Phytotoxic and Photosynthetic Activities of Maduramicin and Maduramicin Methyl Ester

Maria Teresa Gutiérrez-Lugo[a], Blas Lotina-Hennsen[b], Amelia Farrés[c], Sergio Sánchez[d] and Rachel Mata[a]

[a] Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, Coyoacán 04510, México, D. F., México
[b] Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, Coyoacán 04510, México, D. F., México
[c] Departamento de Biotecnología, Facultad de Química, Universidad Nacional Autónoma de México, Coyoacán 04510, México, D. F., México
[d] Departamento de Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Coyoacán 04510, México, D. F., México

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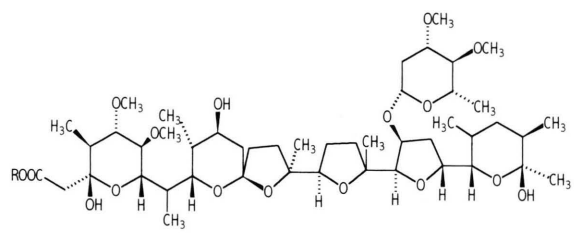
Maduramicin, Photophosphorylation, Uncoupler, Phytotoxicity, Polyether Antibiotic

The polyether antibiotic maduramicin and its methyl ester derivative inhibited photophosphorylation and proton uptake in isolated spinach chloroplasts. Both compounds also enhanced basal and phosphorylating electron transport and stimulated Mg^{2+}-dependent ATPase activity, therefore, they behave as uncouplers of photophosphorylation being the methyl ester derivative more potent than the parent compound. On the other hand, maduramicin inhibited germination and radicle elongation of several crop and weed species. In addition, the antibiotic caused phytotoxic injury and fresh weight reduction to 4-to-6 week old seedlings of two weed and two crop species when applied at 10^{-4} M by foliar application in the greenhouse.

Introduction

In the course of our search for potential herbicidal agents from natural sources we have isolated maduramicin (**1**) in high yields from the actinomycete MIV2B(31) obtained from a soil sample collected in a volcanic area of the State of Baja California, Mexico.

Maduramicin is a polyether antibiotic that belongs to the lipophilic class of antibiotics known as ionophores. These compounds form electrically neutral complexes with monovalent and divalent cations. The lipophilic character of these antibiotics render them soluble in lipid components and they catalyze electrically silent exchanges of cations for protons or other cations across a variety of biological membranes. The ionophorous nature of these antibiotics results in a wide range of biological activities (Reed, 1982).



1, R=Na
1a, R=CH_3

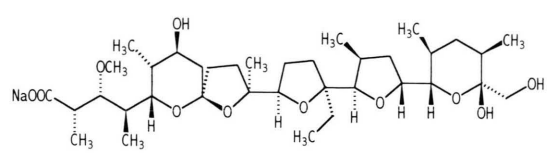


Fig. 1. Chemical structures of maduramicin sodium salt **1**, maduramicin methyl ester **1a** and monesin sodium salt **2**.

Reprint requests to Dr. Lotina-Hennsen or Dr. Rachel Mata.
Fax: (525) 622 53 29
E-mail: blas@servidor.unam.mx
rachel@servidor.unam.mx

Maduramicin is an antibiotic of significant commercial importance to the poultry industry for combating coccidiosis; the ammonium salt of the free acid of maduramicin is marketed as Cygro®, this product has shown to be more active than other

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polyether antibiotics currently used to control poultry coccidia (Liu *et al.*, 1981; 1983; Labeda *et al.*, 1983).

Studies related with the herbicidal effects of polyether antibiotics have shown that some of them such as nigericin and monesin possess pre or post-emergent herbicidal activity to several weed and crop species (Heysey and Putnam, 1986; 1990; Hoagland, 1996). It has been also reported that monesin, nigericin (X-464), lasalocid A, antibiotic X-206, dianemycin and laidlomycin inhibit photophosphorylation in isolated chloroplasts by uncoupling synthesis of ATP from electron flow (Shavit *et al.*, 1967; 1970, Kida and Shibai, 1986). It was found that the dissipation of the proton gradient occurs via K^+/H^+ exchange or antiport (Shavit *et al.*, 1970).

Nigericin has been shown to stimulate ATPase activity in microsomal vesicles of tobacco callus (Sze, 1980). The antibiotic also affect the kinetics of ATP synthesis in heart submitochondrial particles (Matsuno-Yagi and Hatefi, 1989) and induce the release of protons from thylakoid lumen (Yamasaki *et al.*, 1991).

As monesin, maduramicin is introduced into environment via animal excreta from extensive use as an antibiotic in broiler chickens and turkeys (McDougald *et al.*, 1990). However, the effect on this ionophore on plant growth has not been investigated. In this context, the objective of this research was to explore the pre and post-emergent phytotoxic properties of maduramicin. The pre-emergent phytotoxic activity was investigated by determining the effect of the antibiotic on germination and radicle growth of five plant species. The post-emergent phytotoxic activity was established by evaluating the effect of foliar application of maduramicin to 4-to-6 week old seedlings of two weed and two crop plant species. In addition, the effect of maduramicin and its methyl ester on several photosynthetic activities associated with the light reaction phase including ATP synthesis, proton uptake, electron transport (basal, phosphorylating and uncoupled conditions) and Mg^{2+}-ATPase activity, was investigated.

Materials and Methods

General

IR spectra were obtained in $CHCl_3$ on a Perkin-Elmer 599B spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR-500S spectrometer. Melting points were determined in a Fisher Johns apparatus and are uncorrected. FAB-mass spectra were obtained with a JEOL DX 300, JMA 3500 system mass spectrometer using a NBA matrix. The X ray-analysis was performed on a Nicolet R3m.

Analytical and preparative TLC were performed on pre-coated silica gel 60 F254 plates (Merck). TLC spots were visualized by spraying with a 3% solution of vainillin-H_2SO_4 reagent followed by heating at 110 °C.

A Beckman model DU 650 spectrophotometer was used to carry out the spectrophotometric measurements in the photosynthesis experiments.

HPLC was performed on a Waters HPLC Instrument equipped with a Waters refractive index detector using a microporasil silica gel 125 A column (10 μm 19 id X 300 mm, Waters). Control of the equipment, data acquisition, processing and management of chromatographic information were performed by the Millennium 2000 software program (Waters).

Producing organism

Actinomycete MIV2B(31) was isolated from a soil sample collected in the volcanic area of Santa Rosalía, State of Baja California, Mexico. The culture has been deposited at Instituto de Investigaciones Biomédicas Culture Collection, Universidad Nacional Autónoma de México, D. F.

Fermentation extraction and preliminary fractionation

Fermentation of actinomycete MIV2B(31) was carried out at 29 °C in a 14 liter fermentor (Drive assembly, New Brunswick Scientific) with agitation (400 rpm) in Arcamone medium consisting of glucose 2.0%, beer yeast 1.25%, NaCl 0.2%, $CaCO_3$ 0.2%, $MgSO_4$ 0.1%, $ZnSO_4$ 0.001%, $FeS0_4$ 0.001% pH 7 (NaOH 1 M) for six days. The filtrate of the fermentation broth (8 l) was extracted twice with 8 l of ethyl acetate and evaporated *in vacuo* to yield 6.3 g of residue. The resulting extract was evaluated for their ability to inhibit both radicle elongation of *Amarathus hypochondriacus* and ATP synthesis in spinach chloroplasts. The active extract was fractionated by column chromatography on silica gel (Merck 60, 0.63–0.2 mm) and

eluted with a gradient of chloroform-methanol starting with chloroform only. Twenty-six fractions were collected and pooled on the basis of their TLC profiles to yield four mayor fractions (FI-FIV). According to the bioautographic phytogrowth inhibitory bioassay the phytotoxic activity was concentrated in FII (5.35 g) and FIII (0.429 g).

Isolation of maduramicin

Antibiotic **1** was isolated from fraction FII by HPLC chromatography using as mobile phase hexane:2-propanol:methanol (89:5.5:5.5 v/v) and a flow rate of 8.3 ml min^{-1}. The compound was recrystallised from acetone and spectroscopic and spectrometric constants were determined.

Synthesis of maduramicin methyl ester derivative (**1a**)

A solution of maduramicin free acid (200 mg) in ether was allowed to react with ethereal diazomethane until nitrogen evolution ceased and the yellow color persisted (Evans *et al.*, 1988). After additional 30 min, the solution was concentrated and the residue was purified by preparative thin layer chromatography. The elution was accomplished with hexane:acetone 7:3 v/v to yield 50 mg (25%) of **1a** as a white solid. **1a**: mp: 112 °C; FAB^+-EM, *m/z* (relative intensity) 953 (11), 895 (4), 737 (7), 719 (11) and 59 (100); IR $\nu_{max.}$ ($CHCl_3$) 2976, 2935, 1715, 1459, 1377, 1096 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.41 (t, 1H, *J*= 4.0 Hz; H-22), 4.4 (dd, 1H, *J*= 5.0 and 2.0 Hz; H-24), 4.39 (dd, 1H, *J*= 9.75 and 2.0 Hz; H-1'), 4.21 (dd, 1H, *J*= 10.75 and 2.0 Hz; H-9), 4.1 (d, 1H, *J*= 3.5 Hz; H-21), 3.95 (dd, 1H, *J*= 10.75 and 2.0 Hz; H-25), 3.94 (d, 1H, *J*= 2.5 Hz; H-11), 3.7 (dd, 1H, *J*= 9.5 and 3.0 Hz; H-7), 3.58 (s, 3H, OMe-4), 3.55 (s, 6H, OMe-5 OMe-6), 3.54 (s, 3H, OMe-1), 3.41 (s, 3H, OMe-3'), 3.34 (dd, 1H, *J*= 10.25 and 8.5 Hz; H-5'), 3.27 (ddd, 1H, *J*=, 11.5, 8.5 and 5.5Hz; H-3'), 3.18 (m, 1H, H-5'), 3.14 (dd, 1H, *J*= 9.5 and 8.5 Hz; H-6), 2.72 (t, 1H, *J*= 9.0 Hz; H-4'), 2.5 (d, 1H, *J*= 12.5 Hz; Ha-2), 2.16 (d, 1H, *J*= 12.5 Hz; Hb-2), 1.89 (dd, 1H, *J*= 14.5 and 3.5 Hz; Ha-12), 1.62 (dd, 1H, *J*= 14.5 and 2.0 Hz; Hb-12), 1.49 (s, 3H, Me-16), 1.29 (s, 3H, Me-30), 1.27 (d, 3H, *J*= 6.0, Me-5'), 1.21 (s, 3H, Me-20), 1.05 (d, 3H, *J*= 6.5, Me-8), 1.01 (d, 3H, *J*= 6.5, Me-4), 0.92 (d, 3H, *J*= 6.0, Me-28), 0.86 (d, 3H, *J*= 6.5, Me-26), 0.85 (d, 3H, *J*= 6.0, Me-10). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 172.6 (C-1), 107.5 (C-13), 97.74 (C-29), 96.0 (C-3), 95.85 (C-30), 86.2 (C-21), 85.0 (C-5), 84.9 (C-16), 83.0 (C-20), 82.0 (C-17), 81.6 (C-6), 78.0 (C-24), 75.6 (C-22), 73.6 (C-25), 69.46 (C-11), 68.34 (C-9), 68.3 (C-7), 60.76 (OMe-C-4'), 60.75 (OMe-C-5), 60.23 (OMe-C-1), 60.22 (OMe-C-6), 56.81 (OMe-C-3'), 45.6 (C-4), 43.14 (C-2), 39.7 (C-28), 38.7 (C-14), 36.6 (C-27) 34.2 (C-15), 33.6 (C-10), 33.35 (C-12), 33.34 (C-8), 33.34 (C-26), 31.91 (C-19), 31.8 (C-23), 28.49, (Me-C-6), 27.1 (C-18), 23.28 (Me-C-20), 17.96 (Me-C-5'), 17.8 (Me-C-26), 14.6 (Me-C-28), 11.96 (Me-C-4), 10.54 (Me-C-8), 10.54 (Me-C-10).

Seed sources and plant growth

Seeds of three weed and two crop species were used to determine the effect of the antibiotics on germination, radicle elongation and biomass production. Seeds of *Amaranthus hypochondriacus* (amaranthus) and *Triticum vulgare* (wheat) were obtained from commercial sources. The seeds of *Echinochloa crus-galli* (barn-yard grass), *Trifolium alexandrinum* (clover) and *Phaseolus aureus* (mung bean) were purchased from Valley Seed Service, Fresno, CA.

Plant growth for biomass production determination was achieved as previously reported (Chen *et al.*, 1991; Hoagland, 1996). Plants were grown in a 80% RH growth chamber for 4-to-6 weeks (12 h, 28 °C light: dark 300 $\mu Em^{-2}\ s^{-1}$ photosynthetically active radiation) before treatment.

Determination of the effect of maduramicin on seed germination and radicle growth

The determination of the effect of the extract and antibiotics **1** and **2** on germination and radicle elongation was evaluated using the Petri dish phytogrowth-inhibitory bioassay (Anaya *et al.*, 1991). The antibiotics were tested at different concentrations (5×10^{-4}, 5×10^{-5} and 5×10^{-6} M). The data were analyzed by ANOVA ($P<0.05$) and IC_{50} (concentration causing 50% reduction of radicle growth) values were calculated by Probit analysis based on percent inhibition obtained. Monesin and 2,4-D (2,4-dichlorophenoxy acetic acid) were used as positive controls. In addition, a direct bioautographic bioassay system was employed to con-

trol fractionation and speed up the isolation of active compound (Castañeda *et al.*, 1996).

Determination of the effect of maduramicin on fresh weight and shoot elongation of plant seedlings

The determination of the effect of **1** on fresh weight and shoot elongation of four plant species was performed as described Hoagland (1996). Three trays of each species (4-to-6 week old) consisting of 6–8 seedlings each were treated foliarly with maduramicin solution (4×10^{-4} M) containing Tween 80 (0.1 ml litre^{-1}) which was used as detergent. Foliar applications were made using a cotton swab and foliage was applied to the point of runoff. In each case Tween 80 (0.1 ml litre^{-1}) and paraquat (10^{-4} M) were used as controls. Visual damage in the foliage was observed after one month of treatment. All experiments were based on a complete random design with a factorial structure. Data on plant elongation and fresh weight were subjected to ANOVA and mean comparisons performed using Fisher's protected LSD.

Chloroplasts isolation and chlorophyll determination

Chloroplast thylakoids were isolated from market spinach leaves (*Spinacea oleracea* L.) as previously described (Calera *et al.*, 1996) and suspended, unless otherwise indicated, in 3 ml of a medium composed of 400 mM sucrose, 5 mM $MgCl_2 \cdot 6H_2O$, 20 mM KCl and buffered with 0.030 M sodium N-tri-(hydroxymethyl)-methyl glycine (Na^+ tricine) at pH 8.0 (KOH 1M). Chlorophyll concentration was measured spectrophotometrically as described by Strain *et al.* (1971).

Measurement of proton uptake, ATP synthesis and electron transport

Proton uptake was measured as the pH value increased between 8.0 and 8.1 (Dilley, 1972) using a combination microelectrode connected to a Corning Potentiometer with expanded scale. The pH changes were registered using a Gilson recorded. The reaction medium was 100 mM sucrose, 5 mM $MgCl_2 \cdot 6H_2O$, 10 mM KCl, 1 mM Na^+ tricine, pH 8 (KOH, 1M). ATP synthesis and non-cyclic electron transport were determined as previously described (Calera *et al.*, 1995; 1996. Mills *et al.*, 1980; Dilley, 1972). Methylviologen (MV) (0.05 mM) was employed as electron acceptor for the Hill reaction.

Light-induced non-cyclic electron transport in the presence of MV was monitored with a YSI (Yellow Springs Instrument C) model 5300 oxygen monitor using a Clark electrode in a temperature regulated flask at 20 °C. The reaction medium was the same as proton uptake assay except for the Na^+ tricine concentration (15 mM) and the presence or absence of 6 mM NH_4Cl (Calera *et al.*,1995; 1996). The results are expressed as percent of activity compared to control (chloroplasts without antibiotic addition). Monesin was used as positive control in all determinations.

Mg^{2+}-*ATPase isolation and assay*

Intact chloroplast isolated from spinach leaves were suspended in 35 mM sorbitol, 2 mM ethylenediaminetetraacetic acid, 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.6. The membrane bound Mg^{2+}-ATPase activity was determined using the technique reported by Mills *et al.* (1980). Monesin and ammonium chloride were used as positive controls. In each reaction, a blank experiment was performed with the isolated chloroplasts in the reaction medium. All reactions were conducted by triplicate and the data analyzed by ANOVA. The percent of activity of the enzyme was calculated comparing the rate values observed in each experiment with that of the control (100% of activity).

Results and Discussion

Isolation and identification of maduramicin

The organic extract derived from the fermentation broth of the actinomycete MIV2B(31) inhibited both radicle elongation of *A. hypochondriacus* (IC_{50} 58.4 μg ml^{-1}) and photophosphorylation in spinach chloroplast (IC_{50} 42.1 μg ml^{-1}).

Bioassay guided fractionation of the active extract using the bioautographic phytogrowth inhibitory bioassay (Castañeda *et al.*, 1996) allowed the isolation of maduramicin (Liu *et al.* 1981, 1983; Labeda *et al.*, 1983), as the active compound. This product was identified by comparison of IR, RMN and EM data of its sodium salt with the informa-

tion previously described in the literature and by X-ray analysis (Westley *et al.*, 1982; Liu *et al.*, 1983; Rajan *et al.*, 1984; Ellestad *et al.*, 1986; Siegel *et al.*, 1987).

Effect of maduramicin on seed germination and radicle growth

Compound **1** was evaluated for its ability to inhibit seed germination and radicle growth of *A. hypochondriacus, E. crus-galli, T. alexandrinum*, *P. aureus* and *T. vulgare*. Table I summarizes the phytotoxic effect of the antibiotic (**1**) which was more potent as growth inhibitor than as germination inhibitor. The weeds *A. hypochondriacus*, *E. crus-galli* and *T. alexandrinum* were more sensitive to the treatment with **1**. It is important to notice that the effect of **1** on *A. hypochondriacus* and *E. crus-galli* was comparable to that of 2,4-D. On the other hand, the seedlings of *P. aureus* and *T. vulgare* were less sensitive to maduramicin, thus indicating some degree of selectivity among crop and weed species.

Effect of maduramicin on 4-to-6 week old seedling after foliar application

When maduramicin was applied at 4×10^{-4} M and higher concentration produced a wide range of injury on shoot elongation and fresh weight accumulation among the species treated. *T. vulgare* exhibited the highest degree of inhibition of shoot elongation (42.2%) and fresh weight accumulation (54.6%). Shoot elongation and fresh weight of the treated *P. aureus* was also reduced by 21% and 36.8%, respectively. In addition, the antibiotic induced injury in *P. aureus* and *T. alexandrinum*, the injury includes chlorosis, necrosis, desiccation and leaf absission.

Effect of maduramicin and maduramicin methyl ester on ATP synthesis and proton uptake in chloroplast thylakoids

Maduramicin (**1**), maduramicin methyl ester (**1a**) and monesin (**2**) significantly inhibited photosynthetic phosphorylation from water to methylviologen in spinach chloroplast thylakoids in a concentration-dependent manner (Fig. 2). The synthesis of ATP was totally inhibited by **1, 1a** and **2** at 500 μM, 25 μM and 30 μM, respectively.

On the other hand, the extent of proton uptake was also diminished by **1, 1a** and **2** in a concentration-dependent manner (Fig. 2). The calculated IC_{50} of the antibiotics for these activities are summarized in Table II.

Mitchell transduction theory (Mitchell, 1967) predicts that the inhibition of proton uptake and ATP synthesis must be to the same extent and in a parallel manner when the tested compound behaves as an uncoupler. Figure 2 shows a behavior which is in agreement with Mitchell transduction theory.

The light-dependent synthesis of ATP on thylakoids may be inhibited by blocking the electron transport, uncoupling ATP synthesis from electron transport or blocking the phosphorylation reaction itself (Good *et al.*, 1981).

In order to distinguish between these three possibilities the effect of maduramicin, maduramicin methyl ester and monesin on photosynthetic electron transport and Mg^{2+}-ATPase activity was tested.

Effect of maduramicin and maduramicin methyl ester on non-cyclic electron transport and Mg^{2+}-dependent ATPase activity on chloroplasts

Figure 3 shows that the non-cyclic electron transport from water to methylviologen in both

Table I. Effect of maduramicin on seed radicle growth expressed by inhibitory concentration values.

Seed	**IC_{50} [M]** Maduramicin	Monesin	2,4-D
Amaranthus hypochondriacus	1.85×10^{-5}	6.70×10^{-6}	1.31×10^{-5}
Echinochloa crus galli	2.02×10^{-5}	9.60×10^{-5}	1.39×10^{-5}
Trifolium alexandrinum	8.40×10^{-5}	4.10×10^{-5}	5.32×10^{-6}
Triticum vulgare	9.71×10^{-4}	4.16×10^{-5}	1.94×10^{-5}
Phaseolus aureus	1.50×10^{-4}	1.79×10^{-5}	1.67×10^{-5}

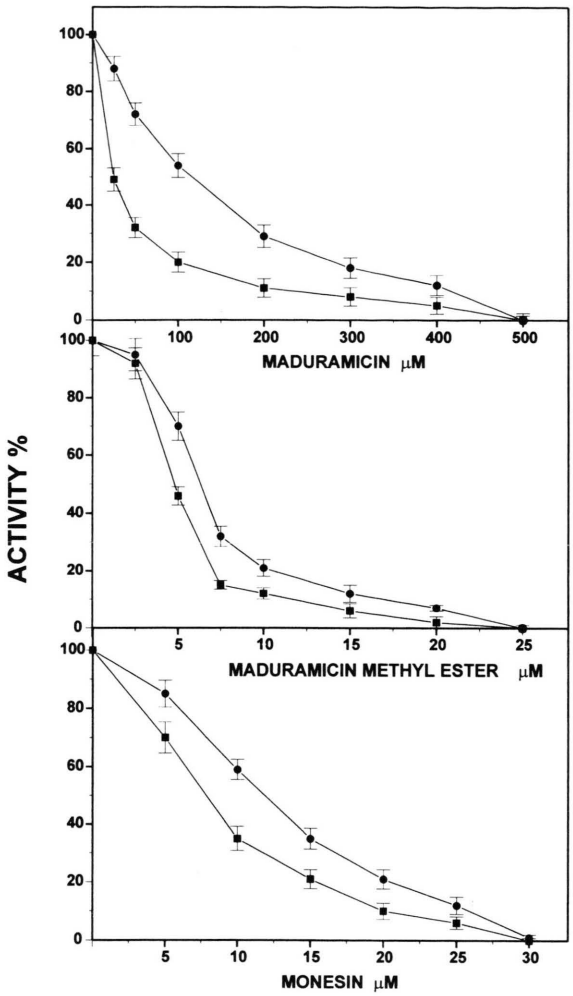

Fig. 2. Inhibitory effect of the antibiotics **1**, **1a** and **2** on photophosphorylation and proton uptake from water to methylviologen in chloroplast thylakoids isolated from spinach leaves. Control value rate for ATP formation (■) and proton uptake (●) were 1633 μmol of ATP h^{-1}· mg Chl^{-1} and 430 μequiv $H^+ \cdot h^{-1}$· mg Chl^{-1}, respectively.

basal and phosphorylating conditions were enhanced by addition of the three compounds. However, uncoupled electron transport was unaffected.

On the other hand, the effect of different concentrations of compound **1, 1a** and **2** on Mg^{2+}-ATPase bound to thylakoids was tested. Table III shows that the three polyether antibiotics stimulated the light-activated Mg^{2+}-ATPase. The activation values range from 279 to 371%.

Chemicals that increase the proton permeability of thylakoid membranes uncouple phosphoryla-

Table II. Effect of maduramicin, maduramicin methyl ester and monesin on ATP synthesis and H^+ uptake in spinach chloroplasts. Control value rate for ATP formation and proton uptake were 1633 μmol of ATP h^{-1} mg Chl^{-1} and 430 μequiv $H^+ \times h^{-1}$ mg Chl^{-1}, respectively.

Antibiotic	IC_{50} [μM] ATP synthesis	H^+ uptake
Maduramicin	20.7	96.6
Maduramicin methyl ester	5.0	6.4
Monesin	8.1	12.1

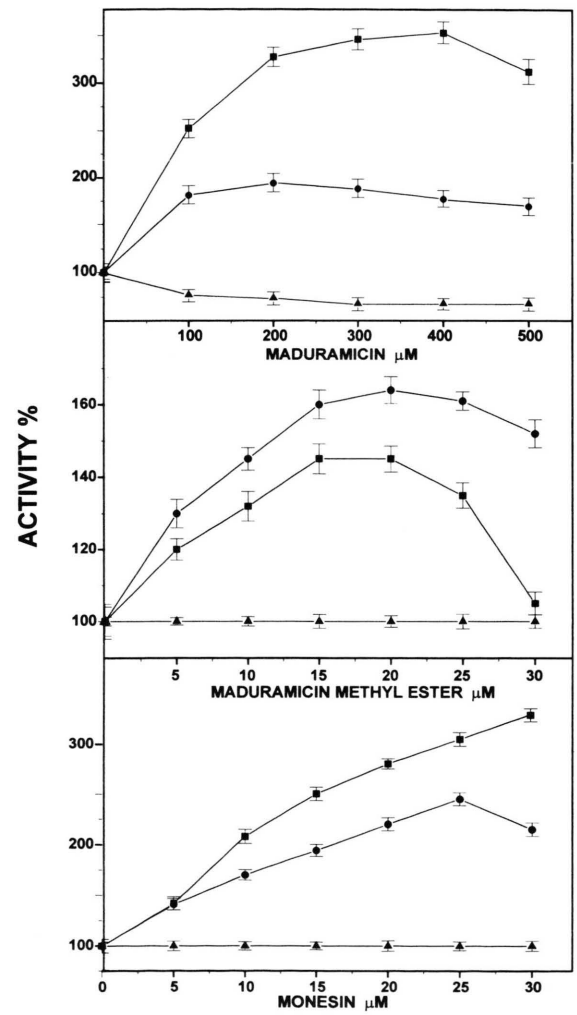

Fig. 3. Noncyclic electron transport (basal ■, phosphorylating ● and uncoupled ▲) from water to methylviologen as a function of antibiotic **1**, **1a** and **2** concentration. Control value rates for basal, phosphorylating and uncoupled electron transport were 167, 833 and 1833 μequiv of $e^- \cdot h^{-1} \cdot mg\ Chl^{-1}$, respectively.

Table III. Effect of maduramicin, maduramicin methyl ester and monesin on the Mg^{2+}-dependent ATPase activity of chloroplasts. 100%-control rate value for the Mg^{2+}-ATPase is 389 μmol Pi·mg Chl·h^{-1} and is equivalent to 100% of activity.

Antibiotic	Concentration [μM]	Mg^{2+}-ATPase activity (% of control)
None	None	100 ± 4
Maduramicin	100	346 ± 12
	200	326± 11
	300	296 ± 9
Maduramicin methyl ester	10	268 ± 9
	20	316 ± 11
	30	371 ± 10
Monesin	10	279 ± 9
	20	353 ± 12
	30	364 ± 12
Ammonium chloride	1.5×10^{3}	317 ± 10
	3.0×10^{3}	487 ± 12

tion from electron flow. Uncoupling agents avoid ATP synthesis by decreasing the proton gradient but allow electron transport to occur at high rates. In addition weakly acidic uncouplers stimulate ATPase when bound to the mitochondrial membrane (F_0F_1-ATPase) (Terada, 1990). Therefore, the inhibitory activity exerted on ATP synthesis and H^+ uptake, as well as the stimulatory effect on basal and phosphorylating electron transport and ATPase activity, indicate that these compounds have uncoupling properties on chloroplast thylakoids.

The effect of maduramicin on ATP synthesis, H^+ uptake and electron flow rate was similar to that previously reported for nigericin (Shavit *et. al.*, 1967, 1970), monesin, lasalocid, dianemycin (Shavit *et al.*, 1970) and laidlomycin (Kida and Shibai, 1986). It has been suggested that these antibiotics increase the permeability of mitochondrial, chloroplasts and artificial membranes to alkali metal cations and protons by inducing a cation↔H^+ exchange (Shavit *et al.*, 1970; Reed, 1979). Therefore, maduramicin is expected to have a similar behavior. On the other hand, maduramicin methyl ester was found to be four times more active as uncoupler than the parent compound. The higher activity could be explained by an increase of lipophylicity of the molecule by the introduction of a methyl group. This fact enhance the diffusion across the thylakoid membrane.

Acknowledgments

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