Inhibition and Uncoupling of Photosynthetic Electron Transport by Diterpene Lactone Amide Derivatives

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Nine diterpene lactone amide derivatives **1**–**9** were synthesized from 6-oxovouacapan-7β,17β-lactone, which was obtained from 6α,7β-dihydroxyvouacapan-17β-oic acid isolated from *Pterodon polygalaeflorus* Benth., and tested for their activity on photosynthetic electron transport. Amide derivatives **3**–**5** behaved as electron transport chain inhibitors; they inhibited the photophosphorylation and uncoupled non-cyclic electron transport from water to methylviologen (MV). Furthermore, **4** and **5** enhanced the basal electron rate acting as uncouplers. Compound **6** behaved as an uncoupler; it enhanced the light-activated Mg^{2+}-ATPase and basal electron flow, without affecting the uncoupled non-cyclic electron transport. Compounds **1**–**2** and **7**–**9** were less active or inactive. Compounds **3**–**5** did not affect photosystem I (PSI); they inhibited photosystem II (PSII) from water to 2,6-dichlorophenol indophenol (DCPIP). Compound **4** inhibited PSII from water to silicomolybdate (SiMo), but it had no effect on the reaction from diphenylcarbazide (DPC) to DCPIP indicating that its inhibition site was at the water splitting enzyme complex (OEC). Compounds **3** and **5** inhibited PSII from water to DCPIP without any effect from water to SiMo, therefore they inhibited the acceptor site of PSII. Chlorophyll *a* fluorescence kinetics confirmed the behaviour of **3**–**5**.

Key words: *Pterodon polygalaeflorus* Benth., Diterpene Lactone Amide Derivatives, PSII Inhibitor, Uncoupler

Introduction

Green plants produce hundreds and thousands of compounds that are not involved in the primary metabolism, and generally have unknown functions. Natural diterpenes isolated from plants contain various interesting biological activities. Different parts of the plant *Pterodon* genus have various uses: *i. e.* seeds infusion is used for the treatment of throat infections, and the fruit oil to inhibit skin penetration of *Schistosoma mansoni cercaria.* The active compound of the oil seems to be 14,15-dihydroxygeranylgeraniol (Mors *et al.*, 1967). 6α,7β-Dihydroxyvouacapan-17β-oic acid was also isolated, and its 6α-hydroxyvouacapan-7β,17β-lactone derivative was prepared. Both compounds exhibit anti-inflammatory, analgesic (Nunan *et al.*, 1982), plant growth regulatory (Demuner *et al.*, 1996, 1998; Castelo-Branco *et al.*, 2006), and allelophatic activities (Demuner *et al.*, 1996). Further studies in our laboratories found that 6α-hydroxyvouacapan-7β,17β-lactone as well as its keto derivative 6-oxovouacapan-7β,17β-lactone (ketolactone) inhibit photosystem II in spinach chloroplasts (King-Díaz *et al.*, 2005, 2006). In this work, our aim was to prepare semisynthetic ketolactone derivatives to test their effect on photosynthesis. The diterpene 6-oxovouacapan-7β,17β-lactone was the starting compound for the development of the synthetic diterpene lactone amide derivatives family

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(Fig. 1) to be further used in studies of structure-activity relationships establishing which part of the molecule is instrumental for the bioactivity in photosynthesis.

Materials and Methods

General

Elemental analyses were performed on a Perkin Elmer 2400 apparatus. Infrared spectra were recorded on a Perkin Elmer PARAGON 1000 grating spectrometer, scanning from 400 to 4000 cm^{-1}. The samples were prepared as KBr disks. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 400 AVANCE (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer. $CDCl_3$ or DMSO-d_6 was used as solvent. Tetramethylsilane was used as the internal standard (δ = 0). Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz. COSY, NOESY, HMQC and HMBC experiments were employed to assign chemical shifts and were carried out using pulse sequence and programs provided by the manufacturer.

*General experimental procedure for the preparation of the diterpene lactone amide derivatives **1–9***

6α,7β-Dihydroxyvouacapan-17β-oic acid used to prepare the lactones was extracted from the fruits of *Pterodon polygalaeflorus* Benth. by a procedure described in the literature (Mahajan and Monteiro, 1973). 6α-Hydroxyvouacapan-7β,17β-lactone was obtained as reported by Rubinger *et al.* (1991). 6-Oxovouacapan-7β,17β-lactone used as starting material was synthesized as reported before (King-Díaz *et al.*, 2006).

Compounds **1–9** were obtained by the reaction of 6-oxovouacapan-7β,17β-lactone with the respective amine, in 55–94% yield. The reaction started with the addition of the amine (1 ml, 40%) (Table I) to a stirred solution of 0.61 mmol of the lactone in 10 ml THF. Then the reaction mixture was incubated at 60 °C (25 °C for compound **2**), for the indicated time (Table I). At the end the temperature was allowed to cool to room temperature. The reaction was monitored by TLC. The mixture was poured into ice to precipitate the product. After filtration, the precipitate was recrystallized from ethyl acetate/hexane except for compound **1**, which was recrystallized from ethanol. The structures of the derivatives **1–9** are shown in Fig. 1.

Table I. Summary of the data for the preparation of amides **1–9**.

Compound	Reagent	Amount [mmol]	Reaction time [h]	Yield (%)
1	Ammonia	42.0	1.5	73
2	Methylamine	6.0	0.17	94
3	Butylamine	6.0	1.0	62
4	Isobutylamine	6.1	2.0	87
5	Benzylamine	20.0	6.5	69
6	Cyclohexylamine	20.0	1.5	59
7	Propylamine	4.8	2.5	61
8	Isopropylamine	5.8	19.0	72
9	Pyrrolidine	20.0	3.0	55

Chloroplast isolation and chlorophyll determination

Intact chloroplasts were isolated from market spinach leaves (*Spinacea oleraceae* L.) as described elsewhere (King-Díaz *et al.*, 2006; González-Vázquez *et al.*, 2006; Mills *et al.*, 1980). Chloroplasts were resuspended in a small volume of the following medium: 400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM KCN, 1 mM *N*-tris(hydroxymethyl)methylglycine (tricine), pH 8.0. Thylakoids were obtained from freshly lysed intact chloroplasts by incubating them in the lysing reaction medium for ATP synthesis measurements or non-cyclic electron transport reaction medium. Thylakoids were immediately used for ATP synthesis or electron transport activity determinations. Chlorophyll concentration was measured as previously described (Strain *et al.*, 1971).

ATP synthesis determination

ATP synthesis was determined titrimetrically in a thylakoid suspension (60 μg of chlorophyll) using a microelectrode (Orion model 8103; Ross, Beverly, MA, USA) connected to a Corning potentiometer model 12 (Corning Medical, Acton, MA, USA), with expanded scale and Gilson recorder (Kipp & Zonen, Bohemia, NY, USA) as reported by Dilley (1972). The reaction medium for ATP synthesis assays was 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 1 mM KCN, 1 mM tricine, pH 8.0, and 50 μM methyl viologen (MV) as an electron acceptor. Increasing contents of each diterpene amide derivative were added to the reaction mixture as indicated. To initiate ATP synthesis, 1 mM ADP and 3 mM Pi were added (Dilley, 1972).

Light-induced non-cyclic electron transport rates from water to MV

It was measured using a Clark-type electrode connected to an oxygraph (Yellow Springs Instrument Co. Inc., USA, Model 5300) as previously published (King-Díaz *et al.*, 2006; González-Vázquez *et al.*, 2006; Mills *et al.*, 1980). Basal electron transport was determined by illuminating chloroplasts (20 μg chlorophyll/ml) in the electron transport medium 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 1 mM KCN, 15 mM tricine, pH 8.0 (adjusted with KOH), 50 μM MV was used as electron acceptor. Reaction time of illumination using saturating white light was 1 min and it was performed under aerobic conditions. The photophosphorylating non-cyclic electron transport reaction was measured as basal non-cyclic electron transport except that 1 mM ADP and 3 mM Pi were added to the electron transport medium (King-Díaz *et al.*, 2006). The uncoupled electron transport reaction was tested under the same conditions as the basal non-cyclic electron transport except for 6 mM NH_4Cl was added as uncoupler (King-Díaz *et al.*, 2006).

Partial reactions determination

To determine the target where the diterpene lactone amide derivatives inhibit the chloroplasts electron transport chain, the activity on uncoupled PSII (photosystem II) and PSI (photosystem I) electron flow and partial reactions was monitored in a similar form as for the uncoupled non-cyclic electron transport. Uncoupled PSII from water to Q_B was measured by the reduction of 50 μM 2,6-dichlorophenol indophenol (DCPIP); 300 μM $K_3[Fe(CN)_6]$, 1 μM 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) to inhibit PSI at b_6f complex level were added to the medium without MV. The partial reaction of uncoupled electron transport from water to sodium silicomolybdate (SiMo) was determined with the same reaction mixture as for PSII, except for 200 μM SiMo, which accepts electrons at Q_A site, and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to inhibit the electron flow by removing Q_B at D1 protein (Giaquinta *et al.*, 1974) were added. The uncoupled PSII partial electron transport rate from diphenylcarbazide (DPC), which donates electrons at P_{680}, to DCPIP was measured using a Beckman DU 650 spectrophotometer and determined with thylakoids previously treated with 0.8 M hydroxymethyl aminomethane (Tris) (pH 8.0) and incubated for 30 min at 4 °C. This treatment inhibited the water splitting enzyme (Vernon and Shaw, 1969).

Measurement of PSI electron flow from Cyt b_6f to MV

The PSI uncoupled electron flow was determined using the basal electron transport medium; 6 mM NH_4Cl, 100 μM DCPIP (reduced with 300 μM sodium ascorbate) as electron donor, 10 μM DCMU to inhibit PSII electron flow and 50 μM MV as electron acceptor were added (Allen and Holmes, 1986). Uncoupled PSI electron flow from tetramethyl-*p*-benzohydroquinone ($TMQH_2$), which donates electrons at PQH_2 level, was determined by polarography. The reaction medium used was the basal reaction medium plus 100 μM $TMQH_2$/300 μM ascorbate; 10 μM DCMU to inhibit the reducing site of PSII and 6 mM NH_4Cl were added as uncoupler (Izawa and Pan, 1978). All reaction mixtures were illuminated with actinic light from a projector lamp (GAF 2660) and were passed through a 5 cm filter of 1% $CuSO_4$ solution. The temperature was 20 °C, and for each reaction a blank experiment was performed with the chloroplasts alone in the reaction medium. The I_{50} value for each activity was determined from the activity plots in the presence of each compound at different concentrations. I_{50} is the concentration producing 50% inhibition.

Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence induction curves were measured at room temperature with a plant efficient analyzer (Handy PEA, Hansatech Instruments UK) in 5 min dark-adapted chloroplasts (20 μg Chl ml^{-1}) (King-Díaz *et al.*, 2006; González-Vázquez *et al.*, 2006). The maximum fluorescence yield from the sample was generated using three light-emitting diodes (broad-band 650 nm). The light (2500 μmol m^2 s^{-1}) was provided by an operating halogen saturation lamp. The pulse duration was 2 s. The reaction medium used was the same employed in basal non-cyclic electron transport measurements. Different photosynthetic parameters associated to PSII activity were evaluated (Strasser *et al.*, 2004): 1) M_0 value, as an indication of the water splitting system function estimated as $M_0 = (F_{300\,\mu s} - F_{50\,\mu s})/(F_M - F_{50\,\mu s}) \cdot 0.25$. 2) Fluorescence quantum yield at J transient as $V_J =$

$(F_{2\,ms} - F_{50\,\mu s})/(F_M - F_{50\,\mu s})$ used as indication for PSII efficiency in primary photochemistry. 3) Electron transport (ET) per active reaction centre (RC) as $ET_0/RC = M_0/V_J \cdot (1 - V_J) = M_0/V_J$. 4) Non-photochemical energy dissipation of an active PSII reaction centre as $DI_0/RC = ABS/RC - (M_0/V_J)$. 5) Absorption of photons (ABS) per active reaction center (RC) estimated by the ratio $ABS/RC = (M_0/V_J)/\Phi_{P\,0}$. 6) Quantum yield at P transient, as indication of the PSII efficiency in the plastoquinone pool reduction evaluated as $\Phi_{P\,0} = (F_M - F_{50\,\mu s})/F_M$.

Mg^{2+}-ATPase activity assays

Mg^{2+}-ATPase activity bound to thylakoid membranes was measured according to Mills and Mitchell (1980). The amount of released inorganic phosphate was measured by colourimetric determination as previously described by Sumner (1994).

Log P value calculations

Log P values for each amide derivative were estimated with the LOGKNOW; KOWWIN program ChemSilico (http://www.logp.com/) and collected in Table I.

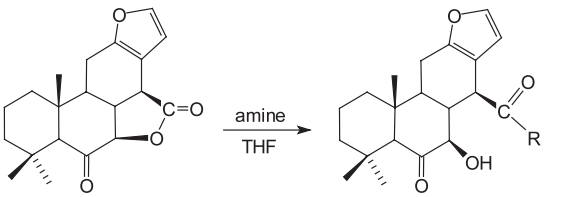
Results and Discussion

*Effect of compounds **1–9** on ATP synthesis*

The diterpene lactone amide derivatives were assayed on the photophosphorylation to know their activity on photosynthesis. The results showed that compounds **3**, **4**, **5** and **6** (Fig. 1) inhibited ATP synthesis coupled to electron transport from water to MV on illuminated freshly lysed intact spinach chloroplasts (Fig. 2). The calculated I_{50} values were 58, 29, 38 and 51 μM for lactone amide derivatives **3**, **4, 5** and **6,** respectively (Table II). However, compounds **2**, **7, 8** and **9** showed inhibitory activity at higher concentrations; the I_{50} values were 243,139, 80 and 189 μM, respectively. Compound **1** did not show any inhibitory effect on the ATP synthesis (data not shown); thus, compounds **1**, **2**, **7**, **8** and **9** were not further studied.

*Effect of compounds **3–6** on electron transport chains of thylakoids*

To elucidate the mechanism of action of the diterpene lactone amide derivatives **3–6** on the ATP synthesis coupled to electron transport, their effect on basal, phosphorylating, and uncoupled



1: R = NH_2
2: R = NHMe
3: R = NHBu
4: R = NHiBu
5: R = $NHCH_2Ph$
6: R = cyclohexylamino
7: R = NHPr
8: R = NHiPr
9: R = pyrrolidyl

Fig. 1. Structures of the amide derivatives of 6-oxo-vouacapan-7β,17β-lactone.

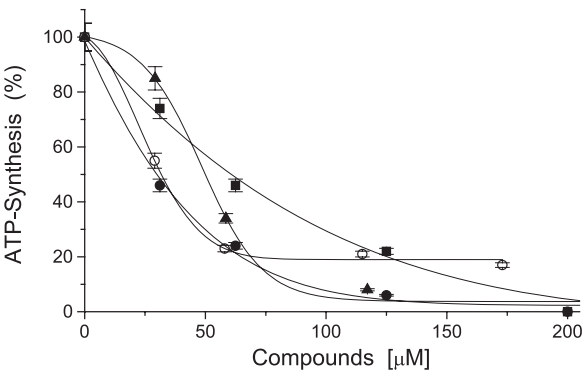


Fig. 2. Effect of amide derivatives **3** (■), **4** (●), **5** (○) and **6** (▲) on ATP synthesis.

electron transport from water to MV was studied on freshly lysed spinach chloroplasts (Table III). Fig. 2 shows the inhibiting effect of **3–5** on phosphorylating and on the uncoupled electron transport (Table III). These results indicate that compounds **3–5** act as Hill reaction inhibitors. Derivative **3** acts as Hill reaction inhibitor by inhibiting ATP synthesis and all electron flow tested. Additionally, amide derivatives **4** and **5** enhanced the basal electron flow (Table III) suggesting that **4** and **5** also act as uncoupler. Derivative **6** enhanced the basal and phosphorylating electron transport rates without any effect on the uncou-

Table II. I_{50} values calculated from ATP synthesis rate inhibition shown in Fig. 2, and Log *P* values calculated for compounds **1**–**9**.

Compound	I_{50} ATP synthesis [μM]	Log *P*
1, R = NH_2	–	0.71
2, R = NHMe	243	1.81
3, R = NHBu	58	3.13
4, R = NHiBu	29	2.81
5, R = $NHCH_2Ph$	38	3.99
6, R = cyclohexyl-amine	51	4.48
7, R = NHPr	139	2.62
8, R = NHiPr	80	2.35
9, R = pyrrolidyl	189	3.53

pled non-cyclic electron transport rate from water to MV (Table III). This could be related with its uncoupling properties by interacting with the CF_1 complex by perturbing the thylakoid membranes.

*Localization of interaction sites of **3**–**5** on PSII, PSI and partial reactions*

To localize the action target of compounds **3**–**5** when they act as Hill reaction inhibitors on thylakoids non-cyclic electron transport chain, their effect on partial reactions of PSII and PSI were tested using artificial electron donors, electron acceptors, and appropriate inhibitors (Allen and Holmes, 1986). Table IV shows that compounds **3**–**5** inhibited PSII from water to Q_B. Derivatives **3** and **5** did not have any effect on partial PSII electron transport rates from water to Q_A where SiMo accepts electrons; moreover, compounds **3** and **5** did not have any effect on the PSI electron transport rate from reduced $TMQH_2$ or reduced DCPIP to MV (data not shown). Therefore, diterpene lactone amide derivatives **3** and **5** have a similar site of interaction and inhibition like herbicides, such as phenylureas and triazines (Trebst and Draber, 1986), which act as inhibitors of the photosynthesis electron transport chain located at the reducing site of PSII. However, derivative **4** inhibited also the partial reaction of PSII from water to SiMo and had no effect on the partial reaction from diphenyl carbazide (an electron donator at P_{680}) to oxidized DCPIP; therefore, the interaction and inhibition site of **4** is located at the water splitting enzyme complex (OEC). Furthermore, derivative **4** inhibited slightly the PSI electron transport rate from $TMQH_2$ (an electron donator at the b_6f complex) to MV (25% at 300 μM), but it had no effect on the PSI electron transport rate from reduced DCPIP to MV. These last results indicate that the second target of **4** is located on the PQH2 oxidation site, at the b_6f complex.

*Effect of the amide derivatives **3**–**5** on fast chlorophyll a fluorescence transient*

To corroborate the amide derivative interaction site of **3**–**5** at the reducing site of PSII, freshly

Table III. Effect of amide derivatives on the non-cyclic electron transport rate in spinach thylakoids.

Concentration [μM]	Basal		Phosphorylating		Uncoupled	
	a	*b*	*a*	*b*	*a*	*b*
Compound **3**						
0	450	100	750	100	1040	100
26	301	67	203	27	364	35
52	149	33	135	18	177	17
Compound **4**						
0	450	100	750	100	1040	100
100	720	160	173	23	697	67
200	180	40	0	0	603	58
Compound **5**						
0	555	100	820	100	1230	100
100	694	125	410	50	873	71
200	971	175	410	50	873	71
300	1249	225	328	40	578	47
Compound **6**						
0	555	100	820	100	1230	100
100	638	115	1123	137	1340	109
200	1271	229	1312	160	1242	101
300	633	114	984	120	1230	100

a = μequiv. $e^- \cdot mg^{-1}$ Chl $\cdot h^{-1}$.
b = %.

Table IV. Effect of compounds **3**–**5** on PSII and PSI electron transport rate in thylakoids and their partial reactions.

Concentration [μM]	PSII H_2O to DCPIP		PSII H_2O to SiMo		PSI $TMQH_2$ to MV		PSI DCPIPred to MV	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Compound **3**								
0	420	100	155	100	860	100	1120	100
100	315	74	155	100	860	100	1120	100
300	181	43	155	100	860	100	1120	100
Compound **4**								
0	420	100	155	100	860	100	1120	100
100	298	71	93	60	860	100	1120	100
300	155	37	39	25	645	75	1120	100
Compound **5**								
0	420	100	155	100	860	100	1120	100
100	210	50	155	100	860	100	1120	100
300	139	33	155	100	860	100	1120	100

a, μequiv · e^- · mg^{-1} Chl · h^{-1}.
b, %.

Table V. F_0, F_m, and F_V/F_m values in thylakoid controls and after treatment with amide derivatives **3**–**5**.

Compound	$F_{50\,\mu s}$	$F_{300\,\mu s}$	$F_{2\,ms}$	F_m	F_V/F_m	Area	M_0	M_0/V_J	DI_0/RC
Control	225	332	571	1000	0.796	45200	0.55	1.24	0.401
10 μM DCMU	320	521	940	1077	0.737	1600			
0.8 M Tris	221	311	451	456	0.553	0	1.53	1.56	1.47
Compound **3** [μM]									
300	251	375	627	1057	0.785	16800			
600	293	401	672	1201	0.774	16300			
900	314	429	707	1098	0.784	15200			
Compound **4** [μM]									
300	233	342	563	947	0.778	13800	0.61	1.32	0.431
600	222	325	536	913	0.782	13600	0.6	1.31	0.417
900	190	280	474	783	0.782	10800	0.66	1.38	0.442
Compound **5** [μM]									
300	300	443	750	1212	0.772	14200			
600	308	451	772	1271	0.777	14800			
900	320	471	822	1253	0.769	13000			

lysed chloroplasts were incubated for 5 min in the dark at room temperature with different concentrations of compounds **3**–**5**. 10 μM DCMU and 0.8 M Tris were used as positive controls (Table V). Fast chlorophyll fluorescence transients of thylakoids from spinach thylakoids, incubated for 5 min with different concentrations of compounds **3**–**5**, were measured. Comparison of the normalized relative variable fluorescence rise of the photochemical phase fluorescence induction curves of thylakoids treated with compounds **3** and **5** with those obtained from samples with authentic herbicide DCMU (10 μM) showed very similar behaviours; the regular OJIP sequence was transformed into an OJ curve (Strasser *et al.*, 1995) (Fig. 3). The initial fluorescence (F_0) of treated samples **3** and **5** was higher than that of the control (Table V). When the concentration of **3** and **5** increased, the area of the J step enhanced similar to DCMU (Fig. 3). It can be concluded that compounds **3** and **5** inhibited the electron transport from Q_A to Q_B on the acceptor site of PSII, similar to the herbicide DCMU which is known to block the electron flow from Q_A to Q_B by displacing Q_B. On the other hand, the fluorescence induction curve form was reduced by the treatment of the amide deriva-

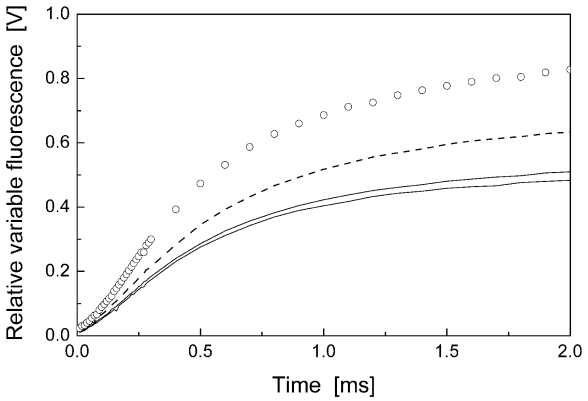


Fig. 3. Normalized relative variable fluorescence rise of the photochemical phase. The curve with the lowest yield represents control thylakoids. Circles represent controls with acceptor impairment after infiltration with 10 μM DCMU. The intermediate curve represents thylakoids exposed to 900 μM **3** and the dashed curve represents thylakoids exposed to 900 μM **5**.

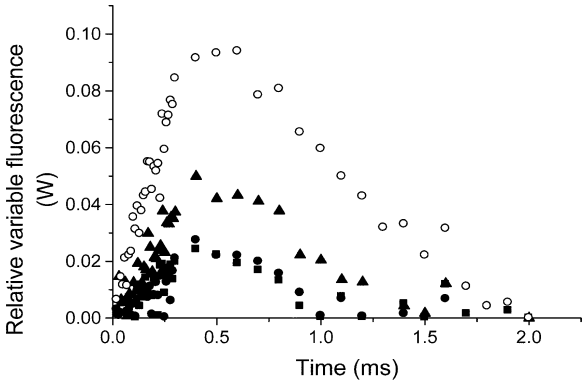


Fig. 4. Appearance of the K band at about 300 μs. Difference of each curve from the control with normalized relative variable fluorescence on the amplitude $F_j - F_0$. Compound **4** at 300 (■), 600 (●) and 900 μM (▲). Broken chloroplasts incubated with 0.8 M Tris (○).

tive **4**. This result indicates a reduction of the maximum fluorescence yield. The K band appeared and a rapid rise to a maximum (at 300 μs) was followed by a decreased fluorescence yield close to the level F_0; the J and I steps were absent from the transient (Fig. 4) (Strasser, 1997; Strasser *et al.*, 2004). Furthermore, under high temperature treatment, very often an increase of M_0 appeared and the V_J values decreased. In this case, when we treated broken chloroplasts with increased concentrations of **4**, M_0 values increased (Table V); so M_0/V_J values, which are equal to TR_0/RC values, increased indicating a damage in the oxygen evolving activity. Thus, the non-photochemical energy dissipation per active reaction centre was constant as shown by the calculated DI_0/RC values. These results indicate that the damage of **4** is stronger at the donor site than at the acceptor site of PSII. This finding is similar to that found with thylakoids treated with Tris, a well-known PSII donor site inhibitor (Rickert *et al.*, 1991), which was used as positive control.

Mg^{2+}-ATPase activity

It is well known that uncouplers such as NH_4Cl, tricolorin A (Achnine *et al.*, 1999) and bullatacin (Chàvez *et al.*, 2001) stimulate Mg^{2+}-ATPase activity. Table VI shows that NH_4Cl enhanced the activity of Mg^{2+}-ATPase, which was used as positive control, and compound **5** enhanced the activity of Mg^{2+}-ATPase by 13% at 300 μM, corroborating that **5** acts partially as uncoupler. In contrast, **4** inhibited the activity of Mg^{2+}-ATPase by 35% at 300 μM indicating that **4** acts partially as energy transfer inhibitor. It also inhibited the phosphorylating electron transport by 100% at 200 μM. However, **4** only inhibited partially the uncoupled noncyclic electron transport from water to MV flow

Table VI. Effect of amide derivatives **4**–**6** and NH_4Cl on the activity of the membrane-bound thylakoid enzyme Mg^{2+}-ATPase.

Concentration [μM]	Activity μM $Pi \cdot mg^{-1}$ Chl $\cdot h^{-1}$]	(%)
Compound **4**		
0	216	100
100	207	96
200	186	86
300	140	65
Compound **5**		
0	239	100
100	284	119
200	304	127
300	270	113
Compound **6**		
0	239	100
100	278	116
200	335	140
300	418	175
NH_4Cl [mM]		
0	223	100
1	303	136
2	384	172
3	420	188

by 42% at 200 μM. On the other hand, **6** enhanced basal and phosphorylating electron transport rates without any effect on the uncoupled electron transport, and testing its effect on Mg^{2+}-ATPase corroborated its behaviour. The results show that **6** enhanced the activity of Mg^{2+}-ATPase (Table VI), indicating its uncoupler properties. The uncoupling properties of **6** may be due to its lipophilic character as shown by a higher partition coefficient that allow to reach and associate with the target on membranes.

*Hydrophobicity of compounds **3**–**6***

From all amide derivatives studied only **3**, **4**, **5** and **6** at low concentrations inhibited ATP synthesis coupled to electron transport from water to MV on illuminated freshly lysed intact spinach chloroplasts. The results show that amides with Log *P* values between 0.71 to 2.62 needed a higher concentration to present inhibitory effects on ATP synthesis or did not present effects (Table II). Therefore, derivatives **1**, **2** and **7**, **8** need to be more lipophilic to reach its target in order to penetrate the thylakoid membrane. Compound **3** is a Hill reaction inhibitor and its action target was found to be between Q_A to Q_B at the acceptor site of PSII, similar to the herbicide DCMU.

Amides **4** and **5** are Hill reaction inhibitors. **4** inhibited the activity of Mg^{2+}-ATPase indicating that **4** acts also as energy transfer inhibitor. **5** enhanced the activity of Mg^{2+}-ATPase, corroborating that it acts as uncoupler too.

The Log *P* values of compounds **3**–**5** were 3.13, 2.81, and 3.99, respectively. **3**–**5** interact with the PSII proteins target for inhibition. Thus, the change of the butyl group in **3** to an *iso*-butyl group in **4** changes the Log *P* value making **4** less lipophilic to reach the donor site of PSII and PSI for inhibition. The Log *P* value of **3** (3.13) allows to reach the reducing side of PSII for inhibition and can not reach PSI. In the same way, **5** has a Log *P* value of 3.99 which allows to inhibit the reducing site of PSII, and it has a different site of interaction than **3**.

However, **6** enhances the basal and phosphorylating electron transport rates without any effect on the uncoupled non-cyclic electron transport rate, indicating that it acts as uncoupler and enhances the Mg^{2+}-ATPase activity. Therefore, for the uncoupling activity of **6**, a higher Log *P* value is required. Diterpene lactone amide derivatives **3**–**6** have different mechanisms and targets of action and promise to be good starting compounds for developing a new prototype herbicide.

Compound **1** is an unsubstituted amide, and this allows it to form two hydrogen bonds, which make it more hydrophilic reflected by its low Log *P* value (0.71). On the other hand compounds **3**, **4** and **5** are monosubstituted amides having aliphatic and aromatic constituents. When the volume of the chain substituent increases from methyl, *iso*-butyl, butyl, to $-CH_2$Phe the hydrophobicity increases as shown by the increasing Log *P* values. The different hydrophobicity properties of the amide derivatives **3**, **4** and **5** allow them to have different action targets on PSII (Hill reaction inhibitor at donor or at acceptor site) and different mechanisms of action: uncoupler and Hill reaction inhibitor (**5**) or energy transfer inhibitor and Hill reaction inhibitor (**4**).

Compounds **5**, **6** and **9** have higher hydrophobicity (Log *P* values 3.99, 4.48 and 3.53, respectively) and different effects on photosynthesis. Derivative **5** is a Hill reaction inhibitor and also an uncoupler; **6** behaves only as an uncoupler compound; and **9** is less active on ATP synthesis. These differences may be due to the fact that **6** and **9** have cyclic rings that may cause some steric hindrance. Compound **6** is an *N*-cyclohexyl amide derivative (*N*-monoalkylamide) and **9** presents an *N*-pyrrolidine-substituted group, being an *N,N*-dialkylamide. However, **6** has similar basicity as **3**, **4** and **5**, but a higher Log *P* value (4.48). On the other hand, the amide derivative with a pyrrolidine group (compound **9**) has also a high Log *P* value (3.53) but is less active than compounds **5** and **6**. Relative to **6**, the behaviour of **9** may be due to the fact that its nitrogen atom as part of the cycle moiety shows more steric hindrance to share the electron pair, thus it acts as a weak base and interacts with the active site, causing less activity.

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