Uncoupling Behavior of the 4-Phenylcoumarins in Spinach Chloroplasts: Structure−Activity Relationships[||]

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4-Phenylcoumarins isolated from *Exostema caribaeum* and *Hintonia latiflora* (Rubiaceae) and some semisynthetic derivatives acted as uncouplers in spinach chloroplasts. The glycoside 5-*O*-*β*-D-glucopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**3**), 5,7,3′,4′-tetrahydroxy-4-phenylcoumarin (**1a**), and 7-methoxy-5,3′,4′-trihydroxy-4-phenylcoumarin (**2a**) inhibited ATP synthesis and proton uptake. On the other hand, basal and phosphorylating electron transport were enhanced by these compounds. The light-activated Mg^{2+}-ATPase was slightly stimulated by coumarins **1a** and **2a**. In addition, at alkaline pH compound **1a** stimulated the basal electron flow from water to methylviologen, but at the pH range from 6.0 to 7.5 the coumarin did not have any enhancing effect. Compound **1a**, which possesses four free phenolic hydroxyl groups, was the most active uncoupler agent. Methylation (**2b**, **4**), acetylation (**2a**), or glycosylation (**1**−**3**) of the phenolic groups at C-3′, C-4′, and C-5 resulted in a reduction or loss of the uncoupling activity. Therefore, the phenolate anions may be the active form responsible for the uncoupling behavior of 4-phenylcoumarins.

Keywords: *Exostema caribaeum; Hintonia latiflora; Rubiaceae; phenylcoumarin; photosynthesis; uncouplers*

INTRODUCTION

The biological role of coumarins, secondary metabolites found in microorganisms, animals, and higher plants (Zobel and Brown, 1995), is not well understood, but they behave as allelochemical agents interfering with the metabolism of other organisms (Einhelling, 1986; Yoshikawa *et al*., 1992; Zobel and Brown, 1995). It has been described that some simple coumarins, furanocoumarins, and pyranocoumarins exerted their phytotoxic activity by inhibiting the energetic metabolism of mitochondria or chloroplasts (Moreland and Novinsky, 1987).

More recently, 5-*O*-*β*-D-galactopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**2**), isolated from *Exostema caribaeum* (Rubiaceae) (Mata *et al*., 1987), was found to act as an energy transfer inhibitor on isolated spinach chloroplasts; the target was localized at the level of CF_0 (Calera *et al*., 1995a). Continuing with our investigations on the biological role of 4-phenylcoumarins, in the present paper we studied the effect of several natural 4-phenylcoumarins (**1** and **3**−4) and some semisynthetic derivatives (**1a** and **2a**−**2c**) on different photosynthetic activities in isolated spinach chloroplasts and the results show that they act as uncouplers.

MATERIALS AND METHODS

Tested Material. Compound **1** was obtained from *Hintonia latiflora* (Sesse ex Mociño ex DC.) Bullock as previously reported (Mata *et al*., 1990). Natural coumarins **2**−**4** were isolated from *E. caribaeum* (Jacq.) Roem. et Schult. (Mata *et al*., 1987, 1988). Derivatives **1a** and **2a**−**c** were prepared from natural products **1** and **2** according to previously described procedures (Mata *et al*., 1987, 1988, 1990). The structures of the 4-phenylcoumarins are shown in Figure 1.

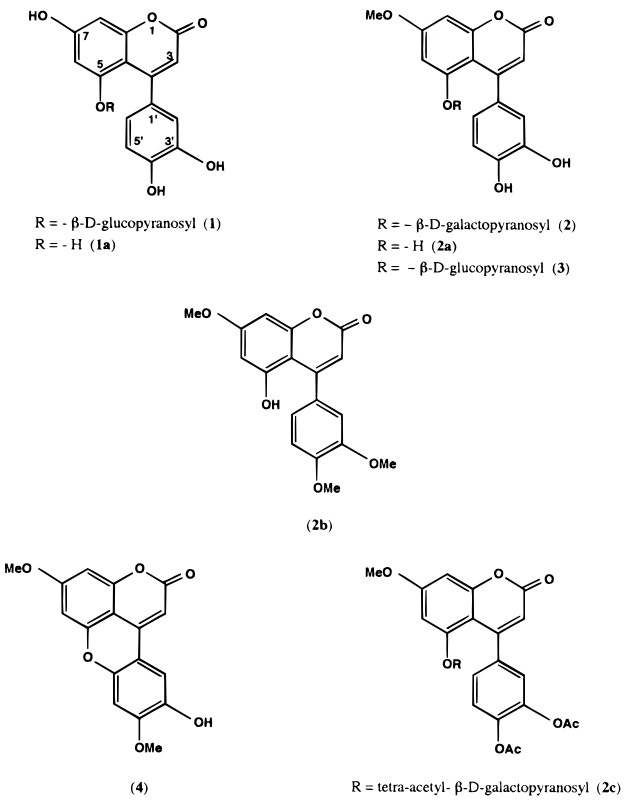

Figure 1. Structures of the tested 4-phenylcoumarins.

Chloroplast Isolation and Chlorophyll Determination. Chloroplasts were obtained from market spinach leaves (*Spinacea oleracea* L.) as described previously (Calera *et al*., 1995a,b), suspended, unless indicated, in 400 mM sucrose, 5 mM $MgCl_2$, and 10 mM KCl, and buffered with 0.03 M Na^+-tricine at pH

[||] This work was taken in part from the Ph.D. thesis of M. R. Calera.

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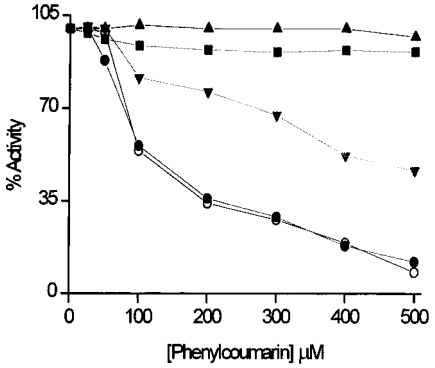

Figure 2. Inhibitory effect of 4-phenylcoumarins on photophosphorylation from water to methylviologen in chloroplast thylakoids isolated from spinach leaves. Photophosphorylation was measured in the presence of 1 mM ADP and 3 mM K_2HPO_4. Each cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions were as described under Materials and Methods. Control value rate was 376.5 μmol of ATP·h^{-1}·mg of Chl^{-1}: **1a** (○), **2a** (●), **3** (▼), **1** and **2b**,**c** (▲), and **4** (■).

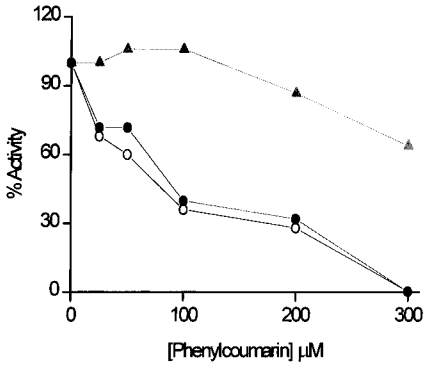

Figure 3. Proton uptake as a function of coumarin concentration: **1a** (○), **2a** (●), and **3** (▼). In each case a cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions were as described under Materials and Methods. Control value rate was 50.0 μequiv of H^+·h^{-1}·mg of Chl^{-1}.

8.0. The chlorophyll concentration was measured spectrophotometrically (Strain, 1971).

Measurement of Proton Uptake, ATP Synthesis, and Electron Transport. Proton uptake was measured as the pH rise between 8.0 and 8.1 (Dilley, 1972) using a combination microelectrode connected to a Corning potentiometer with expanded scale. The pH changes were recorded (Gilson recorder). The reaction medium was 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 1 mM Na^+-tricine, pH 8.0 (KOH). ATP synthesis was measured as in proton uptake conditions in the presence of 1 mM ADP and 3 mM KH_2PO_4 (Calera *et al.*, 1995a,b). Methylviologen (0.05 mM) was added as electron acceptor for the Hill reaction.

Photosynthetic noncyclic electron transport activity from water to methylviologen was determined with an oxygraph 5300. The reaction medium was the same as in the proton uptake assay except that the tricine concentration was 15 mM and the presence or absence of 6 mM NH_4Cl (Calera *et al.*, 1995a,b). All reaction mixtures were illuminated with actinic light of a projector lamp (GAF 2660) passed through a 5 cm filter of a 1% $CuSO_4$ solution.

ATPase Isolation and Assay. Mg^{2+}-ATPase activity bound to thylakoid membranes (Mills *et al.*, 1980) and released inorganic phospahete were measured as reported (Sumner, 1944).

RESULTS AND DISCUSSION

Effect of 4-Phenylcoumarins on ATP Formation. To understand the role of 4-phenylcoumarins as potential natural herbicide agents, their effects on different photosynthetic activities were tested. Photosynthetic phosphorylation from water to methylviologen in freshly lysed intact spinach chloroplasts was inhibited by the tested 4-phenylcoumarins (Figure 2). Compounds **1a**, **2a**, and **3** (Figure 1) inhibited ATP synthesis in a concentration-dependent manner, reducing it by 92, 88, and 54% at 500 μM, respectively. The IC_{50} values for these compounds were 103, 114, and 391 μM, respectively.

The other 4-phenylcoumarins tested (compounds **1**, **2b**,**c**, and **4**; Figure 1) have negligible inhibition effects on photophosphorylation (Figure 2). These findings suggest that the free hydroxyl groups at C-3′ and C-4′ are an important structural requirement for the observed inhibitory effect on ATP synthesis. Methylation or acetylation of the hydroxyl groups significantly reduced their inhibitory activity. The C-5 hydroxyl was also an important structural feature for activity because *O*-glycosilation at this position, as in the case of compound **3**, diminished the inhibitory effect on photophosphorylation.

Effect of 4-Phenylcoumarins on Proton Uptake and Electron Transport Rate. The light-dependent synthesis of ATP on thylakoids may be inhibited by blocking the electron transport, uncoupling ATP synthesis from the electron transport, or blocking the phosphorylation reaction itself (Good *et al.*, 1981).

In order to distinguish between these three possibilities, the effect of the coumarins on the light-dependent proton uptake and electron transport was tested. Figure 3 shows that the proton uptake was completely inhibited by compounds **1a** and **2a** at 300 μM (IC_{50} = 72 and 86 μM, respectively), but phenylcoumarin **3** had only a minor effect at 300 μM. The other 4-phenylcoumarins (compounds **1** and **2b**,**c**) did not exert any significant proton uptake inhibition (data not shown). According to Mitchell's transduction theory (Mitchell, 1961), proton uptake and ATP synthesis inhibition are expected to occur in a stoichiometric manner when the tested compound behaves as an uncoupler. It is remarkable that proton uptake (see IC_{50}) was more strongly inhibited than ATP synthesis, which is not in agreement with Mitchell transduction theory that predict, that the inhibition of both activities must be to the same extent and in a parallel manner. One possible explanation is that ATP synthesis occurs with proton domains which are embedded inside the thylakoid membranes.

To further characterize the mechanism of action of 4-phenylcoumarins, their effect on electron flow was investigated. Figure 4 and Table 1 show that the noncyclic electron transport from water to methylviologen in both basal and phosphorylating conditions were enhanced by addition of compounds **1**, **1a**, **2a**, and **3**, but compounds **2b**,**c** have no effect on the activities (data not shown). On the other hand, uncoupled electron transport was unaffected by all tested coumarins (data not shown).

The inhibitory activity of the 4-phenylcoumarins on ATP synthesis and H^+ uptake, as well as the stimulatory effect on basal and phosphorylating electron transport, indicates that **1a** and **2a** have uncoupling properties on freshly lysed chloroplasts. This behavior is similar to that found for uncouplers like FCCP (carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone), ammonium chloride, and alkylamines (Good *et al.*, 1981 and references therein; Lotina-Hennsen *et al.*, 1987). Like other coumarins (Moreland and Novitzky, 1987;

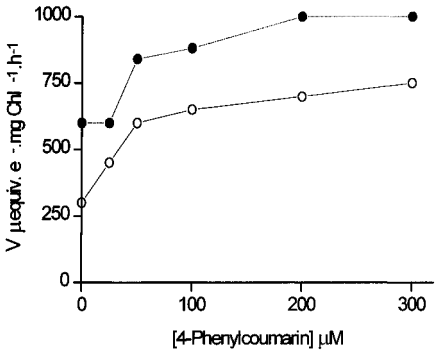

Figure 4. Noncyclic electron transport (basal and phosphorylating) from water to methylviologen as a function of the 5,7,3′,4′-tetrahydroxy-4-phenylcoumarin (**1a**). Photophosphorylating electron transport was measured in the presence of 1 mM ADP and 3 mM K_2HPO_4. Each cuvette contained 20 μg of chlorophyll/mL in the reaction medium. Other conditions were as described under Materials and Methods. Control value rates for basal (○) and phosphorylating (●) electron transport were 300 and 600 μequiv of $e^-\cdot h^{-1}\cdot$mg of Chl^{-1}, respectively.

Table 1. Activity of the Effects of 4-Phenylcoumarins on Electron Flow in Isolated Chloroplasts Compared to Control (100% Activity)[a]

	electron flow (%)					
concn (μM)	**1**	**1a**	**2a**	**2b**	**2c**	**3**
			Basal			
0	100	100	100	100	100	100
25	100	150	112	100	100	100
50	100	200	138	100	100	100
100	100	217	150	100	100	100
200	100	233	150	100	100	100
300	100	250	162	100	100	112
			Phosphorylating			
0	100	100	100	100	100	100
25	100	100	133	100	100	120
50	100	140	141	100	100	120
100	117	147	150	100	100	120
200	117	167	158	100	100	100
300	117	167	167	100	100	100

[a] Control value rates were 180.0 ± 3.1 and 480.0 ± 2.7 μequiv of $e^-\cdot h^{-1}\cdot$mg of Chl^{-1} for basal and phosphorylating electron flow, respectively.

Calera *et al.*, 1995), compounds **1a** and **2a** stimulated only slightly the Mg^{2+}-ATPase (20−30% at 300 μM) from bound membrane thylakoid membranes.

Activity of Coumarin on Basal Electron Transport at Different pHs. To determine if 4-phenylcoumarins suffer acid−base reaction during uncoupling, their effects on electron flow at different pHs were tested. Figure 5 shows the pH dependence of the effect of compound **1a** on basal electron flow. These data indicate that the coumarins enhanced basal electron transport only at alkaline pHs (7.75, 8.0s and 8.5), attaining a maximal increase (150%) at pH 8.0. The concentrations of 4-phenylcoumarins **1a** and **2a** that activate both basal and phosphorylating electron flow are not the same as those required for inhibition of proton uptake and ATP synthesis; therefore, 4-phenylcoumarins act as nonclassical uncouplers of photosynthetic phosphorylation.

Synthetic phenols and natural 4-hydroxycoumarins have been demonstrated to act as uncouplers (Terada, 1990 and references therein). It has been proposed that the uncoupling effect of phenol SF6847 (2,6-di-*tert*-butyl-4-(2,2-dicyanovinyl)phenol) is attributable to its protonophoric action on chloroplast membranes (Terada, 1990). The same type of mechanism has also been

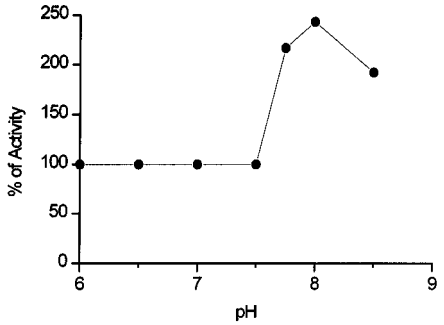

Figure 5. pH dependence of basal electron transport flow in the presence of 200 μM of 5,7,3′,4′-tetrahydroxy-4-phenylcoumarin. Each cuvette contained 20 μg of chlorophyll/mL and either MES (2-(*N*-morpholino)ethanesulfonic acid) (pH 6.0−6.5), HEPES (*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid)) (pH 7.0−7.75), or Tricine (*N*-tris(Hydroxymethyl)-methylglycine; *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-glycine) (pH 8.0−8.5) in the reaction medium. Other conditions were as descibed under Materials and Methods.

suggested for dicoumarol (bishydroxycoumarin) (Jagendorf and Neuman, 1965). Notably, the protonophoric action of SF6847 relies on the presence of the hydroxyl phenolic group, which mediates the interconversion of the compound between the anionic and neutral forms of the molecule (Terada, 1990). In the case of the 4-phenylcoumarins examined in the present study, the free hydroxyl groups had a marked influence on the uncoupling effect, since glycosilation of the hydroxyl group at position C-5 diminished (compound **3**) or abolished (compound **1**) the uncoupling action of these 4-phenylcoumarins. Also the free hydroxyl groups at positions C-3′ and C-4′ of compound **2a** were essential for the uncoupling activity since methylation (compound **2b**) or acetylation (compound **2c**) led to inactive derivatives. On the other hand, the highest uncoupling activity for compound **1a** was observed at pH 8.0; therefore, we suggest that the active species of coumarins **1a** and **2a** are the corresponding phenolates (anionic form).

In conclusion the sensitivity of photosynthetic energy conservation machinery to 4-phenylcoumarins found in this work and that reported previously (Calera *et al.*, 1995a) may be related to their allelopathic role in plants which biosynthesized these compounds. The uncoupler and energy transfer inhibition activities displayed by 4-phenylcoumarins could be useful for the development of new herbicides.

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