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Siderin from *Toona ciliata* (Meliaceae) as photosystem II inhibitor on spinach thylakoids

Thiago A.M. Veiga [a], Raquel González-Vázquez [b], João Oiano Neto [a], Maria F.G.F. Silva [a], Beatriz King-Díaz [b], Blas Lotina-Hennsen [b,*]

[a] *Departamento de Química, Universidade Federal de São Carlos (UFSCar), 13565-905, São Carlos, S.P., Brazil*
[b] *Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México (UNAM), Ciudad Universitaria, 04510 México D.F., Mexico*

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Abstract

Four natural products were isolated from plants of the Rutaceae and Meliaceae families and their effect on photosynthesis was tested. Siderin (**1**) inhibited both ATP synthesis and electron flow (basal, phosphorylating, and uncoupled) from water to methylviologen (MV); therefore, it acts as Hill reaction inhibitor in freshly lysed spinach thylakoids. Natural products **2**–**4** were inactive. Secondary metabolite **1** did not inhibit PSI electron transport. It inhibits partial reactions of PSII electron flow from water to 2,6-dichlorophenol indophenol (DCPIP), from water to sodium silicomolybdate, and partially inhibits electron flow from diphenylcarbazid (DPC) to DCPIP. These results established that the site of inhibition of **1** was at the donor and acceptor sides of PSII, between P_{680} and Q_A. Chlorophyll *a* fluorescence measurements confirmed the behavior of the *Toona ciliate* coumarin **1** as P_{680} to Q_A inhibitor by the creation of silent centers. May be this is the mechanisms of action of **1** and is the way in which it develops a phytotoxic activity against photosynthesis.

Keywords: Siderin; *Toona ciliata* (Meliaceae); Water-splitting enzyme inhibitor; P_{680} to Q_A inhibitor; PSII inhibitor; Spinach thylakoids; Photosynthesis

Coumarins are secondary metabolites containing a 2*H*-1-benzopyran-2-one or benzopyrone moiety [1]. These compounds affect the energetic metabolism of plants including the process of photosynthesis [2–5]. For example, Einhelling [5,6] found that esculetin and scopoletin suppressed photosynthesis of *Lemma minor* L. at concentrations from 100 to 2000 μM similar to those required for growth inhibition. Scopoletin significantly depressed the photosynthetic rate in tobacco (*Nicotiana tabacum* L.), sunflower (*Helianthus annus* L.), and pigweed (*Amaranthus retroflexux* L.) seedlings at 500 μM. In addition, some natural 4-phenyl-coumarins and imperatorin, a furanocoumarin, behaved as photophosphorylation uncouplers (0–300 μM) or energy transfer inhibitors [3,4,7,8] (0–300 μM) in spinach chloroplasts.

Continuing with our studies on natural products, we isolated the coumarin siderin (**1**) from *Toona ciliata* (Meliaceae). In the present work, we describe for the first time the effect of siderin (**1**) on several photosynthetic activities in isolated spinach chloroplasts at increasing concentrations, measured with polarographic techniques and fluorescence induction curves of chlorophyll *a* of photosystem II (PSII)[1]. Measurements of changes to the chlorophyll fluorescence induction curve (Kautsky curve) have been used in photosynthesis research, i.e., for the study of the effect of PSII-inhibiting compounds [9,10]. Generally, changes in the fluorescence yield reflect changes in photochemical efficiency and heat dissipation [11]. Illumination with

* Corresponding author. Fax: +52 55 5622 5329.
E-mail address: blas@servidor.unam.mx (B. Lotina-Hennsen).

[1] *Abbreviations used:* PSII, photosystem II; MV, methylviologen; DCPIP, 2,6-dichlorophenol indophenol; DPC, diphenylcarbazid; DCMU, 3-(3,4-dichlorophenyl)-1,1 dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-*p*-benzoquinone; YSI, Yellow Springs Instrument.

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650 nm of dark-adapted thylakoids produces a rise in chlorophyll fluorescence emission with rapid transient phases called OJIP test [9] (Strasser). These phases are interpreted as follows: (i) The O–J phase corresponds to a complete reduction of the primary electron acceptor Q_A of PSII, (ii) the J–I phase corresponds to electron transfer from Q_A to Q_B (furthermore, the release of fluorescence quenching during the J–I phase is controlled by the PSII donor side, the water-splitting enzyme activity), and (iii) the I–P phase indicates full reduction of the electron carriers involved. Thus, the J–I phase is a useful indicator of water-splitting enzyme activity, although the exact mechanism involved remains to be established [10,11]. To know the mechanism and the action site of the coumarin (**1**), we tested its effect on the J–I phase and I–P phase by the JIP test.

Materials and methods

Tested plant material

Four natural coumarins [siderin (**1**), scaparon (**2**), bergapten (**3**), and 5-methoxy-seselin (**4**)] (Fig. 1) were tested their effect on photosynthesis. The natural product **1** was isolated from the methanolic extract of *T. ciliata* (Meliaceae) leaves as previously published [12,13]. Compounds **2**–**4** were isolated from the plant *Hortia* ssp. Stock solutions for compounds **1**–**4** were prepared using DMSO as solvent, and its maximum concentration in the media was less than 1%.

Isolation of siderin

The methanolic extract of the leaves (120.0 g) of *T. ciliata* was subjected to column chromatography over silica gel (230 mm, 1500 g) and the elution was carried out with hexane, dichloromethane, ethyl acetate, and methanol gradients to give a group of 38 fractions. The fraction 14 (3.3 g) was subjected to another column chromatography over silica gel (320 mm, 70 g) and the elution was carried out with hexane, dichloromethane, and acetone to produce 9 fractions, the fraction 4 was subjected to thin layer chromatography with benzene/dichloromethane/ethyl acetate/methanol (10:10:5:0.5) to produce the compound **1** that was identified with spectroscopic and spectrometric techniques: ^1H and ^{13}C NMR, IR and MS as previously reported [12,13].

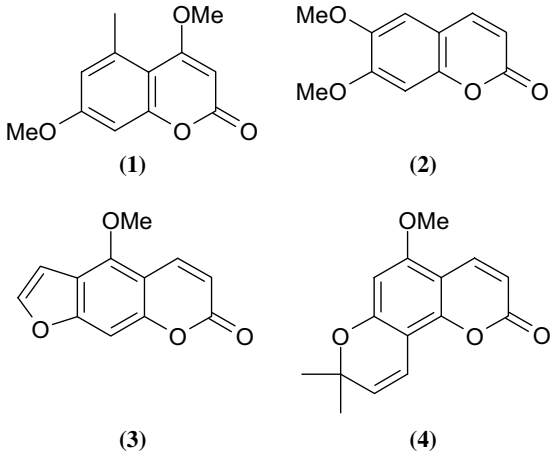

Fig. 1. Structure of the coumarins tested on different photosynthesis activities.

Chloroplasts isolation and chlorophyll determination

Intact chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L.) obtained from a local market as previously described [14,15]. Chloroplasts were suspended in the following medium: 400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, and 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 buffer (pH 8 with the addition of KOH). Chlorophyll concentration was measured spectrophotometrically as reported [16].

Measurement of ATP synthesis

ATP synthesis was determined titrimetrically using a microelectrode Orion model 8103 Ross connected to a Corning potentiometer model 12, with expanded scale as reported [17]. The ATP synthesis reaction medium contained 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, 50 μM methylviologen (MV) was used as electron acceptor and 1 mM Na^+-tricine (pH 8.0) in addition of 20 μg/mL of chlorophyll, where the intact chloroplasts were freshly lysed.

Measurement of non-cyclic electron transport rate

Light-induced non-cyclic electron transport activity from water to MV was determined by using a Clark type electrode, as published, in the presence of 50 μM of MV as electron acceptor [18]. Basal electron transport was determined by illuminating chloroplasts during 1 min (equivalent of 20 μg/mL of chlorophyll) were lysed in 3 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 [19]. Phosphorylating non-cyclic electron transport was measured as basal non-cyclic 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 non-cyclic electron transport medium, and 6 mM NH_4Cl was added as uncoupler. All reaction mixture was illuminated with actinic light of a projector lamp (GAF 2660) passed through a 5 cm filter of a $CuSO_4$ solution for 1 min.

Uncoupled photosystem II (PSII) electron flow determination

Electron transport activity was monitored with an oxygen monitor YSI (Yellow Springs Instrument) model 5300 using a Clark type electrode. The reaction medium was the same as in electron transport assay. Uncoupled PSII from $H_2O \rightarrow$ DCPIP was measured by the reduction of DCPIP supported O_2 evolutions monitored polarographically, DCPIPox accept electrons at the D1 protein, thus measure the electron transport from water to Q_B. 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 reduced (with ascorbate) DCPIP to MV was determined in a similar form to basal non-cyclic 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 for reduction of DCPIP, and 6 mM NH_4Cl, an uncoupler. 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.

Chlorophyll a fluorescence of PSII

Chlorophyll *a* fluorescence induction curves were measured at room temperature with a Hansatech Handy PEA (Plant Efficient Analyzer) as previously described by Strasser et al. [9] and King-Díaz et al. [20]. The maximum fluorescence yield from the sample was generated using the light