Effect of Selected Coumarins on Spinach Chloroplast Photosynthesis†

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Xanthyletin (**1**), 3-(1′,1′-dimethylallyl)xanthyletin (**2**), and chalepensin (**3**), the major coumarins isolated from *Stauranthus perforatus*, inhibit ATP synthesis from water to methylviologen in spinach thylakoids in a concentration-dependent manner. At low concentration chalepensin (**3**) inhibits basal and phosphorylating electron flow from water to $K_3[Fe(CN)_6]$ without affecting uncoupled electron flow but accelerating Mg^{2+}-ATPase activity. Thus, at low concentration the compound behaves as an energy transfer inhibitor. However, at higher concentrations this coumarin acts as an uncoupler because it enhances basal and phosphorylating electron transfer. On the other hand, coumarins **1** and **2** act as Hill reaction inhibitors, although **2** exhibited also uncoupler properties because it induces stimulation of basal and phosphorylating electron flow from water to ferricyanide. The site of interference of xanthyletin was located at the b_6f-PC level of the electron transport chain.

Keywords: *Stauranthus perforatus Lundell (Rutaceae); chalepensin; xanthyletin; 3-(1′,1′-dimethylallyl)xanthyletin; phosphorylation inhibitor; Hill reaction inhibitor; uncouplers*

INTRODUCTION

Coumarins are secondary metabolites containing a 2*H*-1-benzopyran-2-one or benzopyrone moiety (Zobel and Brown, 1995). These compounds affect the energetic metabolism of plants including the process of photosynthesis (Tissut et al., 1980; Moreland and Novitzky, 1987; Calera et al., 1995, 1996; Mata et al., 1998; Einhelling, 1995). For example, Einhelling (1986, 1995) found that esculetin and scopoletin suppressed photosynthesis of *Lemma minor* L. at concentrations 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. In addition, we have reported that some natural 4-phenylcoumarins and imperatorin, a furanocoumarin, behaved as photophosphorylation uncouplers or energy transfer inhibitors (Calera et al., 1995, 1996; Mata et al., 1996, 1998; Lotina-Hennsen et al., 1998b) in spinach chloroplasts. Continuing with our studies on natural coumarins, the present investigation describes the effects of two pyranocoumarins [xanthyletin (**1**) and 3-(1′,1′-dimethylallyl)xanthyletin (**2**)] and a furanocoumarin [chalepensin (**3**)] on several photosynthetical activities in isolated spinach chloroplasts.

EXPERIMENTAL PROCEDURES

Tested Material. Two pyranocoumarins [xanthyletin (**1**) and 3-(1′,1′-dimethylallyl)xanthyletin (**2**)] and a furanocoumarin [chalepensin (**3**)] (Figure 1) were isolated from the roots of *Stauranthus perforatus* Lundell (Rutaceae), a Mexican plant, as reported (Rudiño-Piñero et al., 1995). Stock solutions for compounds **1**−**3** were prepared using a mixture of methanol/water (1:1). The compounds are sparingly soluble in water but soluble in the mixture above indicated, and in all cases the maximum concentration of solvent mixture introduced in the media was 1%.

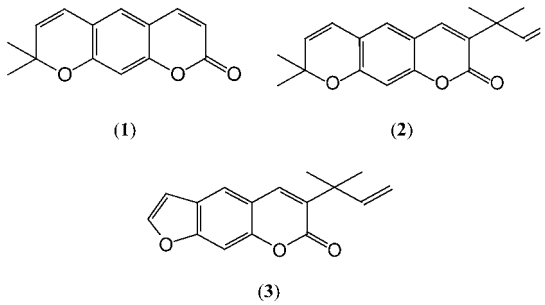

Figure 1. Structures of coumarins.

Chloroplasts Isolation and Chlorophyll Determination. Intact chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L.) obtained from a local market as previously described (Lotina-Hennsen et al., 1998a; Mills et al., 1980). 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 (Strain et al., 1971; Lotina-Hennsen et al., 1998a).

Measurement of ATP Synthesis. For ATP synthesis determination electron transport medium was used but tricine buffer concentration was 1 mM, and 50 μM methylviologen (MV) was used instead of $K_3[Fe(CN)_6]$ as electron acceptor (Dilley, 1972). ATP synthesis was determined titrimetrically using a microelectrode Orion model 8103 Ross connected to a Corning potentiometer model 12, with expanded scale as reported (Dilley, 1972). The pH changes were registered using

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† Taken in part from the M.S. theses of I.S.R. and M.L.M.

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10.1021/jf981121+ CCC: $18.00
Published on Web 04/17/1999

a Gilson recorder. The ATP synthesis reaction medium used contained 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, 50 μM MV, and 1 mM HEPES−KOH (pH 8.0), where the intact chloroplasts were freshly lysed.

Measurement of Noncyclic Electron Transport Rate. Noncyclic electron transport activity from water to potassium ferricyanide was determined through reduction of $K_3[Fe(CN)_6]$, which was added as electron acceptor. Reduced $K_3[Fe(CN)_6]$ was measured spectrophotometrically (Beckman DU 650) at 420 nm (Allen and Holmes, 1986). Basal electron transport was determined as follows: the intact chloroplasts (equivalent of 20 μg of chlorophyll/mL) were 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 300 μM $K_3[Fe(CN)_6]$ at pH 8.0]. The sample was illuminated during 1 min in the presence or absence of 6 mM NH_4Cl (Lotina-Hennsen et al., 1998a; King-Díaz et al., 1998). 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 transport medium, and 6 mM NH_4Cl was added. All reaction mixture was illuminated with actinic light of a projector lamp (GAF 2660) passed through a 5 cm filter of a 1% $CuSO_4$ solution for 1 min.

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 electron transport assay. Uncoupled photosystem II was measured by the reduction of DCPIP-supported O_2 evolutions monitored polarographically. The reaction medium for assaying PSII activity contained the same whole-chain electron transport medium ($H_2O \rightarrow K_3[Fe(CN)_6]$) in the presence of 1 μM DBMIB, 100 μM DCPIP, and 6 mM NH_4Cl. Photosystem I electron transport was determined in a similar form to noncyclic electron transport. The following reagents were added: 10 μM DCMU, 100 μM DCPIP, 50 μM MV, 300 μM ascorbate, and 6 mM NH_4Cl, without $K_3[Fe(CN)_6]$. Uncoupled electron transport from reduced PMS to MV was determined using KCN-poisoned chloroplasts. The reaction medium was the same as in photosystem I except that 500 μM PMS/100 μM ascorbate was used as electron donor to P700, MV as PSI electron acceptor, 10 μM DCMU as inhibitor to PC, and 6 mM NH_4Cl to uncouple 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 (King-Díaz et al., 1998). Moreover, EPR spectroscopy confirmed the ability of reduced PMS to interact directly with P700 (Izawa et al., 1973).

The I_{50} value for each activity was extrapolated using the graph of percent activity versus concentration of compounds **1**, **2**, and **3**. I_{50} is the concentration producing 50% inhibition.

Mg^{2+}-ATPase Assay. Mg^{2+}-ATPase activity bound to thylakoid membranes was measured according to the technique reported by Mills et al. (1980). The amount of P_i generated was quantified according to the procedure of Sumner (1944).

RESULTS AND DISCUSSION

ATP Synthesis. Photosynthetic phosphorylation from water to methylviologen in isolated freshly lysed intact spinach chloroplasts was completely inhibited by all coumarins in a concentration-dependent manner (Figure 2). The I_{50} values for ATP synthesis inhibition were 33, 60, and 30 μM for xanthyletin (**1**), 3-(1′,1′-dimethylallyl)xanthyletin (**2**), and chalepensin (**3**), respectively.

Eletron Flow. Basal and phosphorylating photosynthetic electron transport from water to ferricyanide in spinach thylakoids was inhibited by coumarins **1** and **3** (Figures 3 and 4) and enhanced by compound **2** (Figure 5). Uncoupled electron transfer, measured as ferrocyanide formed, was not significantly affected by **3** (Figure 4) but was partially inhibited by **1** and **2** (Figures 3 and 5). The highest inhibitory effect (75%) caused by compound **3** was observed at a concentration of 150 μM. In the case of compound **1**, the maximum inhibitory effect (100%) was achieved at 175 μM. These results indicated

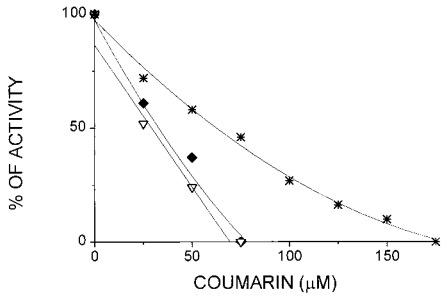

Figure 2. Inhibitory effect of coumarins on photophosphorylation coupled to electron transfer from water to methylviologen. Each cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions were described under Experimental Procedures. Control value rates for chalepensin (▽), xanthyletin (*), and 3-(1′,1′-dimethylallyl)xanthyletin (◆) were 296.3 ± 3.5, 290 ± 3.1, and 292.6 ± 2.8 μmol of ATP·h^{-1}·mg of Chl^{-1}, respectively. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts.

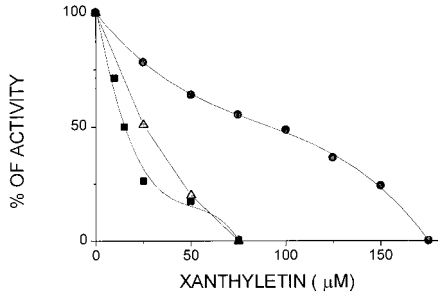

Figure 3. Noncyclic electron transport from water to $K_3[Fe(CN)_6]$ as a function of xanthyletin (**1**) concentration in chloroplast thylakoids isolated from spinach leaves (*S. oleracea* L.). Each cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions were described under Experimental Procedures. Control value rates for basal (■), phosphorylating (▲), and uncoupled (●) electron transport were 292.3 ± 3.3, 338.9 ± 3.1, and 464.8 ± 3.5 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.

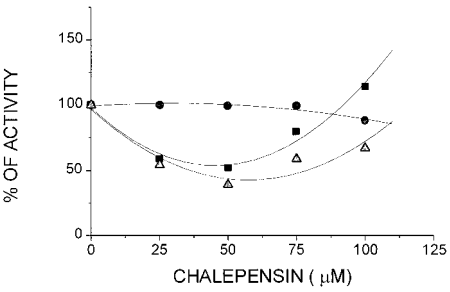

Figure 4. Noncyclic electron transports from water to $K_3[Fe(CN)_6]$ as a function of chalepensin (**3**) concentration in chloroplast thylakoids isolated from spinach leaves (*S. oleracea* L.). Each cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions were described under Experimental Procedures. Control value rates for basal (■), phosphorylating (▲), and uncoupled (●) electron transport were 334.2 ± 4.2, 633.2 ± 2.1, and 1141.2 ± 2.8 μequiv·e^-·h^{-1}·mg of Chl^{-1}, respectively. Each point represents the mean of five determinations. Each repetition was made in differents batches of chloroplasts.

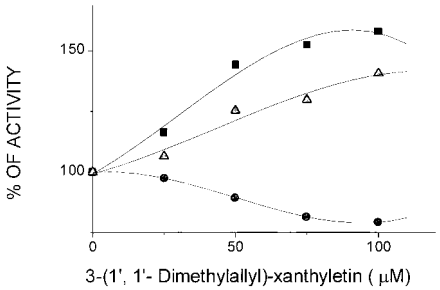

Figure 5. Noncyclic electron transport from water to $K_3[Fe(CN)_6]$ as a function of 3-(1′,1′-dimethylallyl)xanthyletin (**2**) concentration in chloroplast thylakoids isolated from spinach leaves (*S. oleracea* L.). Each cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions were described under Experimental Procedures. Control value rates for basal (■), phosphorylating (▲), and uncoupled (●) electron transport were 202.8 ± 2.2, 371.1 ± 3.1, and 745.5 ± 2.5 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.

Table 1. Effect of the Xanthyletin on Uncoupled Photosystem I and II Electron Transport Rate[a]

	reactions		
	PSII	PSI	
concentration, μM	H_2O to DCPIP	DCPIPred to MV	PMSred to MV
0	100 ± 0.0	100 ± 0.0	100 ± 0.0
25		66.6 ± 2.1	
50		50 ± 1.3	
75		15 ± 41	
100	99.7 ± 4.2	00 ± 0.0	101.7 ± 3.4
125			
150			
175	101 ± 4.3		99.3 ± 5.1

[a] Control value rates μequiv·e^-·h^{-1}·mg of Chl^{-1} from reduced PMS (from H_2O to DCPIP), PSI (DCPIP to MV, PMS to MV): 375.5 ± 2.5, 230.8 ± 1.5, and 305.5 ± 2.5, respectively. Each point represents the mean of five determinations. Each repetition was made in different batches of chloroplasts.

that compound **1** acts as a Hill reaction inhibitor and coumarin **2** as mild uncoupler−Hill reaction inhibitor.

According to the results shown in Figure 3 the target of xanthyletin (**1**) is exposed when the thylakoid is energized (phosphorylating and basal state) because in this condition the inhibition is stronger than when the thylakoid is unenergized (uncoupled state). To determine the site of inhibition, the effect of **1** on partial reactions (photosystems I and II) was measured using artificial electron donors, acceptors, and inhibitors (Lotina-Hennsen et al., 1998a). Uncoupled photosystem II electron flow from water to DCPIP/$K_3[Fe(CN)_6]$ in the presence of 1 μM DBMIB, used as PSI electron flow inhibitor, and uncoupled photosynthesis I from reduced PMS to MV were not affected as the concentration of **1** increased. However, uncoupled PSI electron flow from reduced DCPIP to MV was completely inhibited (100%) in the presence of **1** (100 μM) (Table 1). Therefore, the target of **1** is localized at the b_6f-PC level of the electron transport chain.

Chalepensin (3). Electron flow is coupled to ATP synthesism and the energy transduction theory of Mitchell (1961) has been proposed to account for the mechanisms of coupled electron transport to ATP synthesis. Thus, any chemical that inhibits basal and phosphorylating electron transport rate without affecting uncoupled electron flow will inhibit photophosphorylation acting as an energy transfer inhibitor. On the other hand, any compound (uncoupler) that enhances basal and phosphorylating electron flow, without affecting uncoupled electron flows, inhibitd ATP synthesis through an activation of the Mg^{2+}-ATPase (Good et al., 1981). Thus, chalepensin (**3**) up to 25 μM (Figure 4) behaves as an energy transfer inhibitor. At 25 μM it inhibited basal and phosphorylating electron flows by 48 and 49%, respectively. However, at higher concentrations (25−100 μM) this coumarin acts as an uncoupler because it induced an increment of the basal and phosphorylating electron flow without affecting the uncoupled electron flow (Figure 4). This behavior of chalepensin is similar to that of the alkaloid ajmaline, an energy transfer inhibitor (Vallejos and Andreo, 1974); however, coumarin **3** activates the Mg^{2+}-ATPase and the alkaloid inhibits the enzyme activity. This observation suggests that in the case of compound **3**, the uncoupling activity is more important than the energy transfer properties. Chalepensin also differs from other photophosphorylation inhibitors such as kaempferol, DCCD, Dio-9, phlorizin, and 5-*O*-β-D-galactopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (Arntzen, 1974; McCarthy et al., 1965; McCarthy and Racker, 1967; Caleta et al., 1995) because it acts as an energy transfer inhibitor−uncoupler.

Table 2. Effect of Increasing Concentrations of Chalepensin on Mg^{2+}-ATPase Activity

μM	μM P_i·h^{-1}·mg of Chl^{-1}	%
0	224	100
10	312	139
25	376	168
50	290	129
(NH_4Cl), 1 mM	420	188

Mg^{2+}-ATPase Activity. Most energy-transfer inhibitors such as phlorizin, DIO-9, chlorotri-*n*-butylin, ajmaline, kaempferol, DCCD, and tryphenyltin block Mg^{2+}-ATPase (McCarthy et al., 1965; Izawa et al., 1966; Arntzen et al., 1974; Vallejos and Andreo, 1974; Gould, 1976). The same behavior was expected for chalepensin at concentrations up to 25 μM. However, we found that compound **3** at all concentrations tested (up to 100 μM) enhances the light-dependent Mg^{2+}-ATPase bound to thylakoid membranes (Table 2), thus behaving as an uncoupler as ammonia. This result suggested that the uncoupling activity of chalepensin is stronger that the energy transfer property.

The presence of the dimethylallyl moiety at C-3 could be related with the uncoupling properties exhibited by compounds **2** and **3**. The uncoupling activity of both compounds could be explained through a chaotropic mechanism due to a high lipophilicity related with the presence of the dimethylallyl chain. On the other hand, the nature of the heterocyclic ring fused at C-6/C-7 of the coumarin core seems to have no effect on the observed acitivities.

In summary, coumarins **1**−**3** interfere with energetic metabolism of plants at the level of photosynthesis with different mechanisms. Compound **3** behaves as an energy-transfer inhibitor (or H^+-ATPase inhibitor)−uncoupler, and coumarins **1** and **2** act as Hill reaction inhibitors, although **1** exhibited also uncoupler properties. The target of xanthyletin is at b_6f-PC.

ACKNOWLEDGMENT

We are grateful to Q. Beatriz King-Diaz for technical assistance.

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Received for review October 4, 1998. Revised manuscript received February 25, 1999. Accepted March 1, 1999. This work was supported by Grant IN205197 from DGAPA, UNAM. I.S.R. and M.L.M. acknowledge the fellowship awarded by CONACyT to carry out graduate studies.

JF981121+