A Diterpene γ-Lactone Derivative from *Pterodon polygalaeflorus* Benth. as a Photosystem II Inhibitor and Uncoupler of Photosynthesis

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6α,7β-Dihydroxyvouacapan-17β-oic acid (**1**) was isolated from *Pterodon polygalaeflorus* Benth. Modification of **1** yielded 6α-hydroxyvouacapan-7β,17β-lactone (**2**) and then 6-oxovouacapan-7β,17β-lactone (**3**). Photosynthesis inhibition by **3** was evaluated in spinach chloroplasts. The uncoupled non-cyclic electron transport rate and ATP synthesis were inhibited by **3**, which behaved as a Hill reaction inhibitor. Furthermore, **3** acted as an uncoupler because it enhanced the basal and phosphorylating electron transport rate on thylakoids. This last property of **3** was corroborated when it was observed that it enhances the Mg^{2+}-ATPase activity. In contrast, **3** did not affect photosystem I (PSI) activity. Analysis of the partial photosystem II (PSII) reactions from water to $DCPIP_{ox}$ and water to silicomolybdate allowed to locate the inhibition sites at the redox components of PSII. The OJIP test of the chlorophyll *a* fluorescence transient confirmed that the inhibition sites were 1.) the oxygen-evolving complex (OEC) and 2.) by the formation of silent centers in the non-Q_A reducing centers.

Key words: PSII Inhibitor, *Pterodon polygalaeflorus* Benth., 6-Oxovouacapan-7β,17β-lactone

Introduction

The genus *Pterodon* comprises five species, among them *Pterodon polygalaeflorus* Benth., known in Brazil as "sucupira branca" which is widely distributed in the west of Minas Gerais and in Goias, Brazil (Correa, 1984). The fruit oil of sucupira branca is used to deter skin penetration by *Schistosome cercariae* (Mors *et al*., 1967; Fascio *et al*., 1976). The alcoholic infusions of the fruits of this plant are used in folk medicine as analgesic, anti-rheumatic and anti-inflammatory treatments (Rubinger *et al*., 1991). Phytochemical studies of the hexane extract of *P. polygalaeflorus* Benth. fruits resulted in identification and isolation of the diterpene 6α,7β-dihydroxyvouacapan-17β-oic acid (**1**) (Fascio *et al*., 1976; Rubinger *et al*., 1991). Compound **1** has analgesic and anti-inflammatory properties (Nunan *et al*., 1985) and behaves as a plant growth regulator and as an allelochemical (Demuner *et al.*, 1996, 1998); it led to the preparation of 6α-hydroxyvouacapan-7β,17β-lactone (**2**) (Fig. 1) (Rubinger *et al*., 1991). Although **2** exhibited allelopathic properties, it was more active than **1** (ca. 40%) (Demuner *et al.*, 1996, 1998).

It was previously reported that in spinach chloroplasts lactone **2** behaves as a photosystem II (PSII) inhibitor interacting at the P_{680}-Q_A segment and at the oxygen-evolving complex (King-Díaz *et al.,* 2005). Also, it was proposed that the lactone group of **2** was important for inhibition (Rubinger *et al*., 1991). In this work we evaluate the importance of the properties of **2**, namely, whether the –OH group at C-6 is required for interaction and whether a 6-oxo derivative of 6-oxovouacapan-7β,17β-lactone (**3**) does interact with PSII. To answer these questions, **2** was used to synthesize **3** (Fig. 1). Then, the effects of **3** were assayed on different photosynthetic activities. Previously, it was published (Demuner *et al.*, 1998), that 0.31 mM **2** stimulates radicle growth in *Cucumis sativus* by 20–40%, and by a different methodology it was recently verified that compounds **1** and **3** stimulate the radicle growth of *Cucumis sativus* by 10% at 10^{-4} M, while compound **2** has an inhibitory effect (32%) at the same concentration (Castelo-Branco,

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2001). The photosynthesis inhibition and uncoupling activity of **3** is part of our search for bioactive natural products exhibiting herbicide activity.

Materials and Methods

General procedures

Melting point determinations were performed on a Mettler AE 166 digital apparatus. IR spectra were registered on a Perkin Elmer FTIR 3000 spectrophotometer, using KBr disks, and scanned in the range 625–4000 cm^{-1}. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 400 *AVANCE* spectrometer (400 MHz and 100 MHz, respectively), using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal reference ($\delta = 0$). Chromatographic purification was carried out using silica gel (70–230 μm). Thin layer chromatography was carried out using a mixture of silica gel $60F_{254}$ and 60G (1:3). The natural diterpene 6α,7β-dihydroxyvouacapan-17β-oic acid (**1**), used as the starting material, was isolated from *Pterodon polygalaeflorus* Benth. as published by Demuner *et al.* (1996), and 6α-hydroxyvouacapan-7β,17β-lactone (**2**) was prepared from **1** as previously described by Rubinger *et al.* (1991).

*Synthesis of 6-oxovouacapan-7β,17β-lactone (**3**)*

A dry, 200 mL, three-necked, round-bottomed flask fitted with a magnetic stirring bar and rubber septum was charged sequentially with 23 mL of dry dichloromethane and 1.2 mL of oxalyl chloride (13.6 mmol), under a nitrogen atmosphere. The stirred solution was cooled down to −60 °C and 2.4 mL (33.7 mmol) of dimethyl-sulfoxide in dichloromethane (23 mL) were added. After 10 min of magnetic agitation, 4.33 g (13.1 mmol) of lactone **2** in dichloromethane (45 mL)/dimethyl-sulfoxide (9.8 mL) were slowly added. The rate of addition was such that the internal temperature of the flask never exceeded −60 °C. The mixture was stirred for further 15 min and triethylamine (13.8 mL, 99.4 mmol) was added, and then it was allowed to warm up to room temperature; the stirring was maintained till TLC indicated that **2** had been consumed (~ 3.5 h). Then, water (60 mL) was added and extractions with dichloromethane (5 × 15 mL) were performed. The combined organic extracts were sequentially washed with sodium hypochlorite (0.1 mol L^{-1}, 4 × 15 mL), a saturated solution of sodium carbonate (3 × 15 mL) and brine (3 × 15 mL), dried over sodium sulfate and concentrated under reduced pressure. The crude product **3** was purified by chromatography in a silica gel column eluted with dichloromethane. The yield was 83% (3.58 g, 10.85 mmol).

*6-Oxovouacapan-7β,17β-lactone (**3**):* White crystals. – M.p. 262.3–264.5 °C. – IR (KBr): ν_{max} = 3450, 3010, 2995, 2915, 2850, 1790, 1725, 1600, 1500, 1460, 1445, 1390, 1360, 1280,1230, 1190, 1110, 1090, 1030, 930, 740, 690 cm^{-1}. – ^1H NMR (400 MHz, $CDCl_3$): δ_H = 0.99 (s, 3H, CH_3-18), 1.00 (s, 3H, CH_3-20), 1.14 (dt, 1H, $J_{3ax-3eq} = J_{3ax-2ax}$ = 13.2 Hz, $J_{3ax-2eq}$ = 3.8 Hz, H-3_{ax}), 1.31 (dt, 1H, $J_{1ax-1eq} = J_{1ax-2ax}$ = 13.2 Hz, $J_{1ax-2eq}$ = 3.8 Hz, H-1_{ax}), 1.36 (s, 3H, CH_3-19), 1.43 (ddt, 1H, $J_{3eq-3ax}$ = 13.2 Hz, $J_{3eq-2ax} = J_{3eq-2eq}$ = 3.2 Hz, $J_{3eq-1eq}$ = 1.5 Hz, H-3_{eq}), 1.53 (quid, 1H, $J_{2eq-2ax}$ = 13.2 Hz, $J_{2eq-1ax} = J_{2eq-1eq} = J_{2eq-3eq}$ = 3.8 Hz, H-2_{eq}), 1.65 (tq, 1H, $J_{2ax-2eq} = J_{2ax-1ax} = J_{2ax-3eq}$ = 13.2 Hz, $J_{2ax-1eq} = J_{2ax-3eq}$ = 3.2 Hz, H-2_{ax}), 1.76 (ddt, 1H, $J_{1eq-1ax}$ = 13.2 Hz, $J_{1eq-2ax} = J_{1eq-2eq}$ = 3.2 Hz, $J_{1eq-3eq}$ = 1.5 Hz, H-1_{eq}), 2.17–2.32 (m, 3H, H-5, H-8 and H-9), 2.57–2.66 (m, 1H, H-11_{ax}), 2.75–2.84 (m, 1H, H-11_{eq}), 3.36–3.44 (m, 1H, H-14), 4.72–4.77 (m, 1H, H-7), 6.58 (d, 1H, J_{15-16} = 2.0 Hz, H-15), 7.31 (d, 1H, J_{16-15} = 2.0 Hz, H-16). – ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = 15.05 (C-18), 18.02 (C-2), 21.94 (C-11), 22.13 (C-19), 32.90 (C-20), 32.99 (C-4), 38.75 (C-1), 42.11 (C-14), 42.68 (C-3), 45.21 (C-9), 46.34 (C-10), 50.04 (C-8), 63.61 (C-5), 83.54 (C-7), 107.63 (C-15), 113.32 (C-13), 142.08 (C-16), 151.84 (C-12), 171.57 (C-17), 200.57 (C-6). – Elemental analysis: found: C, 73.10; H, 7.43%; calcd. for $C_{20}H_{24}O_4$: C, 73.15; H, 7.37%.

Chloroplast isolation and chlorophyll determination

Intact chloroplasts were prepared from market spinach leaves (*Spinacea oleracea* L.) as reported previously (Macias *et al.*, 1999; Mills *et al.*, 1980). Chloroplasts were resuspended in a small volume of 400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl and 30 mM *N*-tris[hydroxymethyl]methylglycine (tricine)-KOH (pH 8.0). They were stored as a concentrated suspension in the dark for 1 h at 4 °C. Intact chloroplasts were efficiently lysed to yield free thylakoids prior to each experiment by incubating them in the following basal electron transport medium: 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN and 30 mM tricine-KOH (pH 8.0). The chlorophyll concentration was determined as published by Strain *et al.* (1971).

ATP synthesis determination

ATP synthesis coupled to electron flow from water to methylviologen (MV) was determined titrimetrically using a microelectrode Orion Mod. 8103 Ross connected to a Corning potentiometer Model 12 with expanded scale as reported by Dilley (1972). The ATP synthesis reaction medium contained 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KCN, 50 μM MV, 1 mM tricine-KOH (pH 8.0) and 20 μg of chlorophyll/mL.

Light-induced non-cyclic electron transport determination was performed with a Clark type electrode as published by Saha *et al*. (1971) in the presence of 50 μM MV as electron acceptor. The basal electron transport was determined by illuminating chloroplasts (20 μg of chlorophyll per mL) during 1 min in the basal electron transport medium as previously published (Macias *et al*., 1999; Saha *et al*., 1971). Phosphorylating non-cyclic electron transport was measured as basal non-cyclic electron transport except that in the first case 1 mM ADP and 3 mM KH_2PO_4 were added (Macias *et al*., 1999; Saha *et al*., 1971). Uncoupled electron transport was tested in the basal non-cyclic electron transport medium by adding 6 mM NH_4Cl as uncoupler (Macias *et al*., 1999; Saha *et al*., 1971).

Uncoupled PSII and PSI electron flow

These determinations were performed as an uncoupled electron transport assay. Uncoupled PSII from water to dichlorophenol indophenol (DCPIP) was measured by the reduction of DCPIP-supported O_2 evolution using a Clark type electrode. 1 μM 2,5-dibromo-3-methyl-6-isopropyl-1,4-*p*-benzoquinone (DBMIB), 100 μM DCPIP, 500 μM $K_3[Fe(CN)_6]$ and 6 mM NH_4Cl were added; MV was omitted.

Uncoupled PSII electron transport from water to sodium silicomolybdate (SiMo) was determined as in PSII except that 200 μM SiMo and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were added (Giaquinta *et al*., 1974). Almost all electron flow activities were followed with a Yellow Springs Instrument (YSI) oxygen monitor, model 2300 using a Clark type electrode.

Uncoupled electron transport from diphenyl carbazide (DPC) to DCPIP was measured spectrophotometrically and determined in thylakoids that were previously treated with 0.8 M (hydroxymethyl)-aminomethan (Tris) (pH 8.0) and incubated 30 min at 4 °C (Vernon and Shaw, 1969). After this treatment, the chloroplasts were centrifuged at 5000 × *g* (Sorvall super T 21) for 2 min. The pellet was suspended with 40 mL of the basal electron transport reaction medium and used for DCP to DCPIP electron flow assay; previously chlorophyll was determined.

Photosystem I (PSI) electron transport was determined in a similar form to basal non-cyclic electron rate. 10 μM DCMU, 100 μM DCPIP, 50 μM MV, 300 μM ascorbate and 6 mM NH_4Cl were added to the medium (Allen and Holmes, 1986).

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

Chlorophyll a fluorescence

Chlorophyll *a* (Chl) fluorescence was measured at room temperature with a Hansatech Fluorescence Handy PEA (Plant Efficient Analyzer) in 5 min dark-adapted chloroplasts (20 μg mL^{-1}) (King-Díaz *et al.,* 1998; Chávez *et al.,* 2001). The maximum fluorescence yield from the sample was generated using six red high intensity light emitting diodes (broad band 650 nm). The pulse duration was 2 s. The reaction medium used was as basal non-cyclic electron transport. To monitor Chl *a* fluorescence transients, induction aliquots of dark-adapted thylakoids containing 15 μg Chl were transferred to a filter paper by gravity and immediately dipped in 3 mL of the different test compounds.

Mg^{2+}-ATPase activity assays

Light-triggered Mg^{2+}-ATPase activity bound to thylakoid membranes was measured according to Mills *et al*. (1980). Pi was determined as described by Sumner (1944).

Results and Discussion

Synthesis

Fig. 1 shows the synthetic route leading from 6α,7β-dihydroxyvouacapan-17β-oic acid (**1**) to 6α-hydroxyvouacapan-7β,17β-lactone (**2**) and then to 6-oxovouacapan-7β,17β-lactone (**3**). Compound **2** was prepared from **1** by treatment with acetic anhydride and sodium acetate in tetrahydrofuran, for 50 minutes at 40 °C; the yield was 87% (Rubinger *et al.*, 1991). To prepare the δ-ketolactone **3**, the lactone **2** was oxidized as described by Omura

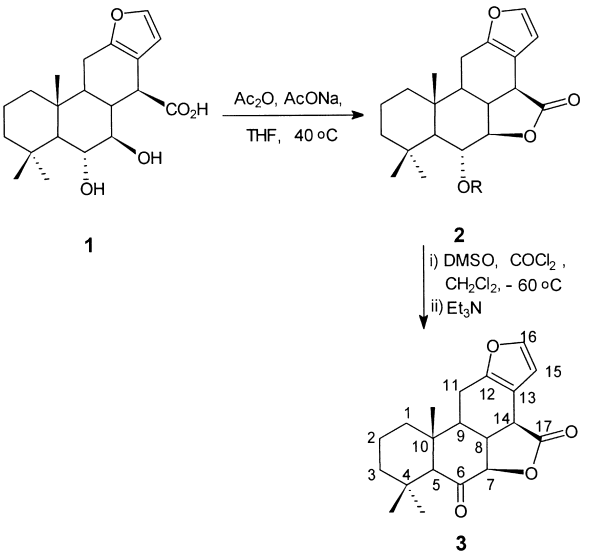

Fig. 1. 6-Oxovouacapan-7β,17β-lactone (**3**) preparation from the natural diterpene 6α,7β-dihydroxyvouacapan-17β-oic acid (**1**) via 6α-hydroxyvouacapan-7β,17β-lactone (**2**).

and Swern (1978). The yield was 83%. The IR spectrum of **3** exhibits two carbonyl absorption bands at 1790 cm^{-1} (lactone) and at 1725 cm^{-1} (ketone), indicating that the desired oxidation was achieved. When comparing the ^1H and ^{13}C NMR spectra of **3** and **2**, some major differences were observed. In **3**, the signal due to C-6 at δ_C 200.57 ppm confirms the oxidation at this position. In addition, the signal due to H-6 observed at δ_H 4.08 ppm in the ^1H NMR spectrum of **2** was not present in the spectrum of **3.**

*Effect of 6-oxovouacapan-7β,17β-lactone (**3**) on ATP synthesis and non-cyclic electron transport rate on spinach chloroplasts*

The effect of **3** on photophosphorylation coupled to electron flow from water to MV was tested on freshly lysed spinach chloroplasts. It was observed that as the concentration of **3** increased, the synthesis of ATP decreased (Fig. 2). The I_{50} value was 91 μM.

ATP synthesis is coupled to electron transport. Thus, it was decided to explore whether the effects of **3** were due to: a) inhibition of an electron transporter within the thylakoid chain, b) inhibition of the H^+-ATPase complex itself or c) dissipation of the H^+ gradient, *i.e.* an uncoupling effect. In order to discriminate the mechanism by which **3** inhibited ATP synthesis, its effect on non-cyclic electron transport from water to MV (basal, phosphorylating and uncoupled) was assayed. The effect of **3** on non-cyclic electron transport rate from water to MV of freshly lysed spinach thylakoids was tested (Fig. 3). Basal and phosphorylating electron transport rates were partially inhibited by up to 50–100 μM **3**. At higher concentrations, **3** behaved as uncoupler as indicated by the acceleration in the

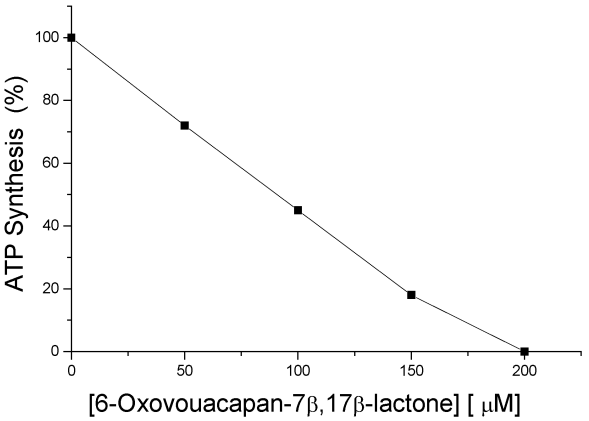

Fig. 2. Effect of 6-oxovouacapan-7β,17β-lactone (**3**) on ATP synthesis coupled to electron transport from water to MV. Experimental conditions are as described in Materials and Methods. Control value was 1280 μM ATP h^{-1} mg^{-1} Chl.

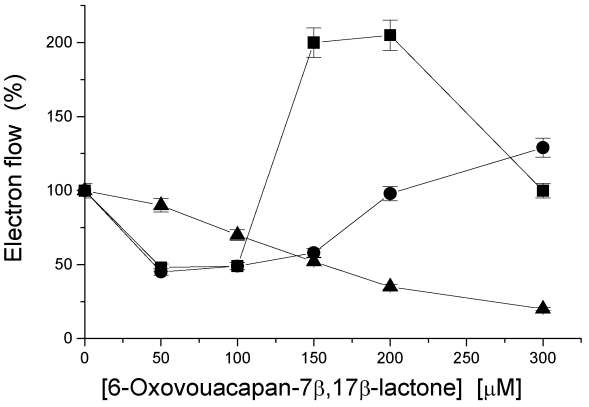

Fig. 3. 6-Oxovouacapan-7β,17β-lactone (**3**) effect on the electron transport rate from water to MV. Experimental conditions are as described in Materials and Methods. (■) Basal, (●) phosphorylating, and (▲) uncoupling conditions. Control values were 580, 845 and 1350 μequiv. e^- h^{-1} mg^{-1} Chl, respectively.

rate of electron transport both during basal and phosphorylating conditions. However, the uncoupled electron transport was inhibited at increasing concentrations of **3**, such that at 300 μM **3** the uncoupled electron transport was inhibited by 80%. This last result indicates that **3** exhibits dual effects, *i.e.* it is an uncoupler and an inhibitor of electron transport. In this regard, the uncoupled noncyclic electron flow from water to MV was inhibited by **3** with an I_{50} of 156 μM, which is higher than the I_{50} for inhibition of ATP synthesis (1.7 times more active). Thus, it is possible that **3** binds to the CF_1CF_0-ATPase complex exerting a direct inhibition of Mg^{2+}-ATPase activity.

*Localization of the PSI or PSII partial reaction where 6-oxovouacapan-7β,17β-lactone (**3**) interacts*

In order to determine the site of inhibition on the thylakoid electron transport chain, the effect of **3** on uncoupled partial reactions of PSI and PSII was determined using appropriate artificial electron donors, acceptors and inhibitors (Allen and Holmes, 1986). Table I shows the inhibited PSII uncoupled electron transport from water to DCPIP and from water to SiMo by **3**. The polarographic measurement indicates that **3** inhibits within the span of water to Q_A of PSII electron transport. Additionally, PSI uncoupled electron transport from $DCPIPH_2$ to MV was unaffected by **3** (Table I).

Chlorophyll a fluorescence

In order to gather further evidence of the 6-oxovouacapan-7β,17β-lactone (**3**)-mediated inhibition of PSII the fluorescence of chlorophyll *a* of PSII was evaluated. Freshly lysed spinach chloroplasts exhibited a polyphasic fluorescence curve with regular OJIP sequence of transients similar to those previously described for several intact organisms (Strasser *et al.*, 1995). Addition of 10 μM DCMU used as positive control, resulted in a fast rise of the fluorescence during the first 2 ms of illumination, transforming the regular OJIP sequence into an OJ sequence (Strasser *et al.*, 1995). Thylakoids were also incubated with 0.8 M Tris, pH 8.0 used as positive control, a well-known donor side inhibitor of PSII, which causes the loss of the electron-donation ability and the loss of the Mn^{2+} complex at the same rate that it inhibits O_2 evolution (Rickert *et al.*, 1991). The Tris-treated chloroplasts exhibit a fluorescence of Chl *a* transient (the OJIP trace) similar to that observed in the heat-treated samples. After the heat (or Tris) treatment of thylakoids, a new step K at about 300 μs appears in the fluorescence induction curve measured under high light illumination by a PEA fluorometer. It was suggested that the appearance of the K step is caused by the inhibition of OEC, which leads to an accumulation of the oxidized secondary electron donor of PSII-Y_Z (Strasser, 1997). Compound **3** had a similar behavior to heat stress or Tris treatment of thylakoids up to 300 μs and thereafter, F_m partially decreases as concentration of **3** increases. Normalizations of all curves between F_0 and F_m show the appearance of the K band, indicating the block of the OEC by **3**. The decreasing F_m and the F_0 values were almost constant (Table II) indicating the creation of "silent centers", *i.e.* non-Q_A reducing centers (Tóth *et al.*, 2005). The results of the K step and the sink-silent centers confirmed the behavior of **3** as a water-splitting enzyme inhibitor and the creation of the

Table I. Concentration-dependent 6-oxovouacapan-7β,17β-lactone (**3**)-mediated inhibition of the uncoupled partial reaction of PSII electron flow from water to DCPIP, from water to SiMo and DPC to DCPIP photoreduction in Tris-treated thylakoids using DPC as electron donor.

	PSII					
Conc. [μM]	H_2O to DCPIP a	%	H_2O to SiMo a	%	DPC to DCPIP b	%
0	467	100	200	100	382	100
25	439	94	184	92	327	86
50	388	83	162	81	264	69
100	290	62	120	60	199	52
150	187	40	74	37	153	40

a, μequiv. e^- h^{-1} mg^{-1} Chl; b, μM DCPIPred h^{-1} mg^{-1} Chl.

Table II. F_0, F_m, and F_v/F_m values in thylakoids after treatment with 6-oxovouacapan-7β,17β-lactone (**3**) and 5 min of incubation.

3 [μM]	F_0	F_m	F_v/F_m
0	448	2193	0.796
25	450	1811	0.752
50	302	1301	0.768
100	350	1370	0.745
200	370	1571	0.764
10 μM DCMU	593	2203	0.731
0.8 M Tris	417	818	0.490

P^{+}_{680} quencher non-Q_A reducing centers which block the non-cyclic electron transport of PSII.

It seems that the lactone group is a requirement for the interaction with the PSII electron transport carrier and CF_0 inhibition (King-Díaz *et al*., 2005; Achnine *et al*., 1999; Calera *et al*., 1995). It was recently published (King-Díaz *et al*., 2005) that 6α-hydroxyvouacapan-7β,17β-lactone (**2**) inhibits PSII electron transport, *i.e.* interfers with OEC function and in the span electron transport of P_{680} to Q_A, and that the lactone group is responsible for inhibition. Here, it is demonstrated that **3** also inhibits the same PSII sites and thus the lactone group is probably important for PSII inhibition (King-Díaz *et al*., 2005). The interaction of **3** with the electron transport chain target is irreversible. This was supported by the finding that when the thylakoid samples with and without 500 μM **3** were illuminated for 1 min and the uncoupled electron flow from water to MV was measured, the electron flow with **3** was inhibited. These samples were washed twice with electron transport medium, and the uncoupled electron flow was measured again with the washed thylakoids. The uncoupled electron transport from water to MV remain inhibited compared with the control. These results indicate that **3** binds covalently with its target. These assays were repeated three times and the results always were reproducible. The assays were done for compound **2** too, and the results indicated the same behavior as for **3**. We propose that the lactone reacts with a nucleophilic group of the thylakoid electron transport carrier like amines, thiols or alcohols from proteins. The lactone is opened when it reacts with amines (to give amides, thiols, alcohols by transesterification). Fig. 4 shows the proposed mechanism for **3** reacting with the amine group of the target. The same is valuable for RSH or ROH. The open derivative is more stable (more favorable entropy). Also, the protein carrier system may not be adequate to favor the reversible reaction.

It seems that the carbonyl group at C-6 of **3** somehow uncouples photophosphorylation as a non-classical uncoupler. Finally, we conclude that the free –OH group at C-6 of **2** is not important for interaction with PSII electron transport carrier.

Mg^{2+}-ATPase activity

It is well known that uncouplers such as tricolorin A, NH_4Cl and FCCP stimulate the activity of the Mg^{2+}-ATPase (Achnine *et al*., 1999). Table III shows that ammonium chloride enhanced the activity of Mg^{2+}-ATPase, which was used as positive control, and that compound **3** enhanced the activity of Mg^{2+}-ATPase, corroborating that **3** acts as an uncoupler. To understand the uncoupler property of **3**, the logarithms of the partition coefficient (Log *P*) of **1**, **2** and **3** were estimated, with values of 1.09, 3.60 and 4.48, respectively (the LOGKOW;KOWWIN program was used). This indicates that **3** was more soluble in the lipid phase than **2**, which could explain the interaction with the H^{+}-ATPase of the thylakoid membranes.

Table III. Effect of 6-oxovouacapan-7β,17β-lactone (**3**) on the activity of the membrane bound thylakoid enzyme Mg^{2+}-ATPase.

3 [μM]	Activity [μM phosphate mg^{-1} Chl h^{-1}]	(%)
0	121	100
100	157	130
200	169	140
300	191	158
NH_4Cl [mM]		
0	121	100
1	207	171
3	263	217
6	152	126

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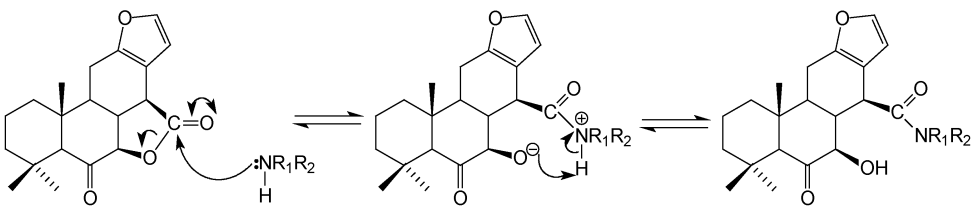

Fig. 4. Mechanism proposed for **3** reacting with the amine group of the target. The same is valuable for RSH or ROH.

Achnine L., Bah M., Pereda-Miranda R., Mata R., and Lotina-Hennsen B. (1999), Tricolorin A, a potential natural uncoupler and inhibitor of photosystem II acceptor side of spinach chloroplasts. Physiol. Plant. **106**, 240–252.

Allen J. F. and Holmes N. G., (1986), Electron transport partial reactions. In: Photosynthesis, Energy Transduction. A Practical Approach (Hipkinns M. F. and Baker N. R., eds.). IRL Press, Oxford, UK, pp. 103–141.

Calera M. R., Soto F., Sánchez P., Bye R., Hernández-Bautista B., Anaya A. L., Lotina-Hennsen B., and Rachel M. (1995), Biochemically active sesquiterpene lactones from *Ratibida mexicana*. Phytochemistry **40,** 419–425.

Castelo-Branco P. A. (2001), Obtenção e estudo químico-biológico de derivados oxidados e nitrogenados de furanoditerpenos de *Pterodon polygalaeflorus* Benth. Ms.Sc. Thesis, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, pp. 77–82.

Chávez D., Mata R., Iglesias-Prieto R., and Lotina-Hennsen B. (2001), Annonaceous acetogenins: Naturally occurring inhibitors of ATP synthesis and photosystem II in spinach chloroplasts. Physiol. Plant. **111**, 262–268.

Correa P. (1984), Dicionário de Plantas Úteis do Brasil e das Plantas Exóticas Cultivadas. Ministério da Agricultura – IBDF, Rio de Janeiro, p. 153.

Demuner A. J., Barbosa L. C. A., Veloso D. P., Alves D. L. F., and Howarth O. W. J. (1996), Structure and plant growth regulatory activity of new diterpenes from *Pterodon polygalaeflorus*. J. Nat. Prod. **59**, 770–772.

Demuner A. J., Barbosa L. C. A, Piló-Veloso D., and Howarth O. W. (1998), Synthesis and plant growth regulatory activity of 6α,7β-dihydroxyvouacapan-17β-oic acid derivatives. Aust. J. Chem. **51**, 61–66.

Dilley R. A. (1972), Ion transport (H^+, K^+, Mg^{2+} exchange phenomena). Methods Enzymol. **24**, 68–74.

Fascio M., Mors W. B., Gilbert B., Mahajan J. R., Monteiro M. B., Santos-Filho M. F., and Vichnewski W. (1976), Diterpenoids furans from *Pterodon* species. Phytochemistry **15**, 201–203.

Giaquinta R. T., Selman B. R., Anderson B. J., and Dilley R. A. (1974), Inhibition of coupling factor activity of chloroplast membrane by diazonium compounds. J. Biol. Chem. **249**, 2873–2878.

King-Díaz B., Barba-Behrens N., Montes-Ayala J., Castillo-Blum S. E., Escartín-Guzmán C., Iglesias-Prieto R., and Lotina-Hennsen B. (1998), Interference by nickel(II) salts and their 5-methylimidazole-4-carboxylate coordination compounds on the chloroplast redox chain. Z. Naturforsch **53c**, 987–994.

King-Díaz B., Pérez-Reyes A., Leite dos Santos F. J., Ferreira-Alves D. L., Piló Veloso D., Uribe C. S., and Lotina-Hennsen B. (2006), Natural diterpene β-lactone derivative as photosystem II inhibitor on spinach chloroplasts. Pestic. Biochem. Physiol. **84**, 109–115.

Macias M. L., Rojas I. R., Mata R., and Lotina-Hennsen B. (1999), Effect of selected coumarins on spinach chloroplast photosynthesis. J. Agric. Food Chem. **47**, 2137–2140.

Mills J. D., Mitchell P., and Shurmann P. (1980), Modulation of coupling ATPase activity in intact chloroplasts. FEBS Lett. **112**, 173–177.

Mors W. B., Santos Filho M. F., Monteiro H. J., and Gilbs B. (1967), Chemoprofilactic agent in schistosomiasis: 14,15-epoxygeranylgeraniol. Science **157**, 950–951.

Nunan E. A., Piló-Veloso D., Turchetti R. M. M., and Ferreira-Alves D. L. (1985), Furano-diterpenos com atividades anti-inflamatoria e pro-inflamatoria. J. Braz. Med. Exp. Sci. **50**, 450.

Omura K. and Swern D. (1978), Oxidation of alcohols by activated dimethylsulfoxide – preparative, steric, and mechanistic study. Tetrahedron **34**, 1651–1660.

Rickert K. W., Sears J., Beck W. F., and Brudvig G. W. (1991), Mechanism of irreversible inhibition of O_2 evolution in photosystem II by Tris [(hydroxymethyl)-aminomethane]. Biochemistry **30**, 7888–7894.

Rubinger M. M. M., Piló-Veloso D., Stefani G. M., and Ferreira-Alves D. L. (1991), Synthesis of 6α,17β-oic acid derivatives. Part I: Hydroxamic acid and amide derivatives. J. Braz. Chem. Soc. **2**, 124–128.

Saha S., Ouitrakul R., Isawa S., and Good N. J. (1971), Electron transport and phosphorylation in chloroplasts as a function of the electron acceptor. J. Biol. Chem. **246**, 3204–3209.

Strain H. H., Cope T., and Svec M. A. (1971), Analytical procedures for the isolation, identification, estimation and investigation of the chlorophylls. Methods Enzymol. **23**, 452–466.

Strasser B. J. (1997), Donor side capacity of photosystem II probed by chlorophyll *a* fluorescence transients. Photosynth. Res. **52**, 147–155.

Strasser R. J., Srivastava A., and Govindjee (1995), Polyphasic chlorophyll *a* fluorescence transients in plants and cyanobacteria. Photochem. Photobiol. **61**, 32–42.

Sumner J. B. (1944), Scientific apparatus and laboratory methods. A method for the calorimetric determination of phosphorous. Science **100**, 413–418.

Tóth S. Z., Schansker G., and Strasser R. J. (2005), In intact leaves, the maximum fluorescence level (F_M) is independent of the redox state of the plastoquinone pool: A DCMU-inhibition study. Biochim. Biophys. Acta **1708**, 275–282.

Vernon L. P. and Shaw E. R. (1969), Photoreduction of 2,6-dichlorophenol-indophenol by diphenylcarbazide: A photosystem 2 reaction catalyzed by Tris-washed chloroplasts and subchloroplast fragments. Plant Physiol. **44**, 1645–1649.