Photosynthetic Inhibitory Activity of Dihydro-β-agarofurans Sesquiterpenes from *Maytenus disticha* and *Maytenus boaria* (Celastraceae)

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Maytenus Species, β-Agarofuran, Hill Reaction Inhibitor

The effects of 9β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran and 9β-furoyloxy-1α, 6β, 8α-triacetoxy-dihydro-β-agarofuran, major phytogrowth inhibitors isolated from the aerial parts of *Maytenus disticha* (Celastraceae) and seeds of *Maytenus boaria* (Celastaraceae), respectively, on different photosynthetic activities of isolated spinach chloroplasts have been investigated. Photophosphorylation and electron transport (basal, phosphorylating and uncoupled) were inhibited in a concentration dependent manner by both compounds, therefore acting as Hill reaction inhibitors. The site of action of these natural compounds was located in the span from P_{680} to Q_A. 9β-benzoyloxy-1,2,6,8,15-pentaacetoxy-dihydro-β-agarofuran was one order of magnitude more potent (I_{50} = 2.6 μM) than 9β-furoyloxy-1,6,8,-triacetoxydihydro-β-agarofuran, suggesting that the substitution at C-9 and the acetoxy groups at carbons 2 and 15 are important structural requirements for the displayed inhibitory activity.

Introduction

The plants from the Celastraceae family have high allelopathic effect on other species with the associated flora, in addition to their antifeedant properties (González *et al*, 1993, 1997a). For instance, the Chinese bittersweet *Celastrum angulatus* Max. has demonstrated activity against several insect species (Wakabayashi *et al*., 1988) and there is a body of work on the antifeedant or insecticidal activity of extracts (Chiu, 1989). Moreover, the antifeedant activity of 15 polyesterified sesquiterpenes from Celastraceae family against the Egyptian cotton leafworm *Spodoptera littoralis* has been documented (González *et al*., 1992).

The genus *Maytenus*, characterized by the occurrence of different bioactive compounds like maytenosides (antitumour activity), is specially rich in β-dihydroagarofurans, sesquiterpene polyol esters (Itokawa *et al*., 1993). Some of these metabolites were isolated from the seeds of *Maytenus boaria* (Alarcón *et al*., 1995). On the other hand, from *Maytenus canariensis* were isolated some sesquiterpenes with β-dihydroagarofuran skeletons which exhibited antifeedant activity on the same insect in an election test (González *et al*., 1993). *Maytenus disticha* (Hook) Urban, a member of Celastraceae family, commonly known as "maitencito" or "romasillo", is a small tree which grows in rainfall forests in the south Pacific slope ranging from Araucanian Region to "Tierra del Fuego" in the Patagonian Region in Chile. From the aerial parts of *Maytenus disticha* were isolated four agarofurans (Alarcón *et al*., 1991); the major component was 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) (Fig. 1). This compound was shown to have antifeedant activity (1 μg/cm^2) against the Egyptian cotton leafworm *S. littoralis* Boisduval (González *et al*., 1997a). *Maytenus boaria*, the sole tree among the Cel-

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCBQ, dichloro-*p*-benzoquinone; DCMU, 3(3,4-dichlorophenyl-1,1-dimethylurea; DCPIP, dichlorophenol indophenol, DPC, diphenylcarbazide, MV, methyviologen; PS, photosystem; SiMo, silicomolybdate.

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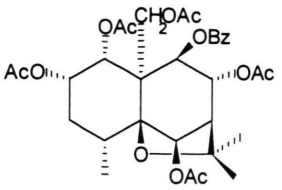

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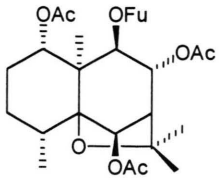

(2)

Fig. 1. Structures of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) and 9-furanoxy-1,6,8-triacetoxy-dihydro-β-agarofuran (**2**).

astraceae family in Chile, usually grows in the arid climate in the slope of mountains. From the seeds of *M. boaria* were obtained four β-agarofuran polyesters and chemically characterized (Alarcón *et al.*, 1991).

Plant natural products, particularly from allelopathic plants, seem to be a good strategy for natural herbicide discovery. In this context, to our knowledge the effect of sesquiterpenes with β-agarofuran skeletons on photosynthesis remains unexplored. Therefore, considering that the process of photosynthesis is the target of a wide range of allelochemicals (Einhellig, 1995; Lotina-Hennsen *et al.*, 1998), the aim of this study was to investigate if the mode of action of the major phytogrowth-inhibitory β-agarofurans from *M. disticha* and *M. boaria* involves an interference with the process of photosynthesis in isolated spinach chloroplasts.

Materials and Methods

General experimental procedures

HPLC was performed on a WATERS Model 600E, equipped with Bondapack RP 18 column, 250 × 8 mm, speed flux 1.5 ml/min, speed paper 0.5 cm/min., U. V. detector 280 nm, mobile phase MeOH/H_2O 7:3 v/v. Analytical and preparative TLC were performed on Silica gel 60 F254 E. Merck plates and the spots were visualized by spraying with a 10% solution of H_2SO_4, followed by heating at 110 °C.

Plant material

Aerial parts (steam, leaf and flowers) from *M. disticha* were collected in Chilean, VIII Region of Chile. A voucher specimens could be found at the ethnobotanical collection of the Herbarium (CONC), Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile. Voucher: R. Rodríguez and C. Marticorena.

Isolation and purification of the β-agarofurans (**1**) *and* (**2**)

9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**). The aerial part (leaves and stems, 1.1 kg, dry weight) of *M disticha* was percolated with methanol. The agarofuran was isolated from n-hexane:EtOAc extract (1:1 v/v) as reported (Alarcón *et al.*, 1991) by conventional chromatographic methods (180 mg). Its molecular formula is $C_{32}H_{40}O_{13}$ M^+ *m/z* 632 and ^{13}C-NMR spectra exhibited 32 carbon signals. All spectral data were identical as in Alarcón *et al.* (1991).

9-β-furoyloxy-1α, 6β, 8α-triacetoxy-dihydro-β-agarofuran (**2**). Seeds (500 g) of *M. boaria* were milled and extracted with MeOH. The extract was solvent partitioned using $CHCl_3$, EtOAc and H_2O. The $CHCl_3$ extract was chromatographed on a silica gel column using petrol-EtOAc mixtures. Polar fractions were combined and separated by TLC (petrol-EtOAc, 1:1 v/v) to give the impure agarofuran, which was further purified by HPLC (RP 18, 250x8 mm, MeOH-H_2O, 7:3 v/v) 45 mg (R_t 8.9 min). The chromatographic and spectral data of the yielded compound (**2**) were identical to those reported by Alarcón *et al.* (1995).

Chloroplast isolation and chlorophyll determination

Intact chloroplasts were prepared from market spinach leaves (*Spinacea oleracea* L.) as previously described (Mills *et al.*, 1980; Saha *et al.*, 1971; Jiménez *et al.*, 1997) and the pellet suspended in the following medium: 400 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl and buffered with 0.03 M Na^+-tricine (N-[tris(hydroxymethyl)methyl]-glycine) at pH 8.0. Chlorophyll concentration was determined according to Strain *et al.* (1971).

Measurement of proton uptake and ATP synthesis

Proton uptake was measured as the pH rose between 8.0 to 8.1 (Dilley, 1972) with a combination of microelectrode connected to a Corning potenti-

ometer (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, pH 8. ATP synthesis was measured titrimetrically according to the procedure of Dilley (1972). Methyviologen (MV) (50 μM) was added as an electron acceptor for the Hill reaction.

Measurement of electron transport

Photosynthetic non-cyclic electron transport rates from water to MV were monitored with a YSI (Yellow Springs Instrument Co., Inc.) Model 5300 oxygen monitor connected to a Clark type electrode. The reaction medium was the same as that used on 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 through a 5 cm filter of 1% $CuSO_4$ solution at 20 °C (Céspedes *et al.*, 1998; Van Gorkom and Gast, 1996).

Photosystems I and II electron transport measurements

Uncoupled PS I electron transport rate from DCPIP to MV was determined in a similar way to uncoupled non-cyclic electron transport (Jiménez *et al.*, 1998). The following reagents were added: 10 μM DCMU, 100 μM DCPIP, 50 μM MV, 500 μM Na^+-ascorbate and 6 mM NH_4Cl. Uncoupled PS II electron flow from water to DCBQ was measured in presence of 200 μM DCBQ, 1 μM DBMIB, 6 mM NH_4Cl and 20 μg Chl/ml. The partial reaction of uncoupled electron transport from water to SiMo was determined with the same reaction mixture as in PS II except that 200 μM SiMo (ALDRICH, Milwaukee, WI) 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, for each reaction a blank experiment was performed with the chloroplasts alone in the reaction medium. I_{50} is the concentration producing 50% inhibition.

Results and Discussion

Structural determination of compounds **1** *and* **2**

The structure of compound **1** (9-β-Benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran) was assigned based on the following evidences: IR absorption bands at 1725 and 1715 (COO) cm^{-1}, a benzoate chromophore in the UV spectrum which was confirmed by the loss of *m/z* 122 (C_6H_5COOH) units in the mass spectrum and signals for five protons at 8.00; 7.44 and 7.58 ppm in the ^{1}H-NMR spectrum. This aromatic substituent was characterized by a large molecular ion peak (Ar-CO) at *m/z* 105 and by elimination of CO to give the phenyl ion *m/z* 77. The five singlets of three protons each of which appears at 1.46; 2.07; 2.10; 2.19; and 2.26 ppm confirm the presence of five acetate groups. These assignments were identical with those previously reported by Alarcón *et al.* (1991).

The structure of compound **2** (9β-furoyloxy-1α, 6β, 8α-triacetoxy-dihydro-β-agarofuran) followed immediately from comparison of the spectral data with those of compound **1**. Accordingly, two acetate signals were missing and aromatic signals indicated the presence of an agarofuran with three acetates and one furanoate. All signals in the ^{1}H-NMR and ^{13}C-NMR spectra were assigned and identical with those reported by Alarcón *et al.* (1995).

Biological activities of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) *and 9-β-furoyloxy-1α, 6β, 8α-triacetoxy-dihydro-β-agarofuran* (**2**)

The effects of these sesquiterpenes on several photosynthetic processes, including ATP-synthesis, electron transport rate (basal, phosphorylating and uncoupled) and partial reactions of the PS I and II, were investigated using freshly lysed spinach chloroplasts (Achnine *et al.*, 1999).

Effect of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) *on photophosphorylation*

This compound was tested for its ability to inhibit ATP formation on freshly lysed spinach chloroplasts from spinach leaves. Photophosphorylation associated with methylviologen reduction was

completely inhibited at 3.5 μM. Only 2.3 μM β-agarofuran (**1**) was required to give 50% inhibition of ATP synthesis (Fig. 2, A).

The inhibitory potential of compound **1** is similar to that shown for the natural product 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene isolated from *Malmea depressa* (Annonaceae) (Jiménez *et al.*, 1998). β-Agarofuran (**1**) was one order of magnitude less potent than sorgoleone, a natural *p*-benzoquinone from root hair of sorghum (González *et al.*, 1997). To the best of our knowledge, these phytotoxic compounds are the most active photophosphorylation inhibitors ever isolated from plants.

Effect of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) *on basal, phosphorylating and uncoupled electron transport*

Photophosphorylation inhibition might be attributed to direct inhibition of the H^+-ATPase complex, blockage of the electron transport, or uncoupling of ATP formation process from the electron transport (Good *et al.*, 1981). To discrimi-

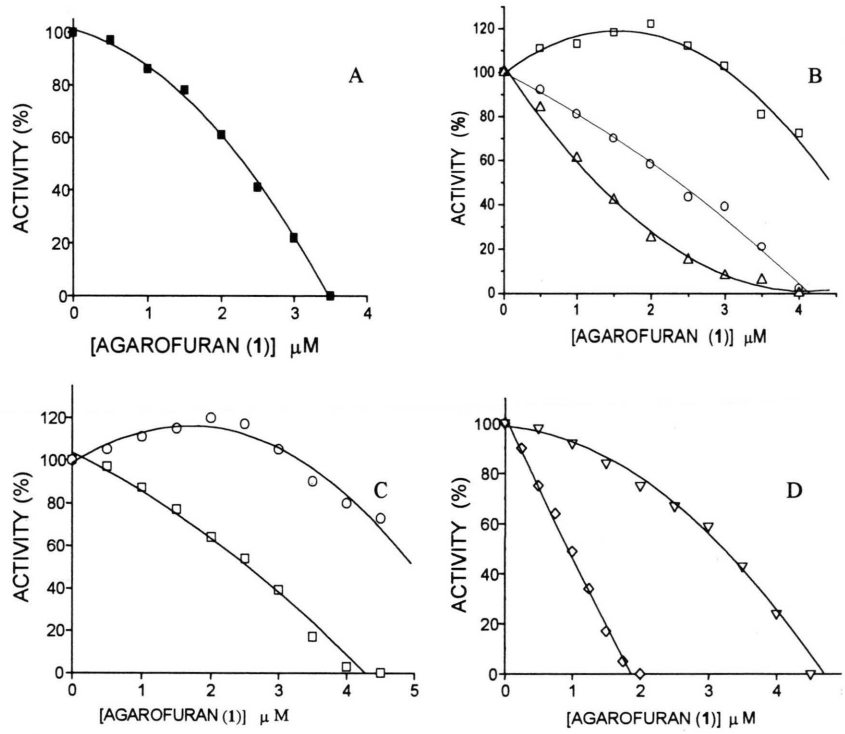

Fig. 2. A.) Effect of increasing concentrations of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) on ATP synthesis (■) in isolated spinach chloroplasts. Control value rate was 210 μmol of ATP mg^{-1} Chl^{-1} h^{-1}. Each cuvette contained 20 μg chlorophyll per ml in the reaction medium.
B.) Noncyclic electron transport (basal (△), phosphorylating (□) and uncoupled (○)) from water to methylviologen as a function of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) concentrations. Phosphorylating electron transport was measured in presence of 1 mM ADP and 3 mM K_2HPO_4. 6 mM of NH_4Cl was used to uncouple basal electron transport. Each cuvette contained 20 mg chlorophyll per ml in the reaction medium. Control value rates for basal, phosphorylating and uncoupled electron transport were 480, 830 and 1680 μequiv. e mg Chl^{-1} h^{-1}, respectively. Each point represents the mean of three determinations.
C.) Uncoupled (6 mM NH_4Cl) photosystem I (O) and II (□) electron transport rates as a function of increasing 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) concentrations. Control value rates, in μequiv. e mg Chl^{-1} h^{-1}, for uncoupled PSI (from DCPIP/H_2 to MV) and PSII (from H_2O to DCBQ) were 1300 and 850, respectively. Each point represents the mean of three determinations.
D.) Effect of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) on photosystem II partial reactions from water to SiMo (◇) and from DPC to DCPIP (▽). Control value rates in μequiv. e mg Chl^{-1} h^{-1} for both partial reactions were 150 and 115, respectively. Each point represents the mean of three determinations.

nate between these possibilities, the effect of compound **1** on non-cyclic electron transport from water to MV in basal, phosphorylating and uncoupled conditions was studied.

Figure 2 (B) shows that the test compound failed to inhibit phosphorylating electron flow, but basal and uncoupled electron transport from water to MV were fully inhibited at 4 μM. Compound **1** stimulated phosphorylating electron flow by 118% at 1.5 μM. These data suggested that the test compound **1** acted as weak uncoupler and strong Hill reaction inhibitor. The calculated I_{50} for basal and uncoupled electron transport was 1.25 and 2.34 μM, respectively. These values are 10 to 100 times lower than those reported for other sesquiterpenes such as cacalol and its derivatives, isoalloalantolactone, zaluzanin C and dehydrozaluzanin C (Aguilar *et al.*, 1996; Calera *et al.*, 1995; Lotina-Hennsen *et al.*, 1992; Galindo *et al.*, 1999), suggesting that compound **1** is a natural powerful Hill reaction inhibitor. Thus, we decided to further explore its site inhibitory mechanism.

Localization of the site of inhibition of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**)

In order to determine the site of inhibition on the electron transport pathway, the effect of compound **1** on partial photosynthetic reactions (PS I and II) was studied using artificial electron donors and acceptors in the presence of appropriate inhibitors (Lotina-Hennsen *et al.*, 1991; Achnine *et al.*, 1998). Uncoupled PS I electron transport, measured from PCPIP/H_2 to MV, was insensitive to the test compound, whereas uncoupled PS II electron flow, measured from H_2O to DCPIP, was completely inhibited at 4 μM of the β-agarofuran **1** (Fig. 2, C).

The calculated I_{50} for PS II inhibition was 2.6 μM. The concentration of compound **1**, needed to induce 100% PS II inhibition is at the same range as that of DCMU or the natural product sorgoleone (Draber *et al.*, 1991; González *et al.*, 1997b). In an effort to define the site (s) of inhibition by compound **1**, we measured the effect (s) of this β-agarofuran on the electron transport reaction catalyzed only by PS II.

Uncoupled (5 mM NH_4Cl) PS II partial reaction, measured from water to SiMo (as electron acceptor) using DCMU as inhibitor of the electron flow between Q_A and Q_B, was inhibited by 76% at 4 μM (Fig. 2, D).

The electron flow from DPC to PCPIP of 0.8 M Tris-washed chloroplasts (Tris-(hydroxymethyl)aminomethane, Merck, Darmstadt) was completely inhibited by compound **1**. It is known that DPC is an electron donor to PSII in Tris washed chloroplasts and Tris wash of chloroplasts blocks the photooxidation of water (Vernon and Shaw, 1969). Since compound **1** inhibited the electron flow from H_2O to SiMo and from DPC to DCPIP, the target was located between the electron carriers P_{680} and Q_A. This target was demonstrated also for several natural products such as, 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene, encecalin, and demethylencecalin, cacalol methyl esters, cacalol acetate, 2-acetylcacalol acetate and isoalloalantolactone (Jiménez *et al.*, 1998; Castañeda *et al.*, 1998; Aguilar-Martínez *et al.*, 1996; Calera *et al.*, 1995), being the first and our test compound the most active.

Comparison of the effects of 9-β-benzoyloxy-1α, 2α, 6β, 8α, 15-pentaacetoxy-dihydro-β-agarofuran (**1**) *and 9-β-furoyloxy-1α, 6β, 8α-triacetoxy-dihydro-β-agarofuran*

Comparing the inhibitory activities of compounds **1** and **2** shows that the latter was less effective inhibitor (Table I). The concentration of 9-β-furoyloxy-1α, 6β, 8α-triacetoxy-dihydro-β-agarofuran (**2**) needed to achieve complete inhibition is

Table I. Comparison of the effects of 9-Benzoyloxy-1,2,6,8,15-penta-acetoxy-dihydro-β-agarofuran (**1**) and 9-furanoxy-1,2,6,8,15-penta-acetoxy-dihidro-β-agarofuran (**2**) on different photosynthetic activities of isolated spinach chloroplasts. I_{50} is the concentration producing 50% of inhibition.

Activities tested	β-Agarofurans (I_{50}, μM)	
	Compound **1**	Compound **2**
Photophosphorylation	2.25	78
Basal electron flow	1.4	63
Phosphorylating electron flow	–	26
Uncoupled electron flow	2.5	65
Photosystem II activity	2.6	72
Photosystem I activity	5.0*	85*

* No-effect concentrations on PS I activity.

~10-fold greater than the concentration of compound **1** required for the same effect. We presume that the benzoyl substituent at C-9, the acetoxy groups at carbons 2 and 15, respectively, seem to be important structural requirements for the displayed inhibitory activities. This high inhibitory potential of compound **1** could be probably due to the presence of acetate esters, since the increase of the number of these substituents was shown to be responsible for the increase of the antifeedant activity of dihydro-β-agarofurans (González *et al.*, 1997). Considering that compound **1** is the major constituent isolated from the aerial parts of *M. disticha* and the low concentration needed to induce the total inhibition of PS II, it is possible to postulate the importance of this allelochemical in *M. disticha* allelopathy.

Acknowledgments

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