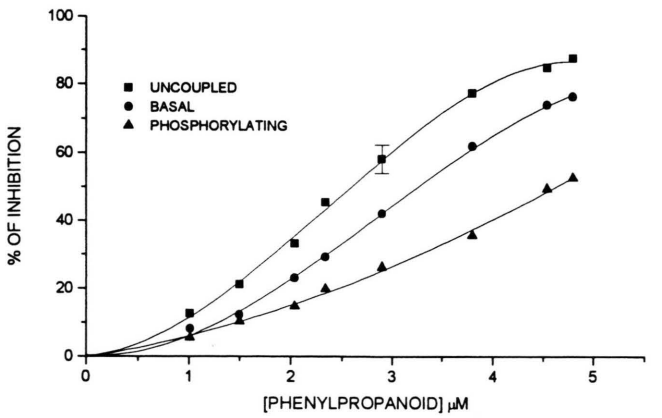

Fig. 2. Noncyclic electron transport (basal, phosphorylating and uncoupled) from water to methylviologen as a function of 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene concentration. Photophosphorylating electron transport was measured in the presence of 1 mM ADP and 3 mM K_2HPO_4. NH_4Cl (6 mM) was added for measuring uncoupled electron transport. Each cuvette contained 20 μg chlorophyll per ml in the reaction medium. Control value rates for basal, phosphorylating and uncoupled electron transport are 580, 488 and 1280, respectively, in $\mu eqe^- \cdot h^{-1} \cdot \mu gChl^{-1}$. Basal (■), phosphorylating (●) μequiv. e^- and uncoupled (△) electron transport.

2.86. Finally, electron transport from DPC to DCPIP was inhibited by 51% at the concentration of 9.08 μM. These results indicate that compound **1** primarily inhibited electron flow from P_{680} to Q_A and partially inhibited that from water to P_{680}. On the other hand, photosystem I electron transport from DCPIP to MV was inhibited 27% by this phenylpropanoid (data not shown). Photosystem II was more sensitive to 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene than photosystem I and is thus the target of compound **1**.

Effect 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene on ATP synthesis and H^+-uptake

ATP synthesis and H^+-uptake (Fig. 3) were also inhibited by phenylpropanoid **1**. The calculated I_{50} values were 1.40 and 2.30 μM, respectively. The concentration of **1** needed to inhibit these activities is lower than that needed to affect electron flow, suggesting that the proton gradient built up by electron transport is not available for ATP synthesis. This conclusion was supported by the fact that Mg^{2+}-ATPase activity was not inhibited by compound **1** (data not shown). It has been previously demostrated that several cinnamic acid derivatives inhibited electron transport and phosphorylation in spinach thylakoids (Einhelling, 1986); the I_{50} displayed by these derivatives ranged between 1 and 10 mM. Therefore, phenylpropanoid **1** was 370 times more potent than these cinnamic acid derivatives.

Conclusion

The interference of compound **1** with energetic metabolism at the level of photosynthesis as a Hill reaction inhibitor might be partially responsible for its phytogrowth inhibitory properties and its possible role as an allelopathic agent. It is important to point out that the target for most commercial herbicides that affect photosynthesis is at the Q_B level; however, this natural phenylpropanoid acts on a different step of the electron transport chain (P_{680} to Q_A).

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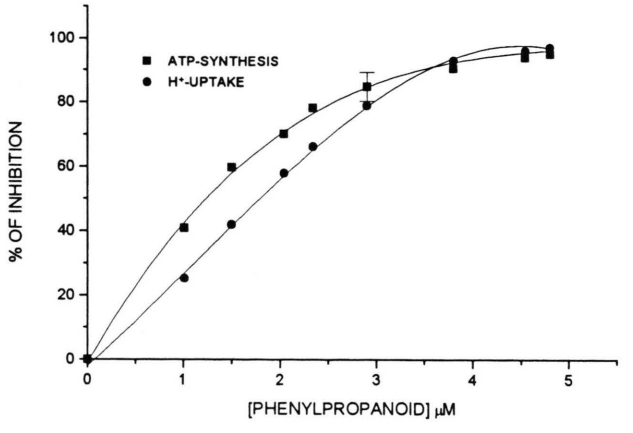

Fig. 3. Proton uptake (●) and ATP synthesis (■) as a function of 1,2,3,4-tetramethoxy-5-(2-propenyl)-benzene concentration. In each case the cuvette contained 20 μg chlorophyll per ml in the reaction medium. Other conditions are described in the experimental section.

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