Sesquiterpenes from *Celastrus vulcanicola* as Photosynthetic Inhibitors

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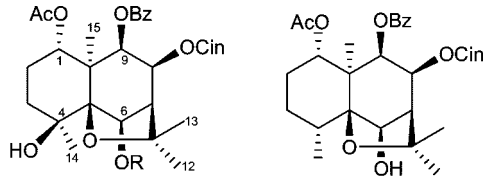
Three new sesquiterpenoids (**1**−**3**) with a dihydro-β-agarofuran skeleton were isolated from *Celastrus vulcanicola*. Their structures were elucidated on the basis of spectroscopic analysis, including homonuclear and heteronuclear correlation NMR experiments (COSY, ROESY, HSQC, and HMBC), and the absolute configurations were determined by circular dichroism and chemical correlations. Their effects on photosynthesis were tested. Sesquiterpene **1** (50 μM) inhibits both light-dependent synthesis of ATP and the electron flow in chloroplasts, whereas at high concentrations the electron flow inhibition was partially reversed. Therefore, **1** behaves as a Hill reaction inhibitor and a weak energy transfer inhibitor and has two targets of interaction: one located at the oxygen-evolving complex, and the other located at the light-activated Mg^{2+}-ATPase. Compound **2** was inactive, whereas **3** acts with the same mechanisms as **1** but was less active. *Celastrus vulcanicola* J. Donnell Smith (Celastraceae) is a subtropical woody vine distributed in Central America and the Caribbean. Its chemical constituents and biological activity have not yet been investigated.

Sesquiterpene esters that are based on a dihydro-β-agarofuran (5,11-epoxy-5β,10α-eudesm-4(14)-ene) skeleton are chemotaxonomic indicators of this family.[1] They have attracted much interest due to their broad spectrum of biological activities, which together with their structural characteristics have permitted dihydro-β-agarofuran sesquiterpenes to be considered as "privileged structures".[2]

As part of an intensive study of the bioactive metabolites from species of the Celastraceae family, the present investigation describes the isolation, structure elucidation, and photosynthetic inhibitory activity of three new sesquiterpenes isolated from *C. vulcanicola*. The structures were established by their physical, spectroscopic (UV, IR, ^{1}H and ^{13}C NMR, and 2D NMR experiments), and spectrometric (EIM and HEIMS) data. Their absolute configurations were determined by applying the exciton chirality method and chemical correlations.

Analysis of whether these sesquiterpenes participate in plant−plant interactions or have an effect on photosynthesis has not been carried out. Therefore, in a search for natural products that affect photosynthetic activities, these three new sesquiterpenes were studied. Compound **1** inhibits several photosynthetic activities, including ATP synthesis and electron flow at low concentrations. At high concentrations it acts as a weak energy transfer inhibitor. Our aim is to employ these sesquiterpenes as natural photosynthetic inhibitors that could assist in the development of "green herbicides".



Results and Discussion

Repeated chromatography of the dichloromethane extract of the leaves of *C. vulcanicola* on Sephadex LH-20 and Si gel afforded the new compounds **1**−**3**. Compound **1** showed the molecular formula $C_{33}H_{38}O_9$ by HREIMS and ^{1}H and ^{13}C NMR data. The IR spectrum showed absorption bands for hydroxy (3425 cm^{-1}) and ester (1724 cm^{-1}) groups. This was confirmed by the ^{1}H and ^{13}C NMR data (Table 1), which included signals for one acetyl group at δ 1.65 (3H, s) and 12 aromatic protons between δ 6.21 and 8.06 for one benzoyl and one *trans*-cinnamoyl group. The presence of two signals at δ 3.19 (1H, s) and 5.10 (1H, d, J = 5.3 Hz) in the ^{1}H NMR spectrum, interchangeable with D_2O, confirm the presence of two hydroxy groups. Two methylene systems at δ 1.50 (1H, m), 1.99 (1H, m) and 1.66 (1H, m), 1.95 (1H, m) were assigned to protons H-2, and H-3, respectively. Resonances were also observed for five methine protons, three forming an ABX system at δ 5.40 (d, J = 6.4 Hz, H-9), 5.55 (dd, J = 2.8, 6.4 Hz, H-8), and 2.58 (d, J = 2.8 Hz, H-7), an oxymethine proton at δ 4.53 (d, J = 5.3 Hz, H-6),[3] and the oxymethine proton of the acetoxy-bearing carbon at δ 5.36 (dd, J = 3.6, 12.3 Hz, H-1). A methyl group at δ 1.62 attached to an oxygen-bearing carbon at δ 72.8 and three methyls at δ 1.43, 1.59, and 1.74, which were confirmed from the ^{13}C NMR spectrum, were also observed. The chemicals shifts for the carbons attached to protons were assigned according to a 2D heteronuclear HSQC experiment. All these data indicate that **1** is a polyester sesquiterpene with a 1,4,6,8,9-pentasubstituted dihydro-β-agarofuran skeleton.

Regiosubstitution of **1** was determined by an HMBC experiment, showing a three-bond correlation between the acetoxy carbonyl resonance at δ_C 169.7 and the proton at δ_H 5.36 (H-1). The attachment of the benzoyloxy group with C-9 was defined by the cross-peak between the carboxyl resonance (δ_C 164.8) and H-9 at δ_H 5.55. The cinnamoyloxy group was located at position C-8 by correlation of the carboxyl resonance (δ_C 165.2) with H-8 (δ_H 5.40).

The relative configuration of **1** was established on the basis of the coupling constants and confirmed by a ROESY experiment (Figure 1). Therefore, in the COSY experiment the coupling constants of H_1−H_2 (J_{1-2} = 3.6, 12.3 Hz) and H_8−H_9 (J_{8-9} = 6.4 Hz) indicated an axial orientation for H-1 and a *cis*-relationship between H-8 and H-9. The ROESY experiment showed associations between H-1 and H-3 and between Me-15 and H-6, H-8, H-9, and Me-14.[2]

The absolute configuration of **1** was resolved by the dibenzoate chirality method, an extension of the circular dichroism exciton chirality procedure.[4] The dihedral angle (43.5°) between the two chromophores (benzoate and cinnamate) was calculated from J values and molecular mechanics calculations, using the PC model.[5]

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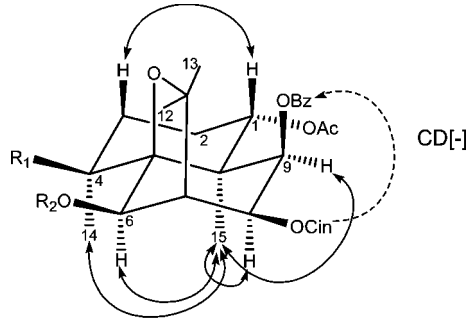
Table 1. $^1H-^{13}C$ NMR (δ, $CDCl_3$, J in Hz in parentheses) Data of Compounds **1**−**3**

	1		**2**		**3**	
position	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]
1	5.36 dd (3.6, 12.3)	72.1 d	5.37 dd (3.7, 12.0)	72.3 d*	6.04 dd (4.0, 11.9)	73.3 d
2	1.99 m, 1.50 m	22.9 t	1.93 m*, 1.51 m	23.3 t	2.15 m, 1.62 m	21.7 t
3	1.95 m, 1.66 m	36.9 t	1.93 m*, 1.70 m	38.6 t	2.40 m*, 1.38 m	26.6 t
4		72.8 s		70.5 s	2.40 m*	33.6 d
5		90.9 s		91.5 s		90.7 s
6	4.53 d (5.3)	78.0 d	5.65 s	77.2 d	3.86 s	75.7 d
7	2.58 d (2.8)	54.7 d	2.54 d (3.1)	53.9 d	2.39 d (2.6)	55.6 d
8	5.55 dd (2.8, 6.4)	69.2 d	5.71 dd (3.1, 6.3)	69.2 d	5.74 dd (2.6, 6.4)	69.8 d
9	5.40 d (6.4)	71.9 d	5.45 d (6.3)	72.3 d*	5.77 d (6.4)	72.9 d
10		48.7 s		50.0 s		48.4 s
11		84.5 s		84.7 s		82.4 s
12	1.74 s	27.0 c	1.72 s	26.5 c	1.97 s	26.8 c
13	1.59 s	30.4 c	1.57 s	30.3 c	1.79 s	31.2 c
14	1.62 s	23.5 c	1.34 s	23.9 c	1.06 d (7.3)	17.2 c
15	1.43 s	19.6 c	1.47 s	19.6 c	1.22 s	18.3 c
OAc-1	1.65 s	20.5 q 169.7 s	1.68 s	20.7 q 169.9 s	1.67 s	20.1 q 168.9 s
OAc-6				21.5 q 170.0 s		

[a] Based on DEPT and HSQC experiments. *Overlapping signals.



1 R_1 = OH; R_2 = H
2 R_1 = OH; R_2 = Ac
3 R_1 = H; R_2 = H

Figure 1. ROE effects (solid line) and CD exciton coupling (dashed line) for compounds **1**−**3**.

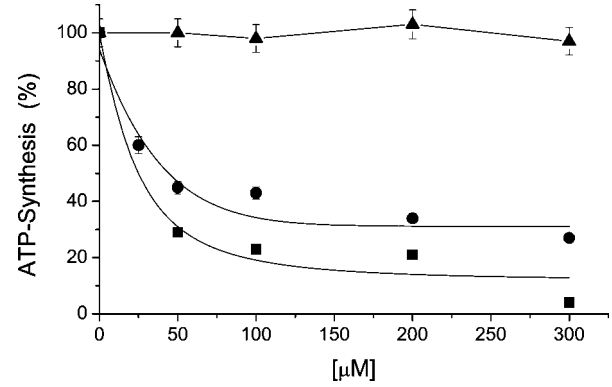


Figure 2. Inhibitory effect of sesquiterpenes **1** (■), **2** (▲), and **3** (●) on photophosphorylation from water to MV. Control rate value was 1190 μM ATP mg^{-1} Chl h^{-1}. Other conditions are as described in the Experimental Section. Data are average of three replicates.

Compound **1** was considered suitable for a CD study, showing a Davidoff-type split curve with a first negative Cotton effect at 266.2 nm ($\Delta\varepsilon$ −5.4) and a second positive effect at 224.0 nm ($\Delta\varepsilon$ +3.3), due to the coupling of the cinnamate and benzoate groups at C-8β and C-9β, respectively (Figure 1). Therefore, the absolute configuration of **1** was established as (1*S*,4*S*,5*S*,6*R*,7*S*,8*S*,9*R*,10*S*)-1-acetoxy-9-benzoyloxy-8-cinnamoyloxy-4,6-dihydroxydihydro-β-agarofuran.

The IR, UV, ^1H and ^{13}C NMR data (Table 1), and 2D of compound **2**, in conjunction with its molecular formula $C_{35}H_{40}O_{10}$ (HREIMS), indicated a dihydro-β-agarofuran sesquiterpene with two acetates, one benzoate, one cinnamate, and one tertiary hydroxy group, located at positions C-1α, C-4β, C-6β, C-8β, and C-9β, respectively. An HMBC experiment established the regiosubstitution patterns, and the relative configuration was solved by analysis of a ROESY experiment (Figure 1). These data revealed that **2** is the 6-*O*-acetyl derivative of **1**. Its absolute configuration was established by chemical correlation with **1**. Acetylation of **1** yielded a compound with spectroscopic data identical to those of **2**.

The structure of compound **3**, with the molecular formula $C_{33}H_{38}O_8$ (HREIMS), was elucidated by spectroscopic methods (Table 1). Its CD spectrum showed a split curve with the first Cotton effect at 265 nm ($\Delta\varepsilon = -5.1$) and the second one at 220 nm ($\Delta\varepsilon = +4.0$). Therefore, **3** was identified as (1*S*,4*R*,5*S*,6*R*,7*S*,8*S*,9*R*,10*S*)-1-acetoxy-9-benzoyloxy-8-cinnamoyloxy-6-hydroxydihydro-β-agarofuran.

The new compounds have the basic polyhydroxy dihydro-β-agarofuran sesquiterpenoid cores of 4β,8β-dihydroxycelorbicol (**1** and **2**) and 8β-hydroxycelorbicol[6] for compound **3**.

Like other natural sesquiterpenes,[7–10] compounds **1** and **3** inhibited the photosynthetic photophosphorylation in isolated spinach chloroplasts from water to MV (methyl viologen) in a concentration-dependent manner (Figure 2), with I_{50} values of 25 and 44 μM, respectively. Compound **2** did not have an effect on ATP formation; therefore, it was not further studied.

The inhibition of ATP synthesis can be explained by three different mechanisms: blocking electron transport, inhibiting the photophosphorylation reaction itself, and uncoupling ATP synthesis from the electron transport. In order to investigate the mechanism of action of **1** and **3**, their effect on noncyclic electron transport from water to MV was evaluated under basal, phosphorylation, and uncoupled conditions. Only data for compound **1** are presented, since compound **3** showed the same behavior but was less active. Figure 3 shows that **1** partially inhibited both the basal and phosphorylating electron transport at 50 μM by approximately 83% and 79%, respectively. However, at higher concentrations it partially reversed both electron transport rates, as the inhibition of both electron flows at 300 μM were 32% and 68%, respectively. Moreover, the uncoupled electron transport rate was inhibited by 26% at 50 μM (maximum inhibition was 52% at 300 μM) (Figure 3). Therefore, **1** has more than one mechanism of action, as a Hill reaction inhibitor and as an uncoupler agent, and its behavior is similar to other natural products, such as 3,4-*seco*-friedelan-3-oic acid, isolated from *Maytenus imbricata* (Celastraceae).[11]

To localize the target of inhibition on the electron transport chain, the effect of **1** on photosystems I (PSI) and II (PSII) and partial

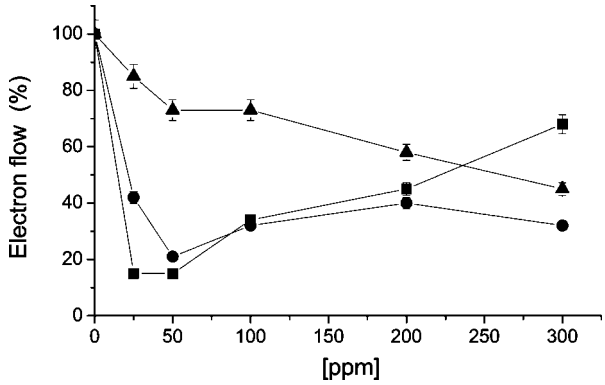


Figure 3. Effect of sesquiterpene **1** on electron flow from water to MV in basal (■), phosphorylating (●), and uncoupled (▲), respectively. Control rate values were 490, 688, and 1133 μequiv e^- h^{-1} mg^{-1} Chl. Other conditions are as described in the Experimental Section. Data are average of three replicates.

reactions were measured, using artificial electron donors and acceptors, as well as appropriate inhibitors.[12] The uncoupled PSI electron transport from reduced DCPIP (2,6-dichlorophenol indophenol) to MV was not affected, as the rate of uncoupled PSI electron flow was 1440 μequiv e^- h^{-1} mg^{-1} Chl, whether in the presence or absence of **1**. However, PSI electron transport from $TMQH_2$ (tetramethyl-*p*-benzohydroquinone) to MV was activated by 31% (Table 2), indicating its uncoupler activity. This compound also inhibited the uncoupled PSII electron transport (36% at 300 μM) from water to DCPIPox/K_3[Fe(CN)$_6$] in the presence of 1 μM DBMIB (2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone, a PSI electron flow inhibitor). It also inhibited the span of electron transport from water to SiMo by 89% at 300 μM (Table 2). However, the PSII electron flow from DPC (donates electron at P_{680}) to DCPIPox (accepted electrons at Q_B site) was not affected (data not shown). These results indicated the first target of **1** was the oxygen-evolving complex (OEC) of the PSII.

To corroborate the interaction site of **1** at PSII, the rapid polyphasic chlorophyll *a* (Chl *a*) fluorescence was measured on freshly lysed chloroplasts. These were incubated for 5 min in the dark at room temperature with different concentrations of **1**, and as positive controls 10 μM DCMU (an herbicide) and 0.8 M Tris were used (Table 3). When the thylakoids are treated with Tris, a well-known donor side inhibitor of PSII,[13] the fluorescence induction curve form is reduced. This results in a reduction of the maximum fluorescence yield and in a rapid rise to a maximum of around 300 μs (K-band) followed by a decrease to a level close to F_0.[14,15] Thylakoids treated with **1** showed a smaller K-band (Figure 4) and a partial decrease in the F_m value (the quantum yield of photochemistry at $F_0 = 0$) (Table 3); therefore, it acts as a weak OEC inhibitor.

Furthermore, the uncoupler activity displayed by **1** may be due to the effect on the enzyme Mg^{2+}-ATPase, as is the case in most classical uncoupler agents. Thus, the effect of **1** on the enzyme Mg^{2+}-ATPase bound to thylakoid membranes during the hydrolysis of ATP was studied. The results showed that H^+-ATPase activity was inhibited by 13% at 300 μM, suggesting that it acts as an energy transfer inhibitor. Thus, this indicates that its second target is the H^+-ATPase of the chloroplasts. NH_4Cl, an uncoupler used as positive control, enhances the Mg^{2+}-ATPase activity as shown in Table 4. H^+-ATPase of the chloroplast is also a target for other secondary metabolites, e.g., 7-oxo-7-deacetoxygedunin.[16]

The active compounds **1** and **3** have a hydroxy group at C-6, whereas the inactive compound **2** possesses an acetate group, suggesting the hydroxy group at this position is necessary for the inhibitory activity.

These sesquiterpenes affect photosynthesis, and it would be desirable to conduct further research in order to characterize their allelophatic behavior. In particular, compound **1** inhibits several photosynthetic activities, including ATP synthesis and electron flow at low concentrations, and acts as an uncoupler at high concentrations. Owing to the fact that natural products are biodegradable and because of their potential allelophatic behavior, our future aim is to employ these sesquiterpenes as natural photosynthetic inhibitors/uncouplers that could lead to the development of "green herbicides".

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter in $CHCl_3$ at 25 °C, and the $[\alpha]_D$ are given in 10^{-1} deg cm^2 g^{-1}. CD spectra were recorded in MeCN on a JASCO J-600 spectropolarimeter. UV spectra were obtained in absolute EtOH on a JASCO V-560 instrument. IR (film) spectra were measured in $CHCl_3$ on a Bruker IFS 55 spectrophotometer. ^1H and ^{13}C NMR spectra were performed on a Bruker Avance 400 at 400 and 100 MHz, respectively, and chemical shifts are shown in δ (ppm) with TMS as an internal reference. EIMS and HREIMS were recorded on a Micromass Autospec spectrometer. Silica gel 60 (15−40 μm) for column chromatography, Si gel 60 F_{254} for TLC, and nanosilica gel 60 F_{25} for preparative HPTLC were purchased from Macherey-Nagel, and Sephadex LH-20 was obtained from Pharmacia Biotech. Compounds used for CD were purified by HPTLC and eluted with a mixture of *n*-hexane−EtOAc (6:4).

Plant Material. *C. vulcanicola*, collected in June 2004 at the Montecristo National Park, province of Santa Ana, El Salvador, was identified by Jorge Monterrosa, and a voucher (J. Monterrosa & RCarballo 412) specimen is deposited in the Herbarium of Missouri Botanical Garden, USA. The dried leaves (1.5 kg) of *C. vulcanicola* were sliced into chips, extracted with EtOH in a Soxhlet apparatus, and concentrated under reduced pressure. The EtOH extract (311.0 g) was partitioned into CH_2Cl_2−H_2O (1:1, v/v) solution. Removal of the CH_2Cl_2 from the organic-soluble extract under reduced pressure yielded 74.8 g of residue, which was chromatographed on a Si gel column using mixtures of *n*-hexane−EtOAc of increasing polarity as eluant to afford seven fractions (A−G). Fraction D was subjected to column chromatography over Sephadex LH-20 using mixtures of CH_2Cl_2−MeOH (1:1) to provide two fractions (D1 and D2). Fraction D1 was further purified by Si gel column chromatography and preparative TLC developed with CH_2Cl_2−Me_2CO (10:1) to yield the new compounds **1** (515.4 mg), **2** (40.8 mg), and **3** (27.4 mg).

(1*S*,4*S*,5*S*,6*R*,7*S*,8*S*,9*R*,10*S*)-1-Acetoxy-9-benzoyloxy-8-cinnamoyloxy-4,6-dihydroxydihydro-β-agarofuran (1): colorless lacquer; $[\alpha]^{25}_D$ −50.3 (*c* 0.3, $CHCl_3$); CD λ_{ext} (MeCN) 266.2 ($\Delta\varepsilon = -5.4$), 232.2 ($\Delta\varepsilon = 0$), 224.0 ($\Delta\varepsilon = +3.3$) nm; UV λ_{max} (EtOH) (log ε) 280 (6.8), 225 (7.6) nm; IR ν_{max} (film) 3425, 2926, 2854, 1724, 1452, 1368, 1277, 1157, 1033, 968, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.19 (1H, s, OH-4), 5.10 (1H, d, $J = 5.3$ Hz, OH-6), 6.21 (1H, d, $J = 16.1$ Hz, OCin), OCin, OBz [7.32 (4H, m), 7.47 (3H, m), 7.60 (2H, m), 8.06 (2H, m)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OCin, OBz [117.4 (d), 127.9 (2 × d), 128.2 (2 × d), 128.5 (2 × d), 129.1 (s), 130.0 (2 × d), 130.2 (d), 133.1 (d), 133.8 (s), 145.3 (d)], 164.8 (s, OCin-8), 165.2 (s, OBz-9), for other signals, see Table 1; EIMS *m/z* (%) 563 [M − 15]$^+$ (10), 545 (21), 518 (1), 441 (9), 430 (2), 248 (5), 202 (13), 166 (6), 131 (91), 105 (100); HREIMS *m/z* 563.2241 [M − CH_3]$^+$ (calc for $C_{32}H_{35}O_9$, 563.2281).

(1*S*,4*S*,5*S*,6*R*,7*R*,8*S*,9*R*,10*S*)-1,6-Diacetoxy-9-benzoyloxy-8-cinnamoyloxy-4-hydroxydihydro-β-agarofuran (2): colorless lacquer; $[\alpha]^{25}_D$ −92.7 (*c* 0.6, $CHCl_3$); UV λ_{max} (log ε) (EtOH) 280 (6.4), 223 (7.0) nm; IR ν_{max} (film) 3555, 3025, 2936, 1736, 1636, 1368, 1278, 1234, 1097, 757, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.85 (1H, s, OH-4); 6.20 (1H, d, $J = 16.0$ Hz, OCin); OCin, OBz [7.33 (4H, m), 7.44 (3H, m), 7.59 (2H, m), 8.06 (2H, m)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OCin, OBz [117.2 (d), 128.1 (2 × d), 128.4 (2 × d), 128.7 (2 × d), 129.5 (s), 130.2 (2 × d), 130.4 (d), 133.3 (d), 134.1 (s), 145.6 (d)], 164.9 (s, OCin-8), 165.5 (s, OBz-9), for other signals, see Table 1; EIMS *m/z* (%) 605 [M$^+$ − 15] (3), 560 (8), 518 (2), 483 (13), 472 (4), 423 (2), 293 (1), 131 (97), 105 (100); HREIMS *m/z* 605.2396 [M − CH_3]$^+$ (calcd for $C_{34}H_{37}O_{10}$, 605.2387).

(1*S*,4*R*,5*S*,6*R*,7*S*,8*S*,9*R*,10*S*)-1-Acetoxy-9-benzoyloxy-8-cinnamoyloxy-6-hydroxydihydro-β-agarofuran (3): colorless lacquer; $[\alpha]^{25}_D$ −40.0 (*c* 0.2, $CHCl_3$); CD λ_{ext} (MeCN) 265 ($\Delta\varepsilon = -5.1$), 230.4 ($\Delta\varepsilon = 0$), 220 ($\Delta\varepsilon = +4.0$) nm; UV λ_{max} (EtOH) (log ε) 279 (6.1), 223

Table 2. Effect of **1** on Uncoupled PSII, PSI Electron Transport Rate and PSII Partial Reactions

	PS II		PS II		PS I		PSI	
	H_2O to DCPIP		H_2O to SiMo		$TMQH_2$ to MV		$DCPIP_{red}$ to MV	
concentration, μM	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0	480	100	190	100	1100	100	1440	100
50	446	93	60	32	1265	115	1520	106
100	384	80	60	32	1419	129	1440	100
200	341	71	40	21	1320	120	1440	100
300	307	64	20	11	1441	131	1440	100

[a] Rate values have the photosystems and partial reactions in μequiv e^- h^{-1} mg^{-1} Chl. [b] 100% is the activity of the control.

Table 3. Effect of **1** and 10 μM DCMU on Fluorescence Parameters on Thylakoids[a]

compound	F_0	F_m	F_V/F_m	area
control	272	1366	0.801	19 800
DCMU 10 μM	310	1198	0.741	1200
Tris 0.8 M	227	527	0.569	0
1				
150 μM	251	1217	0.794	10 400
300 μM	265	1261	0.790	11 800
600 μM	183	875	0.791	6000

[a] Thylakoids were previously incubated for 5 min in the dark with 0.8 M Tris, pH 8.0.

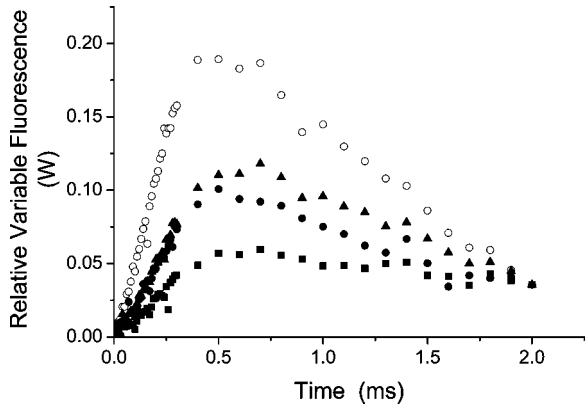


Figure 4. Appearance of the K-band at about 300 μM. Difference of each curve from the control with normalized relative variable fluorescence on the amplitude $F_j - F_0$. **1** at 150 (■), 300 (●), 600 μM (▲) and chloroplasts incubated with 0.8 M Tris, which inhibits OEC activity (○). Data are average of three replicates.

Table 4. Effect of **1** and NH_4Cl on the Light-Activated Thylakoids Membrane-Bound Mg^{2+}-ATPase

compound	μM Pi released/mg Chl h	(%)
1 (μM)		
0	320	100
100	291	91
200	285	89
300	277	87
NH_4Cl (mM)		
0	320	100
1	746	233
3	819	256
6	704	220

(7.9) nm; IR ν_{max} (film) 3506, 3025, 2927, 1720, 1636, 1452, 1386, 1367, 1281, 1166, 1101, 1027, 964, 759, 713 cm^{-1}; ^1H NMR (C_6D_6) δ OCin [6.48 (1H, d, J = 16.0 Hz), 7.72 (1H, d, J = 16.0 Hz)], OCin, OBz [6.97 (4H, m), 7.05 (1H, m), 7.16 (3H, m), 8.45 (2H, m)], for other signals, see Table 1; ^{13}C NMR (C_6D_6) δ OCin, OBz [117.5 (d), 127.7 (2 × d), 128.0 (2 × d), 128.2 (2 × d), 129.7 (2 × d), 129.9 (s), 130.2 (2 × d), 132.6 (d), 133.9 (s), 145.1 (d)], 164.8 (s, OCin-8), 165.7 (s, OBz-9), for other signals, see Table 1; EIMS *m/z* (%) 562 $[M]^+$ (2), 547 (22), 502 (2), 487 (9), 440 (1), 414 (2), 412 (2), 339 (9), 249 (4), 131 (100), 105 (84); HREIMS *m/z* 562.2585 (calcd for $C_{33}H_{38}O_8$, 562.2567).

Acetylation of 1. A mixture of Ac_2O (4 drops), compound **1** (12.3 mg), and DMAP (4.0 mg) in Py (10 drops) was stirred at room temperature for 16 h. The mixture was evaporated to dryness, and the residue was purified by preparative TLC (CH_2Cl_2−Me_2CO, 9:1) as eluant to yield a product (8.3 mg), whose spectroscopic data were identical to those of **2**.

Chloroplast Isolation and Total Chlorophyll Determination. Intact chloroplasts were isolated from spinach leaves (*Spinaceae oleraceae* L.) obtained from a local market,[17] and total chlorophyll concentration was measured spectrophotometrically as previously described.[18]

ATP Synthesis. The ATP formation was determined by the procedure of Dilley.[19] The 0.05 mM MV (methyl viologen) was employed as electron acceptor for the Hill reaction. Intact chloroplasts (20 μg chlorophyll/mL) were broken by osmotic rupture in nonbuffered solution with 100 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, and 0.001 M Na^+-tricine, and 1 mM ADP and 3 mM Pi were added for phosphorylation conditions.[20] The linear alkalization in light was calibrated by back-titration with saturated HCl, and the ATP formed (μmol ATP mg Chl^{-1} h^{-1}) was calculated. This ATP synthesis was determined titrimetrically using a microlelectrode (Orion Mod. 8103 Ross) connected to a Corning potentiometer (model 12) with expanded scale.

Electron Transport Determination. Light-induced noncyclic electron transport activity from water to MV was monitored with a Yellow Springs Instrument model 5300 oxygen monitor and a Clark type electrode. Basal electron transport was determined by illuminating chloroplasts (20 μg chlorophyll per mL) during 1 min in the basal electron transport medium (3 mL).[21] Photophosphorylating noncyclic electron transport was measured as basal noncyclic electron transport except that 1 mM ADP and 3 mM Pi were added. Uncoupled electron transport was tested in the basal electron transport; medium and 6 mM NH_4Cl were added as uncoupler. The reaction mixture was illuminated with actinic light of a projector lamp (Gaf 2669) passed through a 5 cm filter of a 1% $CuSO_4$ solution for 2 min.[22]

Uncoupled Photosystem II and Photosystem I Activity Measurements. Uncoupled PSII from water to DCPIP was measured by the reduction of DCPIP-supported O_2 evolution, monitored polarographically.[20,21] In the basal electron transport medium (3 mL), 300 μM $K_3[Fe(CN)_6]$ was used instead of MV, and the following reagents were added: 1 μM DBMIB, 50 μM DCPIP, and 6 mM NH_4Cl. The partial PSII reaction of uncoupled electron transport from water to SiMo (sodium silicomolybdate) was determined with the same reaction mixture as uncoupled electron flow without MV, except that 200 μM SiMo and 10 μM DCMU ([3-(3,4-dichlorophenyl)-1,1-dimethylurea]) were added.[23] Uncoupled PSII electron transport rate from DPC (diphenyl carbazide) to DCPIP was determined at 600 nm, using a spectrophotometer Beckman DU 650. PSII activity was determined in thylakoids, previously treated with 0.8 M Tris [(hydroxymethyl)aminomethane] (pH 8.0) and incubated for 30 min at 4 °C.[24,25] The reaction medium contained 200 mM DCP, 100 mM DCPIP, 1 mM DBMIB, and 6 mM NH_4Cl.

PSI electron flow from cyt b_6f to MV was determined using 3 mL of the basal uncoupled electron flow medium. To this medium were added 100 μM DCPIP (reduced with 300 μM sodium ascorbate) as electron donor, 10 μM DCMU to inhibit PSII electron flow, 6 mM NH_4Cl as uncoupler, and 0.05 mM MV as electron acceptor. The uncoupled PSI electron flow from tetramethyl-*p*-benzohydroquinone ($TMQH_2$) to MV was determined polarographically,[26,27] adding 100 μM $TMQH_2$/300 μM ascorbate, 10 μM DCMU, and 6 mM NH_4Cl to 3 mL of the basal electron medium. The temperature was 20 °C, and for each reaction a blank experiment was performed with the chloroplasts alone in the reaction medium. The IC_{50} value for each activity

was determined from plots of the activity in the presence of each compound at different concentrations. IC_{50} is the concentration producing 50% inhibition.

Chlorophyll *a* Fluorescence Measurements. The rapid polyphasic chlorophyll *a* fluorescence kinetics of PSII was measured at room temperature with a Hansatech Handy PEA (plant efficient analyzer) in 5 min dark-adapted chloroplasts (20 μg Chl mL^{-1}).[13] 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 (3 mL) was the same as that employed in basal noncyclic electron transport measurements.

Mg^{2+}-ATPase Activity Assays. Mg^{2+}-ATPase activity bound to thylakoid membranes was measured according to Mills.[17] The amount of released inorganic phosphate was measured by colorimetric determination as described by Sumner.[28]

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