Phytochemical and Biochemical Characterization of Epimeric Photogedunin Derivatives. Their Different Sites of Interaction on the Redox Electron Transport Carrier of *Spinacea oleracea* L. Chloroplasts†

Carlos L. Céspedes,‡ José S. Calderón,‡ Beatriz King-Diaz,§ and Blas Lotina-Hennsen*,§

Instituto de Química, Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de Mexico, Ciudad Universitaria, 04510 México DF, México

Bioactivity-directed fractionation of the dichloromethane extract of the heartwood of *Cedrela salvadorensis* L. resulted in the isolation of two epimeric photogedunins and in the acquirement of two bioactive epimeric photogedunin acetates **3** (β) and **4** (α) at the C-23 position. Compounds **3** and **4** inhibited photophosphorylation, H^+-uptake, and noncyclic electron flow from water to methyl viologen (basal, phosphorylating, and uncoupled). Inhibiton of all photosynthetic activities require 3 min of preincubation in the light before inhibition is observed. The results indicate that compounds **3** and **4** behave as Hill reaction inhibitors. α-Photogedunin acetate **3** inhibits PS II electron flow from H_2O to DCBQ without affecting PS I electron transport from reduced DCPIP to MV and PSII from water to SiMo. Therefore, its target is localized at the Q_B level. β-Photogedunin acetate **4** inhibits PS I electron flow from reduced DCPIP to MV, PMS_{red} to MV, and $TMQH_2$ to MV without affecting PS II electron flow, interacting at the b_6f level and in the span of P_{700} to F_x.

Keywords: *α- and β-photogedunin acetate; Cedrela salvadorensis; Meliaceae; photosystem I and II inhibitors; herbicide target*

INTRODUCTION

Several limonoids have been isolated from the Mexican species *Cedrela salvadorensis* Standley (*Meliaceae*) (Segura et al., 1994; Toscano et al., 1996). *C. salvadorensis* is a small tree which grows on the dry pacific slope ranging from Jalisco to Chiapas in Mexico, through Central America, and up to the north of Panama.

A number of limonoids are noted because of their potent insect antifeedant characteristics. These compounds have also shown to have potential for the development of "green herbicides" in photosynthetic studies. Some of these compounds possessing novel structures significantly inhibited several photosynthetic activities in spinach chloroplasts (Mata et al., 1996).

A phytochemical study of *Cedrela odorata* led to the isolation of photogedunin (Burke et al., 1969) without determination of the stereochemistry at C-23. We describe the isolation from *C. salvadorensis* Standley of the epimeric mixture and the acquirement of photogedunin acetates **3** and **4** (Figure 1). In addition, the behavior of compounds **3** and **4** on different photosynthetic activities was investigated.

In our group, it was demonstrated that diverse secondary metabolites behave with different mechanisms of action and have different targets of interaction on photosynthetic apparatus (Calera et al., 1996; Jimenez et al., 1997, Aguilar et al., 1996; Lotina-Hennsen et al., 1991, 1992, 1998, Mata et al., 1996). As far as we know, the effect of **1**−**4** on photosynthesis had not been investigated.

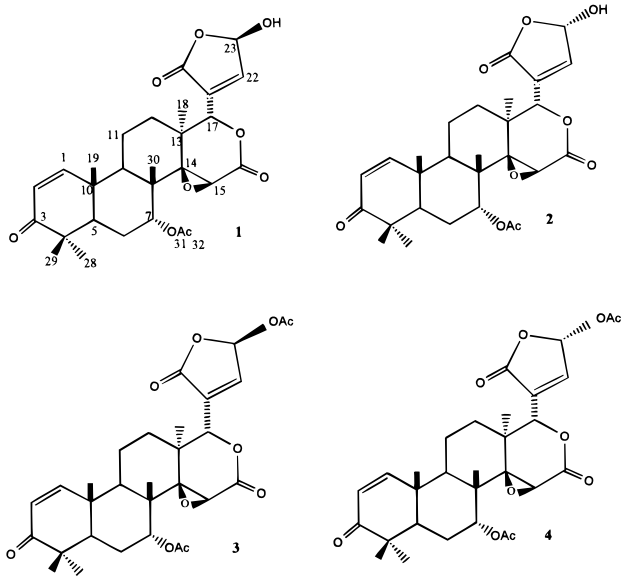

Figure 1. Structures of photogedunins **1** and **2** and acetate derivatives **3** and **4**.

MATERIALS AND METHODS

Chemicals and Solvents. All reagents used were commercially available. Tricine, MV, sorbitol, ADP, DCPIP, DBMIB, DCBQ, DCMU, and SiMo were from Sigma Chemical Co. Methanol, $CHCl_3$, CH_2Cl_2, KCl, $CuSO_4$, NH_4Cl, $MgCl_2$, pyridine, acetic anhydride, silica gel GF_{254} analytical chro-

* To whom correspondence should be addressed [e-mail blas@servidor.unam.mx; fax (52-5) 6225329].

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‡ Instituto de Química.

§ Facultad de Química.

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matoplates, silica gel grade 60, 70−230, 60Å, for column chromatography, *n*-hexane, and ethyl acetate were from Merck. Pyridine and acetic anhydride were distilled prior to use.

Apparatus. ^{1}H NMR and ^{13}C NMR, spectra were registered at 300 and 500 MHz, respectively, on Varian VXR-300S and VXR-500S spectrometers, chemical shifts (ppm) are related to $(CH_3)_4Si$ as an internal reference, and $CDCl_3$ and acetone-d_6 were used as solvents from Aldrich Chemical Co. Coupling constants are quoted in Hz. IR spectra were obtained in KBr on Perkin-Elmer 283-B and FT-IR Nicolet Magna 750 spectrophotometers. Mass spectra were taken on a JEOL JMS-SX102A (70 eV). UV spectra were determined on a Shimadzu UV-160 spectrophotometer. Optical rotation was determined on a JASCO DIP-360 spectropolarimeter. Melting points were obtained on a Fisher-Johns with calefactor plates apparatus and remain uncorrected. Electron transport was determined with a Clark type electrode connected to an oxygraph YSI model 5300. X-ray analysis was done on a Nicolet P3F diffractometer using Ni-filtered Cu Kα radiation. Data were corrected for *LP* effects.

Plant Material. The plant material heartwood was collected in Morelia, State of Michoacán, Mexico, in February 1997. A voucher sample can be found at the ethnobotanical collection of the National Herbarium (MEXU), Instituto de Biología, UNAM. Voucher: M. T. German and P. Tenorio No. 2.174. Register number: 800.192.

Isolation and Purification of Photogedunin. The photogedunin epimeric mixture was isolated and purified by chromatography. The milled heartwood of a young tree of *Cedrela salvadorensis* L. (7.5 kg) was percolated with CH_2Cl_2 (10 L). The resulting crude CH_2Cl_2 extract was evaporated to dryness at room temperature under vacuum. Then the oil residue (377 g) was partitioned between *n*-hexane (4 × 50 mL) (F_0-1) and CH_2Cl_2 (F_0-2). Elimination of the solvent yielded residues F_0-1 (331 g) and F_0-2 (45.5 g). The original extract primary fractions F_0-1 and F_0-2 were biologically evaluated for their potential phytogrowth-inhibitory property. The initial phytogrowth-inhibitory activity was evaluated on seeds of *Lolium multiflorum*, *Physalis ixocarpa*, *Triticum vulgare*, and *Trifolium alexandrinum*, by using the Petri dish germination bioassay (Hasegawa et al., 1982). According to the results, fraction F_0-2 was the most bioactive and was further fractionated by column and thin-layer chromatographies over silica gel (60/254, Merck), eluted with *n*-hexane/ethyl acetate (9:1) mixture gradient polarity, and underwent phytogrowth-inhibitory germination bioassay as directed fractionating. These procedures yielded an α- and *β*-photogedunin epimeric mixture (3.98 g) in the *n*-hexane/ethyl acetate (3:7) elution system of fraction (F_0-3), and then they were purified on TLC (preparative plates Macherey-Nagel, precoated TLC plates SIL G-100 UV_{254}, 1.0 mm). The TLC R_f were identical with those from authentic samples isolated from *C. oaxacensis* (Lopez, 1996).

Structural Elucidation of Photogedunin Epimeric Mixture. By means of repeated crystallizations, crystals from MeOH as colorless prisms were obtained; one of them was analyzed by X-ray diffraction and corresponded to the majority α-photogedunin **2** (Figure 2). Crystal data: $C_{28}H_{34}O_9$; crystal dimensions 0.24 × 0.22 × 0.04; monoclinic; a = 8.950(5) Å, b = 11.275(5) Å, c = 12.733(5) Å; V = 1282.1(6) Å^3; space group $P2_1$; Z = 2; $F(000)$ = 550.76. Using ω scans, 1507 reflections were collected at 293 K. The structure was solved by direct methods using the program SIR92 (Burla et al., 1992). Hydrogen atoms at calculated positions with a common U = 0.06 Å^2. The non-hydrogen atoms were refined (SHELXTL) (Sheldrick, 1990) with anisotropic thermal parameter and refinement converged at R = 6.24 and wR = 7.99. The function minimized was $\sum [w(F_o - F_c)^2]$, where $w = [\sigma^2(F_o) + 0.0043(F_o)^2]^{-1}$. The final difference map showed no features greater than ±0.16 e ±$^{-3}$. It was not necessary to perform an absorption correction.

The remaining crystals were analyzed including proton and ^{13}C NMR, showing the following data. Mp: 285 °C (dec). $[\alpha]_D$ +15° ($CHCl_3$; *c* 0.02). UV: λ_{max} 224 nm (ϵ 14 550). IR (ν_{max}, KBr, cm^{-1}): 3421 (OH), 1755 (C═O of *γ*-lactone, α-, *β*-unsaturated), 1707 (C═O of acetate and *δ*-lactone), 1660 (C═O of cyclohexenone) and 1590 cm^{-1}. MS: [*m/e* (rel int)]: 516 [M + 2]$^+$ (0.01), 515 [M + 1] (0.04), 514 [M$^+$] (11) ($C_{28}H_{34}O_9$), 454 [M$^+$ − AcOH] (13), 436 [M$^+$ − AcOH − H_2O (6), 313 (17), 299 (12). ^{1}H NMR ($CDCl_3$, ppm, 300 MHz): Table 2. ^{13}C NMR ($CDCl_3$, ppm, 75 MHz): Table 3.

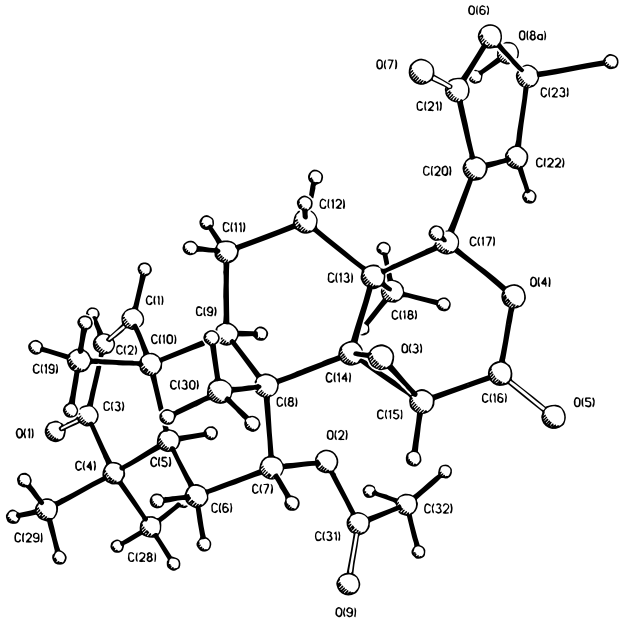

Figure 2. Stereoscopic view of photogedunin **2**.

Table 1. Effect of α- and *β*-Photogedunin Acetates on Photosynthesis Electron Transport Rate Measurements in Intact Freshly Lysed Spinach Chloroplasts

	IC_{50}[a] (μM) of inhibition (pI_{50})		
compd	basal	uncoupled	phosphorylating
3		18.5 (4.921)	11.0 (4.959)
4	12.0 (4.921)	18.0 (4.745)	19.0 (4.721)

[a] IC_{50} = 50% inhibitory concentration.

Acetylation of Photogedunin Epimeric Mixture. The α- and *β*-photogedunin epimeric acetates were obtained by acetylation of the photogedunin epimeric mixture and then separated and purified by thin-layer chromatography using silica gel GF_{254} preparative chromatoplates, 2 mm, Macherey-Nagel. An epimeric mixture of photogedunin (140 mg) was treated with Ac_2O (2 mL) and pyridine (2 mL) at room temperature for 48 h. The reaction mixture was worked up as usual to yield 43 mg of **3** and 85 mg of **4**, which were compared on TLC with the epimeric mixture, where **4** showed the higher R_f (0.65). The purification of both acetate epimers was carried out with $CHCl_3$/AcOEt (9:1) as the eluent system; both separated epimers were crystallized from acetone as plates. *β*-Photogedunin acetate (**3**): R_f = 0.45; $[\alpha]_D$ = +61° ($CHCl_3$; *c* 0.02); mp 289−292 °C (dec); UV λ_{max} 224 nm (ϵ 14 550); IR ν_{max} 1750 (C═O of *γ* lactone, α-, *β*-unsaturated), 1710 (C═O of acetates and lactones), 1660 (C═O of cyclohexenone) and 1590 cm^{-1}; MS *m/e* 556 [M$^+$] ($C_{30}H_{36}O_{10}$), 512 [M$^+$ − 44], 496 [M$^+$ − 60], 478 [M$^+$ − 78]; ^{1}H NMR ($CDCl_3$, ppm, 500 MHz) see Table 3; ^{13}C NMR ($CDCl_3$, ppm, 125 MHz) see Table 2. α-photogedunin acetate (**4**): $[\alpha]_D$ = +62° ($CHCl_3$; *c* 0.02); mp 289−292 °C (dec); UV, IR, and MS identical to compound **3**; ^{1}H NMR ($CDCl_3$, ppm, 500 MHz) see Table 2; ^{13}C NMR ($CDCl_3$, ppm, 125 MHz): see Table 3.

Chloroplasts Isolation and Chlorophyll Determination. Intact chloroplasts were prepared from market spinach leaves (*Spinacea oleracea* L.) as described (Mills et al., 1980; Saha et al., 1971; Jimenez et al., 1997), and the pellet was suspended in the medium 400 mM sorbitol, 5 mM $MgCl_2$, and 10 mM KCl and buffered with 0.03 M Na^+−tricine at pH 8.0. Chlorophyll concentration was determined according to Strain et al. (1971).

Table 2. ^{1}H NMR Chemicals Shifts[a] for a Photogedunin Epimeric Mixture and 3 and 4

H position	photogedunin[b] (epimeric mixture)[e]	**3**[c]	**4**[d]
1	6.99 (d) (10.2)	7.10 (d) (10.5)	7.10 (d) (10.5)
2	5.74 (d) (10.2)	5.88 (d) (10.5)	5.87 (d) (10.5)
5	2.01 (dd) (12.4; 3.0)	2.01 (dd) (13.5; 3.5)	2.02 (dd) (13.5; 3.5)
6	1.86 (m)	1.91 (ddd) (13.5, 2.5, 3.5)	1.87 (ddd) (13.5, 2.5, 3.5)
6′		1.89 (ddd) (13.5; 2.5; 3.5)	1.78 (ddd) (13.5; 2.5; 3.5)
7	4.41 (dd) (2.5; 2.0)	4.54 (dd) (2.5, 2.0)	4.54 (dd) (2.5, 2.0)
9	2.45 (dd) (12.5, 3.0)	2.31 (dd) (12.5, 6.0)	2.45 (dd) (12.5, 6.0)
11′	2.36 (m)	2.33 (ddd) (12.5, 12.5, 3.5)	2.35 (ddd) (12.5, 12.5, 3.5)
11		2.18 (ddd) (12.5, 12.5, 3.5)	2.141 (ddd) (12.5, 12.5, 3.5)
12	1.55 (dd) (12.5, 3.0)	1.61 (ddd) (12.5, 12.5, 3.5)	1.59 (ddd) (12.5, 12.5, 3.5)
15	3.37 (s)	3.52 (s)	3.52 (s)
17	5.42 (s)	5.60 (d) (1.2)	5.57 (t) (1.5)
18	1.12 (Me, s)	1.08 (Me, s)	1.08 (Me, s)
19	1.10 (Me, s)	1.26 (Me, s)	1.26 (Me, s)
22	7.12 (s)	7.35 (t) (1.2)	7.34 (t) (1.5)
23	6.07 (s)	7.03 (d) (1.2)	6.91 (t) (1.5)
28	0.93 (Me, s)	1.07 (Me, s)	1.07 (Me, s)
29	0.94 (Me, s)	1.16 (Me, s)	1.17 (Me, s)
30	1.03 (Me, s)	1.23 (Me, s)	1.23 (Me, s)
32	1.97 (OAc, s)	2.11 (OAc, s)	2.10 (OAc, s)
34		2.19 (OAc, s)	2.18 (OAc, s)

[a] δ values in ppm downfield from $SiMe_4$ in $CDCl_3$. [b] 200 MHz. [c] 300 MHz. [d] 500 MHz. [e] 8.30 (br s, exchange D_2O, OH-23).

Table 3. Carbon Chemical Shifts[a] for a Photogedunin Epimeric Mixture and 3 and 4

carbon	photogedunin[b] (epimeric mixture)	**3** and **4**[b]
1	156.50 (d)	157.24 (d)
2	125.22 (d)	125.68 (d)
3	203.12 (s)	204.42 (s)
4	43.36 (s)	43.93 (s)
5	45.36 (d)	45.87 (d)
6	22.59 (t)	22.99 (t)
7	72.54 (d)	73.16 (d)
8	41.97 (s)	42.52 (s)
9	38.74 (d)	39.26 (d)
10	39.45 (s)	39.94 (s)
11	14.20 (t)	14.66 (t)
12	24.66 (t)	25.27 (t)
13	38.68 (s)	39.36 (s)
14	69.03 (s)	69.46 (s)
15	56.03 (d)	56.42 (d)
16	166.27 (s)	166.80 (s)
17	75.25 and 75.46 (d)	76.17 and 75.99 (d)[c]
18	16.48 and 16.60 (q)	17.03 and 17.18 (q)[c]
19	19.19 (q)	19.55 (q)
20	132.11 (s)	134.30 (s)
21	170.00 (s)	169.85 (s)
22	150.99 and 150.12 (d)	148.63 and 148.11 (d)[c]
23	97.52 and 96.93 (d)	92.81 and 92.01 (d)[c]
28	26.54 (q)	26.87 (q)
29	20.63 (q)	21.03 (q)
30	17.71 (q)	18.13 (q)
31	169.07 (s)	168.59 (s)
32	20.48 (q)	20.44 (q)
33		168.22 (s)
34		20.87 (d)

[a] δ values in ppm downfield from $SiMe_4$. [b] In $CDCl_3$. [c] Signals at right side are of majority compound **4**.

Measurement of Proton Uptake and ATP Synthesis. Proton uptake was measured as the pH rose between 8.0 and 8.1 (Dilley, 1972) with a combination microelectrode connected to a Corning potentiometer (model 12 Research pH meter) with expanded scale and registered in a Gilson recorder. The reaction medium was 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, and 1 mM Na^+−tricine at pH 8. ATP synthesis was measured titrimetrically according to the procedure of Dilley (1972). MV (50 μM) was added as an electron acceptor for the Hill reaction.

Measurement of Electron Transport. Photosynthetic noncyclic electron transport rates from water to MV were monitored with a YSI (Yellow Spring Instrument) model 5300 oxygen monitor connected to a Clark type electrode. The reaction medium was the same as that used on the H^+-uptake assay except for the tricine concentration (15 mM), and in the case of the uncoupled electron transport measurement, 6 mM NH_4Cl was added. All reaction mixtures were illuminated with actinic light from a projector lamp (GAF 2660) passed trough a 5 cm filter of 1% $CuSO_4$ solution at 20 °C (Calera et al., 1995a, 1996; Van Gorkom and Gast, 1996).

Photosystems I and II Determination. Uncoupled photosystem I electron transport rate from DCPIP to MV was determined in a similar way to uncoupled noncyclic electron transport (Jimenez et al., 1997). The following reagents were added: 10 μM DCMU, 100 μM DCPIP, 50 μM MV, 500 μM ascorbate, and 6 mM NH_4Cl. Uncoupled photosystem II electron flow from water to DCBQ was measured in the presence of 200 μM DCBQ, 1 μM DBMIB, 6 mM NH_4Cl, and 20 μg of chl/mL. The partial reaction of uncoupled electron transport from water to SiMo was determined with the same reaction mixture as in photosystem II except that 200 μM SiMo and 10 μM DCMU were added (Giaquinta et al., 1974) and DCBQ was omitted. 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 extrapolated using the graph of percent activity vs concentration of photogedunin derivatives. I_{50} is the concentration producing 50% inhibition.

RESULTS AND DISCUSSION

Photogedunin Epimeric Mixture. The purification of a photogedunin epimeric mixture was carried out by TLC-preparative methods of the mother liquor; the R_f, IR, UV, ^{1}H NMR, and ^{13}C NMR data are in accordance with an authentic sample (Lopez, 1996). In the chromatography plates we were observed two plots very close to each other corresponding to the epimeric mixture, and it was not posible to separate them. Because of this fact the photosynthetic studies were not carried out with this mixture.

Transformation of Photogedunin Epimeric to Photogedunin Acetates 3 and 4. The chromatographic procedures on the active photogedunin epimeric mixture yield a product that was then acetylated; the products obtained were β-photogedunin acetate (**3**) and α-photogedunin acetate (**4**). Their spectral data were compared with a photogedunin epimeric mixture. Each

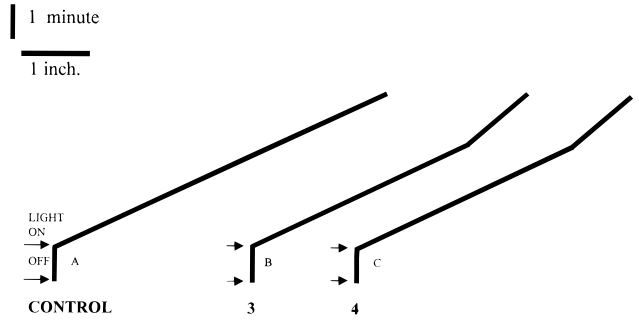

Figure 3. Polarographic traces showing the inhibition of photosynthetic electron transfer of compounds **3** and **4** at 11 and 19 μM, respectively, compared with control from water to MV. The concentration used is the IC_{50} for compounds **3** and **4** obtained by extrapolation in Figure 5, under phosphorylating conditions. Measurements were made by monitoring the change of oxygen concentrtion in the medium using a Clark electrode attached to a YSI oxygraph model 5300. The reaction mixture contained 0.1 M sucrose, 5 mM $MgCl_2$, 10 mM KCl, 1.0 mM ADP, 3.0 mM Pi, 15 mM tricine−KOH buffer (pH 8.0), and thylakoids equivalent to 20 μg/mL of chlorophyll (reaction volume, 3.0 mL; 20 °C). Arrows indicate onset illumination.

compound fragmentation pattern was matched with a typical one for limonoid types (Budzikiewicz, 1964) and provided a parent peak at *m/z* 556, supporting the molecular formula $C_{30}H_{36}O_{10}$, and included fragments at *m/z* 512 ($C_{29}H_{38}O_8$, $[M^+ - 44]$) and *m/z* 496 ($C_{29}H_{36}O_7$, $[M^+ - 60]$), which indicate photogedunin acetate.

*Compound **3***. ^{1}H NMR assignments were all established. A doublet at δ 7.03 corresponding to the 23-H α to the 23-β-acetoxyl group was observed. Other recognizable signals included δ 5.60 (1H, H-17, d), 5.88 (H-2, d), 7.10 (1H, H-1, d), and 7.35 (1H, H-22, t). ^{13}C NMR chemical shift assignments (Table 2) were established rigorously through DEPT experiments and compared well with literature values.

*Compound **4***. ^{1}H NMR assignments were all established. A triplet at δ 6.91 corresponding to the 23-H β to the 23-α-acetoxyl group was observed. Other recognizable signals included δ 5.57 (1H, H-17, t), 5.87 (H-2, d), 7.10 (1H, H-1, d), and 7.34 (1H, H-22, t). ^{13}C NMR chemical shift assignments (Table 2) were established rigorously through DEPT experiments and compared well with literature values (Lopez, 1996; De Paula et al., 1997).

Light-Dependent Inhibition of Electron Transport Rate by Photogedunin Acetate Derivatives. Figure 3 shows that the inhibiting effects of photogedunin acetates **3** and **4** on the whole electron transport chain from water to MV, measured as oxygen uptake, are light-dependent, due to the fact that these compounds have no effect on thylakoid O_2-uptake in the first 3 min of illumination or 10 min of incubation in the dark as compared with a control (Figure 3, trace A versus trace B or C). Therefore, for all the experiments reported in this work chloroplasts were preilluminated for 3 min and then the effect of compounds studied was recorded. Figure 5 shows that β-photogedunin acetate (**3**) inhibits phosphorylating electron flow more strongly than **4** suggesting that besides inhibiting electron flow at the same percentage, both epimers may also inhibit the H^+-ATPase. The light-dependent inhibition of these compounds is similar to those found in some substituted 4-pyridone-3-carboxanilide derivatives (Osabe et al., 1992).

Effect of Photogedunin Acetates on ATP Formation. To understand the role of β-photogedunin acetate (**3**) and α-photogedunin acetate (**4**) on photosynthesis, their effects on different photosynthetic activities were tested. Photosynthetic phosphorylation from water to methyl viologen in freshly lysed intact spinach chloroplasts was inhibited by the tested photogedunin acetates **3** and **4** (Figure 4). Compounds **3** and **4** inhibited ATP synthesis in a concentration-dependent manner, reducing it by 98 and 96% at 35 μM, respectively. The acetoxy group at the C-23 position is an important structural requirement for the observed inhibitory effect on ATP synthesis, and this fact suggests that a hydroxyl group at this position significantly reduced the inhibitory potency of **3** and **4** on photophosphorylation.

Effect of Photogedunin Acetates 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 (Izawa and Good, 1972; Skulachev, 1998). To distinguish between these three possibilities, the effect of the photogedunin acetates on the light-dependent H^+-uptake and electron transport rate was tested. The results show that β-photogedunin acetate (**3**) inhibited light-dependent proton uptake completely at 32 μM (IC_{50} = 17 μM), but α-photogedunin acetate (**4**) had only a minor inhibiting effect, i.e., 33% at 32 μM (Figure 4), which suggest the importance of the stereochemistry of the configuration of acetoxyl group in both the diasteromeric epimers at C-23 for ATP inhibition.

Compound **3** is a more potent H^+-uptake inhibitor than **4**. These results also show that the target of **3** in the thylakoid membranes either is exposed in the energized state (basal condition), as indicated by the

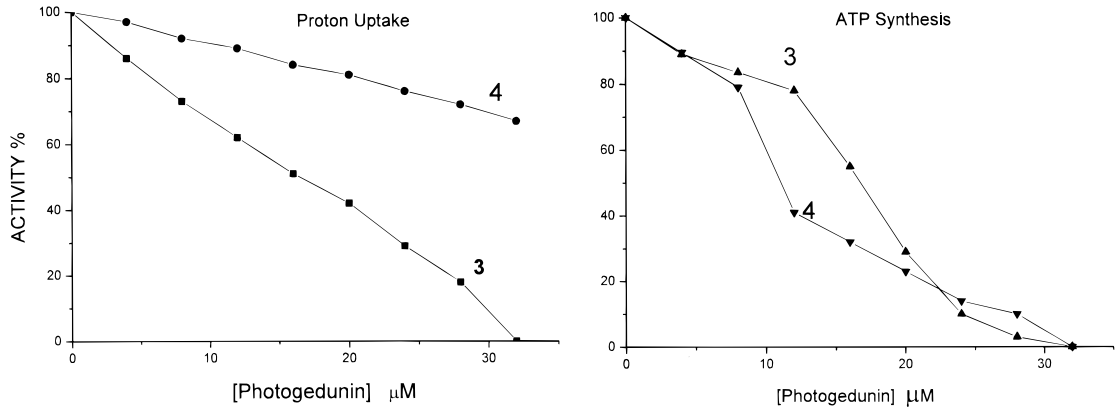

Figure 4. Rate of ATP synthesis and proton uptake as the concentrations of **3** and **4** increase.

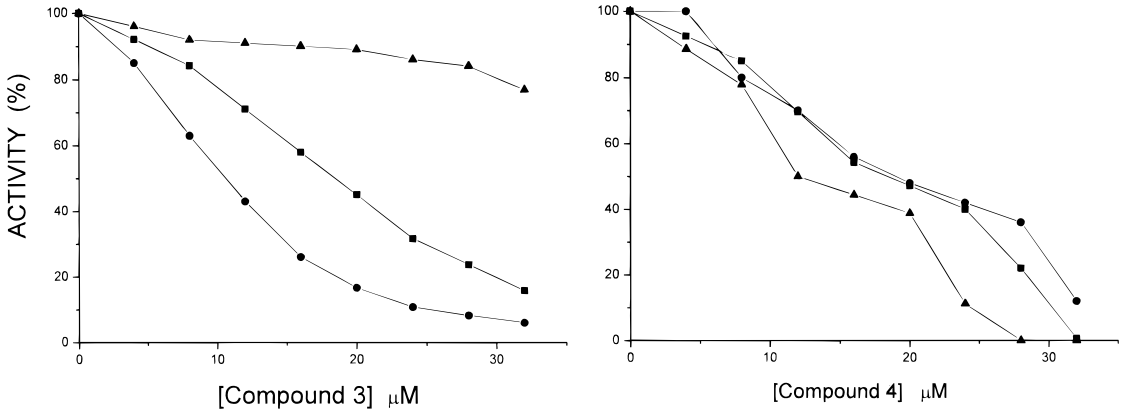

Figure 5. Effects of photogedunin acetates **3** and **4** on electron transport rate: ■, uncoupled; ●, phosphorylating; ▲, basal. Control rate values in μequiv e h^{-1} (mg of chl)$^{-1}$ are 200, 700, and 1100, respectively.

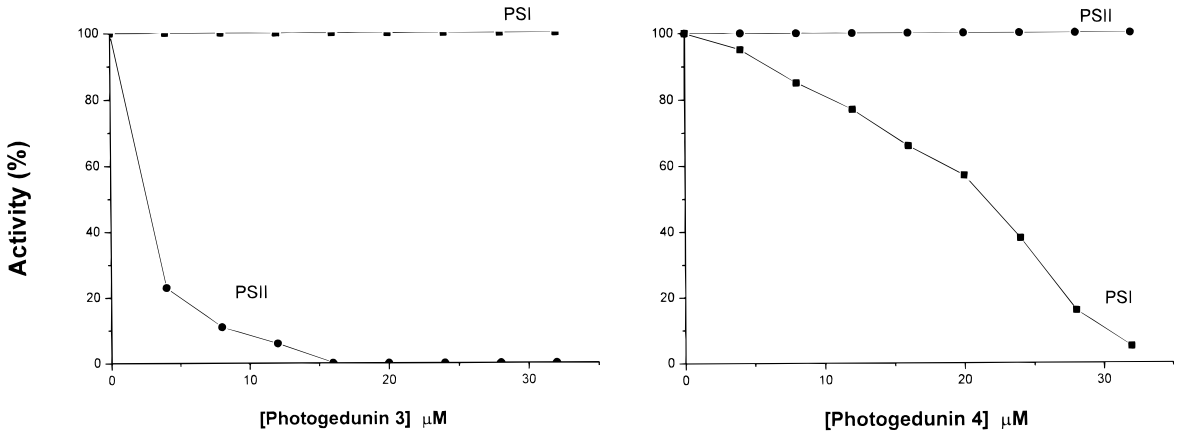

Figure 6. Behavior of photogedunin acetates **3** and **4** on the rate of PS I and PS II electron transport. Control rate values in values in μequiv e h^{-1} (mg of chl)$^{-1}$ are 1200 and 700, respectively.

lower CI_{50} value being obtained by this state for **3** than **4**, or the stereospecificity of C-23 position in the δ-lactone ring is important for inhibition H^+-uptake. According to Mitchell's transduction theory (Mitchell, 1961), H^+-uptake and ATP synthesis inhibition are expected to be affected in a stoichiometric manner when the tested compound behaves as an uncoupler, Hill reaction inhibitor, or in some cases behaves as energy transfer inhibitor. Figure 5 shows that the noncyclic electron transport from water to MV in both phosphorylating and uncoupled conditions was inhibited by addition of compounds **3** and **4**, phosphorylating electron flow being the most inhibited by **3**. On the other hand, basal electron transport was unaffected by **3** (Figure 5) but inhibited by **4**. The inhibition of electron flow (uncoupled and phosphorylating), ATP synthesis, and H^+-uptake from water to MV indicate that both epimeric compounds act as Hill reaction inhibitors. Noteworthy, **3** is sensitive to energization of the thylakoid membranes, since it only interacts with the electron transport chain, if it is de-energized (phosphorylation and uncoupled electron flow condition). The target of **3** is not exposed when the membrane is energized (basal electron flow conditions) due to the existence of a different conformational state of the thylakoid membranes in these two energy state conditions. However both compounds **3** and **4** have the same potency as Hill reaction inhibitors demonstrated by uncoupled electron flow inhibition (see Figure 5). This behavior of photogedunin acetate **4** is similar to that found for the herbicide DCMU and other urea type herbicides that interact with the D_1 protein (Calera et al., 1996; Bowyer and Camilleri, 1987), but it differs from DCMU herbicide behavior because photogedunin acetate derivatives require a 3 min of preillumination period before their inhibitory effect is observed. On the other hand, β-photogedunin acetate (**3**) also differs from DCMU herbicide compared with the urea type herbicide, since basal electron flow is unaffected by **3**. The I_{50} values for basal, uncoupled, and phosphorylating activities were 12, 18, and 19 μM, respectively, for α-photogedunin acetate (**4**) and for phosphorylating and uncoupled electron flow were 11 and 18.5 μM, respectively, for β-photogedunin acetate (**3**). Therefore, both epimers have a similar potency on Hill reaction inhibition.

Effect of Photogedunin Acetates 3 and 4 on PS II and PS I. To study the interaction site of compounds **3** and **4** on redox electron transport chain, the PS II and PS I and partial reactions on photosystems on broken spinach chloroplasts were assayed using artificial electron donors, acceptors, and inhibitors (Lotina-Hennsen et al., 1991; Calera et al., 1995a). The results show that **3** inhibits uncoupled PS II electron flow from H_2O to DCBQ, without affecting PS I electron transport from DCPIP/asc to MV, and uncoupled PS II from water to SiMo (Figures 6 and 7), indicating that **3** inhibits electron transport at the Q_B level, on the reducing side of PS II. The I_{50} is 3 μM for **3** on PS II electron flow. On the other hand, **4** inhibited totally the uncoupled PS I electron flow from DCPIP/Asc to MV, while it does not affect PS II electron transport (Figures 6 and 7). To localize the site of **4** inhibition on PSI redox enzymes, the partial reaction on PS I was investigated. It was found that the electron flow from PMS/Asc to MV and

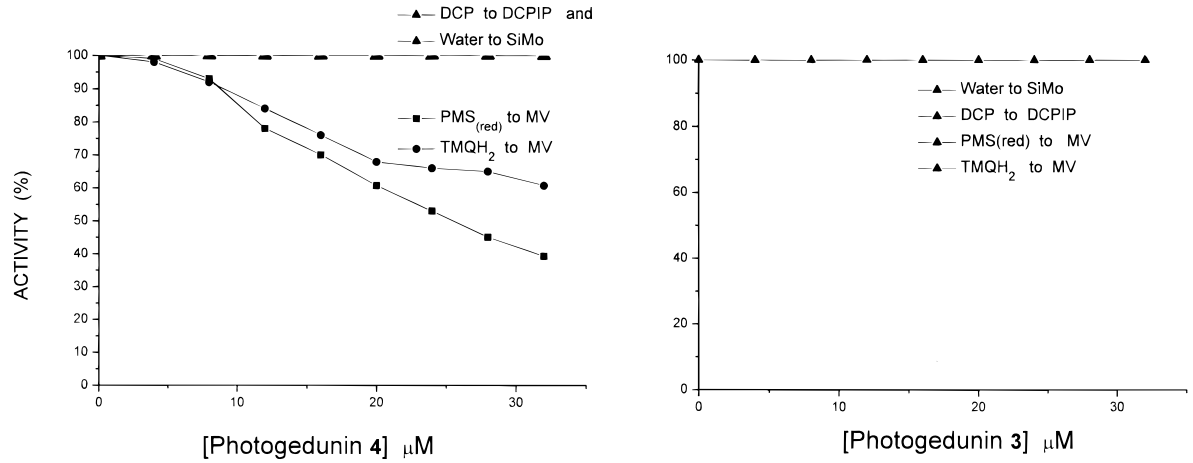

Figure 7. Effects of photogedunin acetates **3** and **4** on PS I and PS II partial reactions.

$TMQH_2$ to MV were partially inhibited by 60 and 40%, respectively, by 32 μM of α-photogedunin acetate (**4**) (Figure 7). The I_{50} for $TMQH_2$ to MV is 26 μM, and for PS I PMS/Asc to MV electron flow is not reached at 32 μM. These data suggest that **4** has two interaction targets, one is at the b_6f level, and the second one is at the span of PS I electron transport from P_{700} to F_x.

CONCLUDING REMARKS

We conclude that photogedunin acetates **3** and **4** are powerful Hill reaction inhibitors of electron transport chain in spinach thylakoids. To show inhibition on photosynthesis both compounds require a preillumination treatment; therefore they are photoactivable compounds. According to their pI_{50}, both compounds **3** and **4** have the same potency as Hill reaction inhibitors similar to some commercial herbicides that affect chloroplast electron flow. Also the pI_{50} values indicate that they are some of the most potent Hill reaction inhibitors, which suggests a role as allelochemicals.

The results show that photogedunin acetate **3** inhibits PS II at the level of Q_B and photogedunin acetate **4** presents one target localized at b_6f and the second site at the level of P_{700} to F_x which strengthens the importance of the configuration of the epimeric photogedunins. However, the significance of this finding in terms of allelochemicals remains undefined.

ABBREVIATIONS USED

Chl, chlorophyll; D_1, a 33 kDa polypeptide which contains the Q_B binding locus and helps form the PSII core; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, $DCIPH_2$, the oxidized and reduced forms, respectively, of 2,6-dichlorophenolindophenol; FeCN, ferricyanide; TLC, thin-layer chromatography; ^{1}H NMR, proton nuclear resonance spectroscopy; PQ, plastoquinone A; PS I, photosystem I; PS II, photosystem II; MV, methyl viologen; DPC, diphenylcarbazide; TMQ, $TMQH_2$, the oxidized and reduced forms respectively, of tetramethyl-*p*-benzoquinone; SiMo, silicomolybdate; PMS/asc, phenazine methosulfate/ascorbate; DCBQ, dichloride-*p*-benzoquinone; DAD, diaminedurene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); frs, fractions.

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