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Pachypodol from *Croton ciliatoglanduliferus* Ort. as Water-Splitting Enzyme Inhibitor on Thylakoids

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A bioactivity-guided chemical study of aerial parts of *Croton ciliatoglanduliferus* Ort. led to the isolation for the first time of the flavonoids retusin (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) (**1**) and pachypodol (5,4′-dihydroxy-3,7,3′-trimethoxyflavone) (**2**) from the *n*-hexane extract. Compounds **1** and **2** were separated by preparative thin-layer chromatography. Compound **2** was the most active compound on ATP synthesis inhibition. The I_{50} value was 51 μM. Pachypodol behaves as a Hill reaction inhibitor. It inhibited the uncoupled electron flow on photosystem II partial reaction from water to dichlorophenol indophenol (DCPIP) and from water to sodium silicomolybdate. However, the uncoupled partial reaction from diphenylcarbazide to DCPIP and the uncoupled photosystem I from DCPIPred to MV were not inhibited by **2**. These results were corroborated by fluorescence decay data. Therefore, pachypodol inhibits the water-splitting enzyme activity. Compound **1** with a 4′-methoxy group was a weak inhibitor, indicating that the 4′ free −OH group is important for strong inhibition.

KEYWORDS: ***Croton ciliatoglanduliferus*** **Ort.; photosynthesis; flavonoids; retusin; pachypodol; photosystem II; OEC inhibitor**

INTRODUCTION

Croton ciliatoglanduliferus Ort. (Euphorbiaceae) is a wild plant, found in the Tehuacan region (Puebla State) and in Guerrero State, in Mexico. It has been used as a repellent against insects and for herbal medicine to cure some diseases (*1*). By a bioactivity-guided chemical study of the hexane extract of leaves of *C. ciliatoglanduliferus*, the mixture of two flavonoids was isolated. They were separated by chromatographic methods and characterized by spectroscopy as retusin (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) (**1**) and pachypodol (5,4′-dihydroxy-3,7,3′-trimethoxyflavone) (**2**). Retusin has been found previously in the genus *Ballota* (*2*), and pachypodol has been found in *Larrea cuneifolia* (*3*), *Ballota inaequidens* (*4*), and *Ballota glandulosissima* (*2*), among other plants. Antifungal (*2*) and antimutagenic (*5*) activities of these compounds have also been reported. However, to our knowledge, the effect on photosynthesis of the chemical constituents of *C. ciliatoglanduliferus* has not been reported.

As a part of our research on bioactive natural products (benzofuranes and benzochromans, monoterpenes, diterpenes, sesquiterpenes, sesquiterpene lactones, nortriterpenoids, phenylcoumarins, phenilpropanoids, etc.), we are conducting a systematic study of their potential herbicide activity (*6*). In this paper, we report the effect of **2** on different photosynthetic activities at increasing concentrations up to 300 μM measured with polarographic techniques and fluorescence induction curves of chlorophyll *a* of photosystem II (PSII). Measurements of changes to the chlorophyll fluorescence induction curve (Kautsky curve) have been used in photosynthesis research, and they can be used for the study of the effect of PSII-inhibiting compounds (*7*, *8*). Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest. Therefore, changes in the fluorescence yield reflect changes in photochemical efficiency and heat dissipation (*9*). Illumination with 650 nm of dark-adapted thylakoids produces a rise in chlorophyll fluorescence emission with three phases based in the OJIP test. 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 (*8*, *9*). To know the site of action of the flavonol (**2**), we discuss its effect on the J−I phase.

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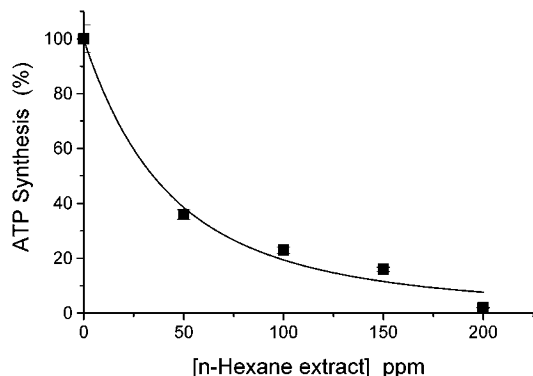

Figure 1. Effect of increasing concentrations of *n*-hexane extract obtained from leaves of *C. ciliatoglanduliferus* on the ATP synthesis rate of spinach thylakoids. The control value was 942 μM ATP mg^{-1} Chl h^{-1}.

MATERIALS AND METHODS

Tested Material. Aerial parts of *C. ciliatoglanduliferus* Ort. were collected in Guerrero State, Mexico, in 2004 (by N.D. and J. R. de Santiago, collection no. R1253), and a voucher specimen was deposited at the Facultad de Ciencias-Herbarium, UNAM, no. 95711.

The air-dried leaves (500 g) were powdered and extracted exhaustively by maceration at room temperature with *n*-hexane. The solvent was evaporated in vacuo to give a crude extract (15 g) that inhibited ATP synthesis (**Figure 1**). The I_{50} value was 42 ppm. This extract was fractionated via column chromatography on silica gel (0.0063−0.200 mm) and eluted with a mixture of hexane−EtOAc with polarity ascendent gradient. From fractions eluted with hexane−EtOAc 80:20, a mixture of **2** and **1** spontaneously crystallized (50 mg). Both compounds were separated by repeated runs in preparative thin-layer chromatography (hexane−ethyl acetate 80:20 v/v), and their structures were confirmed by comparison of their physical (melting points) and spectroscopical properties (UV, ^{1}HNMR, ^{13}CNMR, HMQC, HMBC, and NOESY data) with those previously published (*3*, *10*, *11*).

Chloroplasts Isolation and Chlorophyll Determination. Intact chloroplasts were isolated from market spinach leaves (*Spinaceae oleraceae* L.) as previously published (*12*, *13*) and suspended in a medium that contained 400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, and 30 mM Tricine−KOH (pH 8.0). They were stored as concentrated suspensions in the dark for 1 h at 4 °C. The chlorophyll (Chl) concentration was measured according to Strain et al. (*14*).

Determination 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 by Dilley (*15*). The ATP synthesis reaction medium contained 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, 1 mM Tricine−KOH (pH 8.0), 50 μM MV (methyl viologen) as an exogenous electron acceptor in the presence of 1 mM ADP, and 3 mM KH_2PO_4. The reaction was started by turning on the light in the presence of chloroplasts (20 μg of chlorophyll per mL).

Light-induced noncyclic electron transport rate from water to MV was determined by using a Clark type electrode connected to YSI (Yellow Spring Instrument) model 530 oxygen monitor. The basal electron transport rate from water to MV was determined by illuminating chloroplasts (20 μg of chlorophyll per mL) during 1 min in the basal electron transport medium as previously published (*16*, *17*). The basal electron transport medium was similar to the ATP synthesis medium except that 1 mM tricine was changed by 15 mM, and both 1 mM ATP and 3 mM KH_2PO_4 were excluded. The phosphorylating noncyclic electron transport rate from water to MV was measured as basal noncyclic electron transport, but in this case, 1 mM ATP and 3 mM KH_2PO_4 were added. Uncoupled noncyclic electron transport from water to MV was tested with the basal electron transport medium, and 6 mM NH_4Cl was used as uncoupler.

Uncoupled PSII and Photosystem I (PSI) Electron Flow. The uncoupled PSII and PSI electron flows were performed as uncoupled electron transport assays. PSII was measured from water to the reduction of DCPIP (dichlorophenol indophenol)-supported O_2 evolution. To perform this, 1 μM DBMIB (2,5-dibromo-3-methyl-6-isopropil-*p*-benzoquinone), 100 μM DCPIP, 500 μM $K_3[Fe(CN)_6]$, and 6 mM NH_4Cl were added; MV was omitted (*13*, *16*).

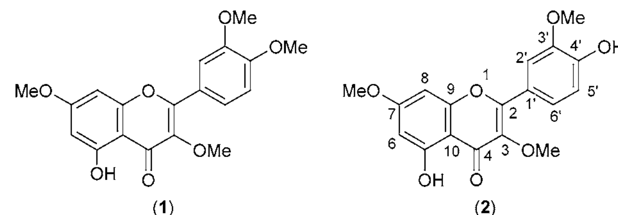

Figure 2. Structure of retusin (5-hydroxy-3,7,3′,4′-tetramethoxyflavone) (**1**) and pachypodol (5,4′-dihydroxy-3,7,3′-trimethoxyflavone) (**2**).

Uncoupled PSII partial reaction from H_2O to SiMo (sodium silicomolybdate) was determined as in PSII except that 1 μM DBMIB, 100 μM DCPIP, and 500 μM $K_3[Fe(CN)_6]$ were omitted, and 200 μM SiMo and 10 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] were added (*17*).

The uncoupled PSII partial electron transport rate from DPC (diphenyl carbazide) to $DCPIP_{OX}$ was measured as the $DCPIP_{OX}$ reduction rate with a spectrophotometer Beckman DU 650 and determined in thylakoids previously treated with 0.8 M Tris (pH 8.0) and incubated for 30 min at 4 °C (*18*).

The uncoupled PSI electron transport rate was determined as oxygen consumption in a similar form to noncyclic basal electron transport rate with 10 μM DCMU, 100 μM DCPIP reduced with 300 μM ascorbate, 50 μM MV, and 6 mM NH_4Cl, which were used in the medium (*19*).

The I_{50} value for each activity is the concentration of the compound producing 50% inhibition. Stock solutions for **1** and **2** were prepared with DMSO (dimethyl sulfoxide). The DMSO concentrations were less than 0.5%.

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. (*7*) and King-Díaz et al., (*20*). The maximum fluorescence yield from the sample was generated using three light-emitting diodes (broad band 650 nm). The pulse duration was 1 s. The reaction medium used was as the one employed in basal noncyclic electron transport measurements. To monitor Chl *a* fluorescence transients, aliquots of dark-adapted thylakoids containing 20 μg of Chl were transferred to filter paper by gravity with a dot-blot apparatus (Bio-Rad, United States) to ensure a homogeneous and reproducible distribution of thylakoids in the filter paper and dipped immediately in 3 mL of medium with different concentrations of the tested compound.

RESULTS AND DISCUSSION

Isolation of 1 and 2 from *n*-Hexane Leaves Extract of *C. ciliatoglanduliferus*. The *n*-hexane extract from the aerial parts of *C. ciliatoglanduliferus* displayed inhibitory activity on ATP synthesis (**Figure 1**). To know the compound responsible for this inhibitory activity, the *n*-hexane extract was fractionated by column chromatography; then by thin-layer chromatography, two compounds were isolated and characterized as **1** and **2** (**Figure 2**) as described in the Materials and Methods (45 and 30% of efficiency, respectively).

Effects of 1 and 2 on Different Photosynthetic Activities. Results showed that both flavonoids **1** and **2** inhibit ATP synthesis coupled to electron transport from water to MV on freshly lysed intact spinach chloroplasts (**Figure 3**). Compound **2** is the most active. The calculated I_{50} value was 51 μM. Compound **1** was not further studied because it did not reach the I_{50} of inhibition. These data indicate that the free −OH group at position 4′ of **2** is an essential requirement for interaction and inhibition of ATP synthesis, whereas in **1**, the position 4′ is methoxylated and thus is inactive as an ATP synthesis inhibitor.

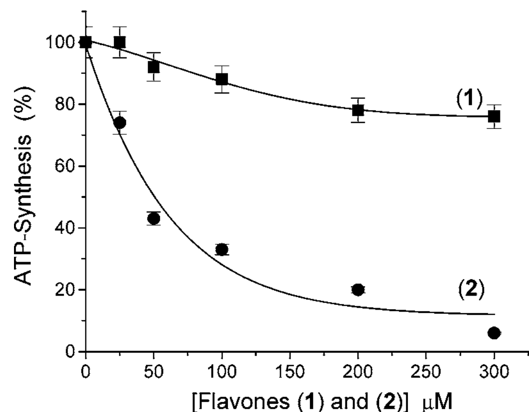

Figure 3. Effect of increasing concentrations of **1** (■) and **2** (●) on ATP synthesis rate of spinach thylakoids. The control value for ATP synthesis was 1200 μM ATP mg^{-1} Chl h^{-1}.

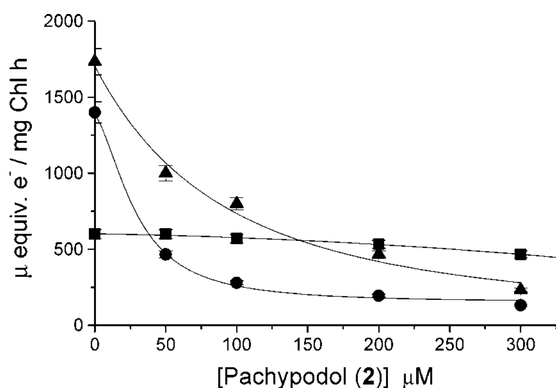

Figure 4. Inhibition of the electron transport chain of spinach chloroplasts measured as basal (■), phoshorylating (●), and uncoupled (▲) electron flow, from water to MV as a function of increasing concentrations of pachypodol. The control value rates were 600, 1401, and 1734 μequiv e$^-$ mg^{-1} Chl h^{-1}, respectively.

The light-dependent photophosphorylation is coupled to electron transport. Thus, ATP formation can be inhibited by either blockage of the electron transport, by direct inhibition of the H^+-ATPase, or by uncoupling of the ATP synthesis process from the electron transport (*21*). To elucidate the mechanism of action of **2** on photosynthesis, its effect on noncyclic electron transport from water to MV (basal, phosphorylating, and uncoupled) was investigated.

Compound **2** inhibited basal, phosphorylating, and uncoupled electron transport in different degrees (**Figure 4**) as concentrations of **2** increased up to 300 μM; phosphorylating and uncoupled electron transport rates were the most inhibited; their I_{50} values were 30 and 76 μM, respectively. However, basal electron transport rate from water to MV was less inhibited (**Figure 4**), and these results suggest that during phosphorylating and uncoupled electron transport rates on thylakoids, the target of **2** is exposed by conformational changes during illumination. However, in basal conditions, the target on the electron transport chain of thylakoids is buried for **2**. Thus, **2** cannot interact with the site of inhibition. All data indicate that **2** acts as a Hill reaction inhibitor.

Localization of 2 Interaction Sites on PSII, PSI, and Partial Reactions. To localize the site of inhibition on thylakoid electron transport chain, the effect of **2** on PSII, PSI, and the partial reactions were tested using artificial electron donors and electron acceptors, as well as appropriate inhibitors (*21*). **Table 1** shows that **2** inhibits both uncoupled PSII from water to DCPIP and the span of electron flow from water to SiMo by 75% at 300 μM. These results indicate that **2** inhibits PSII at the span of electron transport from water to Q_A.

Table 1. Effect of **2** on Uncoupled PSII, PSI, Electron Transport Rate, and PSII Partial Reactions

	PSII H_2O to DCPIP		H_2O to SiMo		DPC to DCPIP		PSI DCPIPred to MV	
concn (μM)	μequiv e$^-$ mg^{-1} Chl h^{-1}	%	μequiv e$^-$ mg^{-1} Chl h^{-1}	%	μM DCPIPred mg^{-1} Chl h^{-1}	%	μequiv e$^-$ mg^{-1} Chl h^{-1}	%
0	488	100	178	100	700	100	1120	100
100	381	78	135	76	635	91	1155	103
200	268	55	89	50	690	99	1153	103
300	132	27	45	25	710	101	1095	98

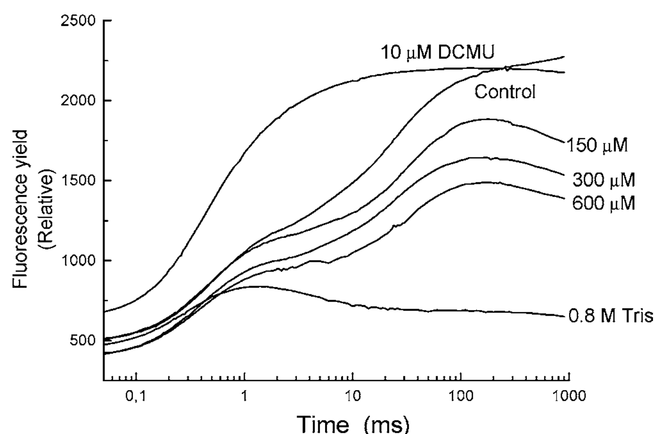

Figure 5. Fluorescence rise kinetics of freshly lysed broken chloroplasts infiltrated with pachypodol, DCMU, and Tris-treated thylakoids. Control chloroplasts are shown for comparison. Chl *a* fluorescence induction curves were measured at room temperature. Details are in Materials and Methods. Data are averages of three replicates.

To determine the site of electron transport inhibition of **2** between water to Q_A, electron flows were measured from DPC to DCPIP in Tris-treated chloroplasts (uncoupled with 6 mM NH_4Cl) in the presence of **2**. Data show that this span of electron transport was not inhibited at all concentrations tested, and neither uncoupled PSI from DCPIP red to MV (**Table 1**). Therefore, the last two results indicate that the target of **2** is located at the donor side of PSII; it interacts at the water-splitting enzyme complex.

Chlorophyll *a* Fluorescence Transient Measurements in the Presence of 2. To corroborate the interaction site of **2** at the donor site of PSII, freshly lysed chloroplasts were incubated for 5 min in the dark at room temperature with different concentrations of **2**, 10 μM DCMU, and 0.8 M Tris, which were used as positive controls (**Figure 5**). The thylakoids control showed a polyphasic fluorescence curve with OJIP sequence of transients similar to that previously described for plants, green algae, and cyanobacteria (*7*) having slower kinetics than leaves. The addition of 10 μM herbicide DCMU induces a fast rise of the fluorescence yield during the first 2 ms of illumination; it transforms the regular OJIP sequence into an OJ curve (*7*). When the thylakoids are treated with Tris, a well-known donor side inhibitor of PSII (*22*), the fluorescence induction curve form is reduced, and this results in a reduction of the maximum fluorescence yield, and the K-band appears and consists of a rapid rise to a maximum (at 300 μs) followed by a decreased fluorescence yield to level close to F_0. All other steps, J and I are absent from the transient (*23*, *25*). The Tris-treated thylakoid conditions are shown also by heat-treated chloroplasts where

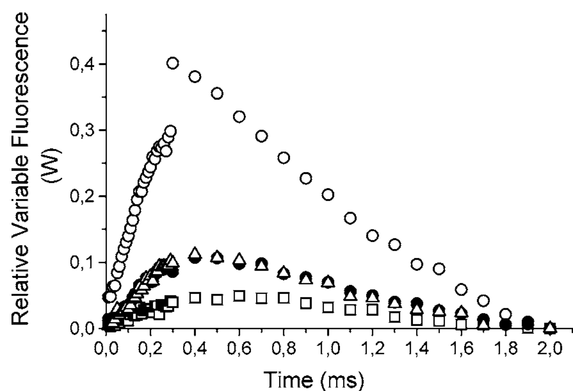

Figure 6. Appearance of the K-band at about 300 μs. Difference of each curve from the control with normalized relative variable fluorescence on the amplitude $F_j - F_0$. Pachypodol at 150 (□), 300 (●) and 600 μM (△). Broken chloroplasts incubated with 0.8 M Tris (○).

Table 2. Effect of Increasing Concentration of **2** on Fluorescence Parameters on 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	468	2275	0.794	69200
10 μM DCMU	593	2203	0.731	3600
0.8 M Tris	427	835	0.489	0
		2		
150 (μM)	467	1883	0.752	24000
300 (μM)	367	1642	0.776	16600
600 (μM)	374	1488	0.749	17200

the OEC damage occurs. Analysis of the difference of each curve from control on relative variable fluorescence when the O−J phase was normalized between $F_j - F_0$, $W_t = F_{v,t}/(F_j - F_0) = (F_t - F_0)/(F_j - F_0)$, shows an increasing suppression of J level (at 2 ms), and a rapid rise at about 300 μs appears when the concentration of **2** increases, indicating clearly the creation of the K-band (**Figure 6**). According to Strasser (*23*), the appearance of the K-band emerges from an imbalance in the electron flow between the donor and the acceptor side of PSII. In this condition, the electron flow from OEC to Z is slower than the electron flow from P_{680} to Q_A. The fast initial fluorescence rise is due to the reduction of Q_A to Q_A^- followed by the reduction of P_{680}^+ by Z without the direct participation of OEC (*23*). Thus, the analysis of Chl *a* fluorescence transient in this work clearly indicates that the water-splitting enzyme was blocked by **2**. When OEC is inhibited, it cannot donate electrons to Y_Z, which in turn cannot reduce $P680^+$; in this way, a maximal accumulation of $P680^+$, $Pheo^-$, and Q_A^- occurs (*24*). Strasser et al. (*25*) proposed the term "silent reaction center" as the centers that can neither reduce Q_A nor back transfer their excitation energy to the antenna; hence, the corresponding PSII units do not contribute to the variable fluorescence and their fluorescence yields are lower. In this work, the F_0 values were almost constant and F_M decreased with increasing concentrations of compound **2**, and the low values of the area above the curve between F_0 and F_M (**Table 2**) indicate that the electron transfer to the quinone pool size is blocked. Then, Q_A of PSII is not reduced, and these results indicate the creation of the silent reaction centers (*25*).

Conclusion. From phytochemical studies, **2** and **1** were found for the first time as constituents of the plant *C. ciliatoglanduliferus* Ort. (Euphorbiaceae). Compound **2** 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*), etc. Polarographic measurements and chlorophyll *a* fluorescence measurements indicate that **2** inhibits the water-splitting enzyme and creates the silent centers. The free hydroxyl group at position 4′ in the structure of **2** is an essential requirement for its action. In this work, we found for the first time that the flavonoid **2** behaves as a Hill reaction inhibitor of the oxygen-evolving complex on chloroplasts.

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NOTE ADDED AFTER ASAP PUBLICATION

The original posting of January 13, 2006 has been corrected. A name in references 2 and 4 was corrected, and a sentence was added to the Acknowledgment in the revised ASAP posting of January 20, 2006.

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