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METHYL-LIMONATE INDUCES A RESTRICTED CHAOS IN PLANT MITOCHONDRIAL MEMBRANES

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Key Word Index—*Solanum tuberosum*; Solanaceae; chaotropic effect; plant mitochondria; monoterpenoids; methyl-limonate.

Abstract—The monoterpenoid methyl-limonate is a highly lipophilic hemisynthetic compound (log $P = 5.8$). In potato tuber mitochondria it induces three types of changes corresponding to a chaotropic effect. Firstly, it is an uncoupler of the oxidative phosphorylations (D_{50} close to 200 μM); secondly it inhibits the electron transfer inside the inner membrane (I_{50} close to 1 mM) with at least one inhibitory target located between the quinone pool and cytochrome c; thirdly, it induces an O_2 consumption in the presence of mitochondria without any respiratory substrate, and of a hydrosoluble lipoxygenase. After concentration of the monoterpenoid inside the mitochondrial membranes, this lipoxygenase is able to reach the constitutive unsaturated fatty acids coming from the phospholipid hydrolysis and finally to lead to the formation of fatty acid hydroperoxides. Methyl-limonate is both the most lipophilic and the most active compound of a series of 10 derivatives including for instance pulegone, limonene, carvone, dihydrocarvone and isopulegol.

INTRODUCTION

Terpenoids, especially monoterpenoids, are well-known secondary plant products and over 22 000 individual compounds of this class are now identified [1]. They are most often emitted by the glandular hairs of the aerial parts or produced by specialized tissues of the stems, roots and leaves in several families such as Labiaceae, Rutaceae, Euphorbiaceae and Myrtaceae, etc. They can also be found in tree resins, as is the case for Pinaceae. In these examples, they seem to play the part either of allelopathic agents [2] or of wood protectants [3]. In human health some of these compounds can act as drugs, but the biotoxicity of some monoterpenoids has been previously demonstrated and β-pinene for instance appeared as a powerful uncoupler [4].

The major compound studied here and named methyl-limonate, is the methyl ester of the 1-methyl-4(1-methylpropyl acid) cyclohexene. It is a synthetic derivative of the well-known natural compound limonene. Among a series of 10 natural or hemisynthetic monoterpenes, it is shown in this work to be the most effective inducer of several changes in the plant mitochondria inner membrane.

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RESULTS AND DISCUSSION

Uncoupling effects of methyl-limonate (ML)

In mitochondria oxidizing a substrate such as NADH or succinate at state IV (without ADP), ML at 0.6 mM induced a slight increase of the oxygen consumption from 30 to 140 nmol O_2 min^{-1} mg^{-1} protein. Thereafter, the addition of the reference uncoupler CCCP (5 μM) did not induce the well-known increase in oxygen consumption corresponding to the breakdown of the H^+ transmembrane gradient. It was therefore suggested that the mitochondria were previously fully uncoupled by ML at 0.6 mM. This point was verified by swelling experiments in NH_4NO_3 or NH_4Cl iso-osmotic media (Fig. 1). A full swelling was obtained with ML at 0.6 mM in NH_4Cl or NH_4NO_3 iso-osmotic media. This showed that H^+ permeabilization of the inner membrane allowed NO_3^- or Cl^- free diffusion into the matrix, inducing an increase of its osmotic pressure and, hence, water entrance. The uncoupling properties of ML at 0.6 mM were, therefore, clearly established.

At that stage, the question arose of the mode of uncoupling due to ML. Did it correspond to the simplest mode shown for lipophilic phenol derivatives and previously described by Terada [5] ? In fact, ML was shown to be a non-ionizable compound at bio-

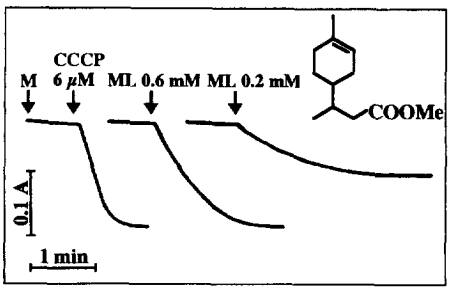

Fig. 1. Spectrophotometric results showing the uncoupling activity of methyl-limonate (ML), as inducing a passive swelling mechanism in potato tuber mitochondria suspended in an iso-osmotic NH_4Cl solution containing 0.1% BSA. M: 0.3 mg protein ml^{-1}.

logical pHs. It was, therefore, unable to ensure a H^+ transmembrane transfer through a change from an anionic to a protonated form. Furthermore, when using a KCl or KNO_3 iso-osmotic medium, the swelling also occurred at the same ML concentration, showing that a K^+ transfer through the inner membrane could be induced by ML. With non-ionizable compounds having a greater molecular size such as sucrose or mannitol, no swelling occurred, even at high concentrations (2–3 mM ML), discounting the possibility of a detergent effect. In the studied series, ML was shown to be the best uncoupler (D_{50} at 0.2 mM), but compounds such as dihydrocarvone (D_{50} = 3 mM), pulegone (D_{50} = 7 mM), limonene-oxide (D_{50} = 7 mM) or carvone (D_{50} = 7 mM) were slightly effective. The lowest effect was obtained with methyl-carvonate.

Inhibition of the electron transfer by ML

The increase in the O_2 consumption rate obtained with ML 0.6 mM in the presence of NADH or succinate as substrates remained much lower than that obtained with CCCP (5 μM). This result suggested that ML might be at the same time uncoupler and inhibitor of the electron transfer. This point was established as shown in Fig. 2. Mitochondria oxidized either NADH, succinate, citrate or malate at pH 6.5 or 7.5. The oxidative rates were measured either at state III (2 mM ADP), or at an uncoupled state (5 μM CCCP). In each case, the electron transfer through the KCN-sensitive pathway was fully inhibited for a ML concentration of 1.7 mM.

These results allowed us to draw several conclusions:

(1) There was no powerful inhibition of the matrix enzymes involved in Krebs cycle, such as malate dehydrogenase or malic enzyme. This is in good agreement with the high lipophilicity of ML, suggesting a high membrane concentration and a very low one in the hydrophilous matrix space.

(2) The similarity of the inhibitory effect between state III and the uncoupled state demonstrated that no decrease of the coupling factor activity was induced by ML at concentrations lower than those which gave the uncoupling effect.

(3) The stable value of the I_{50}, whatever the substrate, suggested that the inhibition could occur at the same point of the electron transfer chain.

When using duroquinol, an electron donor at the level of the quinones, the same inhibition was obtained, showing that the inhibitory point was downstream from the quinone pool. Furthermore, with the ascorbate-tetramethyl-*p*-phenylene diamine electron donor system, which is able to reduce cytochrome c, no inhibitory effect of ML was obtained. The complex IV activity was, therefore, unaffected by ML and the inhibitory target of this product was located between the quinone pool and cytochrome c, most probably at the level of complex III. The same type of inhibition was previously obtained for lipophilic phenols such as pentachlorophenol [6]. A similar inhibitory mechanism was obtained with the other compounds of the studied series of monoterpenes. However, their effect was much lower, with an I_{50} value five times higher in the case of pulegone and more than 10 times higher for the others.

ML induction of mitochondrial O_2 consumption without respiratory substrate

ML is a chemically stable compound which was not oxidized or oxygenated, when it was dissolved in the medium used for studying mitochondrial activities. Furthermore, the comparative TLC analysis of ML before and after incubation with potato mitochondria failed to show any structural change in this product. However, the addition of 2 mM ML to a mitochondrial suspension containing 0.3 mg protein ml^{-1} induced an O_2 consumption reaching 10 ± 0.3 nmol O_2 min^{-1} mg^{-1} protein, no respiratory substrate being added. This consumption was obtained after 3–12 min of latency, depending on ML concentration (between 1 and 3 mM). This consumption was insensitive to antimycin A (3 μM) and KCN (30 μM) (Fig. 3). However, the addition of salicylhydroxamate (SHAM) at 15 mM stopped this consumption. As this study was carried out without adding any type of respiratory substrate, the hypothesis that ML could create a new KCN-insensitive pathway as was the case for platanetin [7], was excluded.

As SHAM is known to be an inhibitor of both the KCN-insensitive electron transfer chain in mitochondria, and of lipoxygenases [8], this type of enzyme was looked for in the mitochondrial pellets obtained from potato tubers, without further purification steps on Percoll gradient. The final pellet indeed contained a lipoxygenase activity as shown by the O_2 consumption when an aliquot of the pellet was stirred in the presence of an unsaturated fatty acid such as linoleic acid (3.4 mM). This consumption was inhibited fully by SHAM (15 mM). Furthermore, it was possible to separate the lipoxygenase activity from the mito-

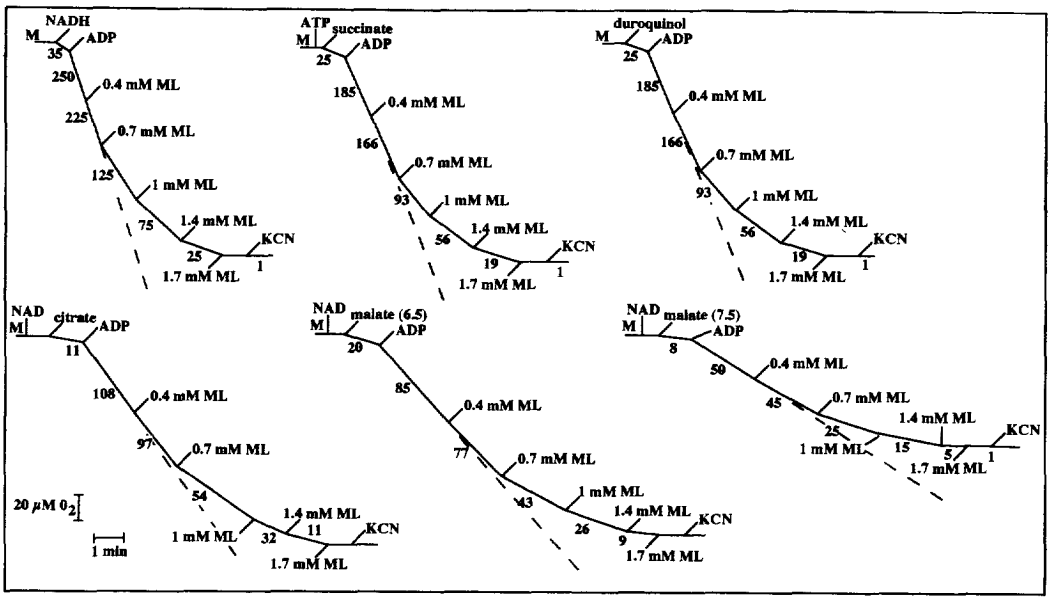

Fig. 2. Inhibitory effect of methyl-limonate (ML) on the electron transfer in potato mitochondria. Substrates: NADH, 1 mM; succinate, 6 mM + ATP 0.3 mM; duroquinol, 0.75 mM; citrate, 10 mM; malate, 15 mM; ADP, 2 mM; NAD, 2 mM. M: 0.3 mg protein ml^{-1}. Dotted lines correspond to reference traces and numbers on traces refer to nmol O_2 consumed mg^{-1} protein min^{-1}.

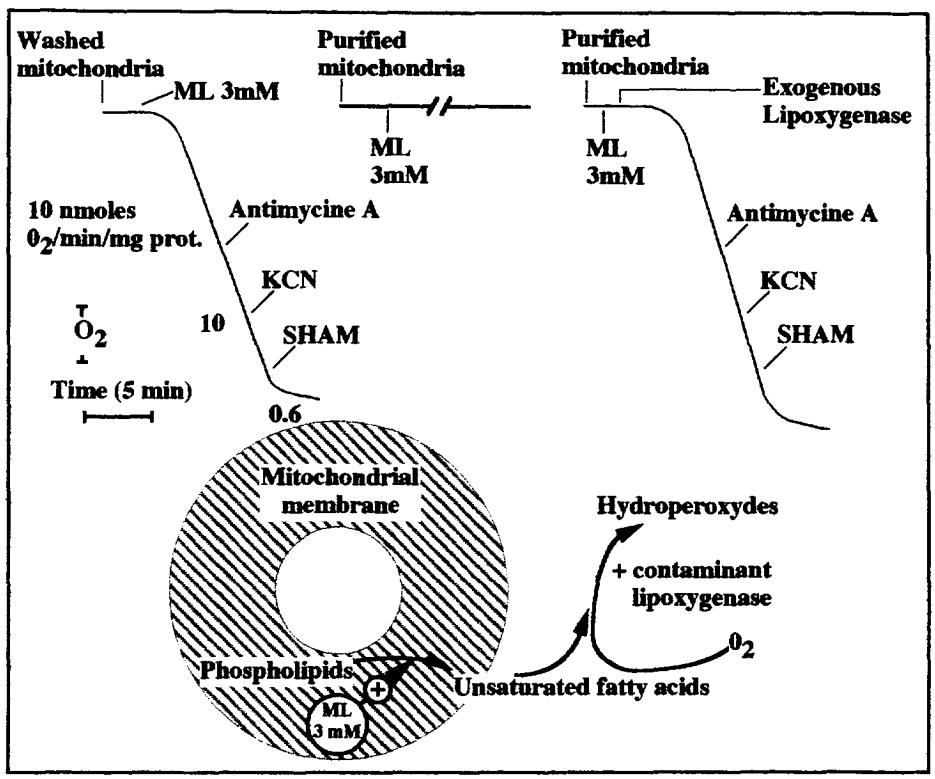

Fig. 3. Induction of a non-respiratory oxidative pathway by methyl-limonate (ML) in potato mitochondria: polarographic traces and interpretative scheme. Numbers on traces refer to nmol O_2 consumed mg^{-1} protein min^{-1}. Antimycine A, 3 μM; KCN, 30 μM; SHAM, 15 mM. Mitochondria, 1.3 mg ml^{-1}.

chondria through the use of a Percoll gradient on the top of which the lipoxygenase remained with the amyloplast band. Mitochondria purified under these conditions, were unable to induce O_2 consumption in the presence of ML. The addition, at this stage, of an aliquot of the aqueous upper band of the gradient restored the O_2 consumption.

A similar result was obtained when pure com-

mercial lipoxygenase (600 units) was added to purified mitochondria in the presence of ML 2 mM. As a whole, it seems that ML 2 mM was able to induce the emission of free unsaturated fatty acids form the mitochondria, allowing their transformation into hydroperoxides in the presence of contaminating lipoxygenase (Fig. 3). Among the mitochondrial phospholipids, one—cardiolipin—is composed of more than 85% of linoleic and linolenic acids [9].

Log P *measurement for the studied compound*

The effect shown here on plant mitochondria for ML was the highest among the other monoterpenoid derivatives studied here. It was interesting to verify if it was associated with an especially high lipophilicity. The octanol/water partition coefficient of these terpenoids was therefore measured through TLC, following Bate-Smith and Westall [10], in the presence of reference compounds (Fig. 4). ML actually proved to be the most lipophilic compound of the series studied here with a log P value reaching 5.8, the less lipophilic one being the methyl-carvonate (log $P = 3.7$).

It is now well established that washed mitochondria suspended in a stirred medium emit spontaneously low amounts of fatty acids, possibly responsible for mitochondrial uncoupling [9]. This effect is strongly alleviated by the addition of serum albumin 0.1% to the medium [11]. ML at 2 mM was shown to be able to increase this process drastically, probably giving access to contaminating enzymes into the membrane inducing phospholipid hydrolysis. In potato tuber preparations, these enzymes were shown to be present as lipolytic acylhydrolases in the membrane fraction coming from amyloplasts [12] where the lipoxygenase was also found.

The ML membrane concentration, for which this effect was induced, was extremely high. A 2 mM concentration in the water medium theoretically corresponds to a concentration in the membrane lipidic phase reaching 1260 M (2 mM × partition coefficient value), since the partition value lipid/water is approximately the same as octanol/water [13]. In fact, under our conditions (1 ml medium for 1.3 mg mitochondrial proteins) ML, when added in the medium at 2 mM at the beginning of the experiment, was submitted to the same partition process with the mitochondria, leading to a severe decrease of the ML concentration in water and giving approximately 3.1 μM in the water and 2 M in the membrane lipids. At this concentration, the first symptom of change in the mitochondrial membrane was the loss of the H^+ impermeability, showing that the integrity of the polar lipid bilayer was no longer maintained. The second symptom of this change was the inhibition of the electron transfer from the quinone pool to cytochrome c. This inhibition is certainly not a specific inhibition of cytochrome b, but has to be understood either as a dilution of the quinone pool or as a change of the position of cytochrome

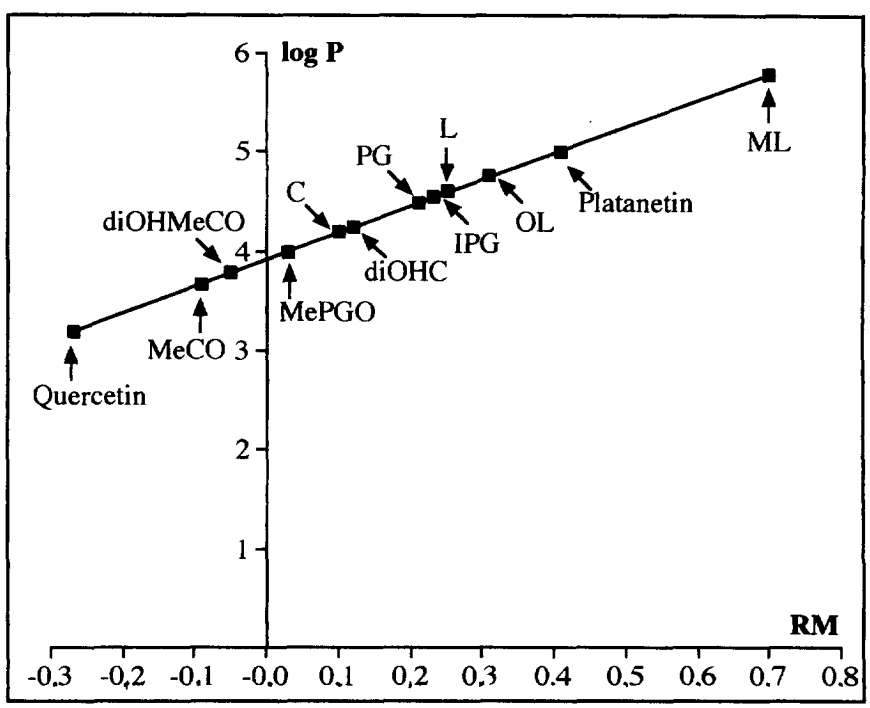

Fig. 4. Evaluation of log P values for the monoterpenoids studied here. The quercetin and platanetin values were experimental ones whereas the others were obtained by TLC experiments as described in the Experimental. MeCO, methyl-carvonate; diOHMeCO, dihydromethyl-carvonate; MePGO, methyl-pulegonate; C, carvone; diOHC, dihydrocarvone; PG, pulegol; IPG, isopulegol; L, limonene; OL, limonene-oxyde; ML, methyl-limonate.

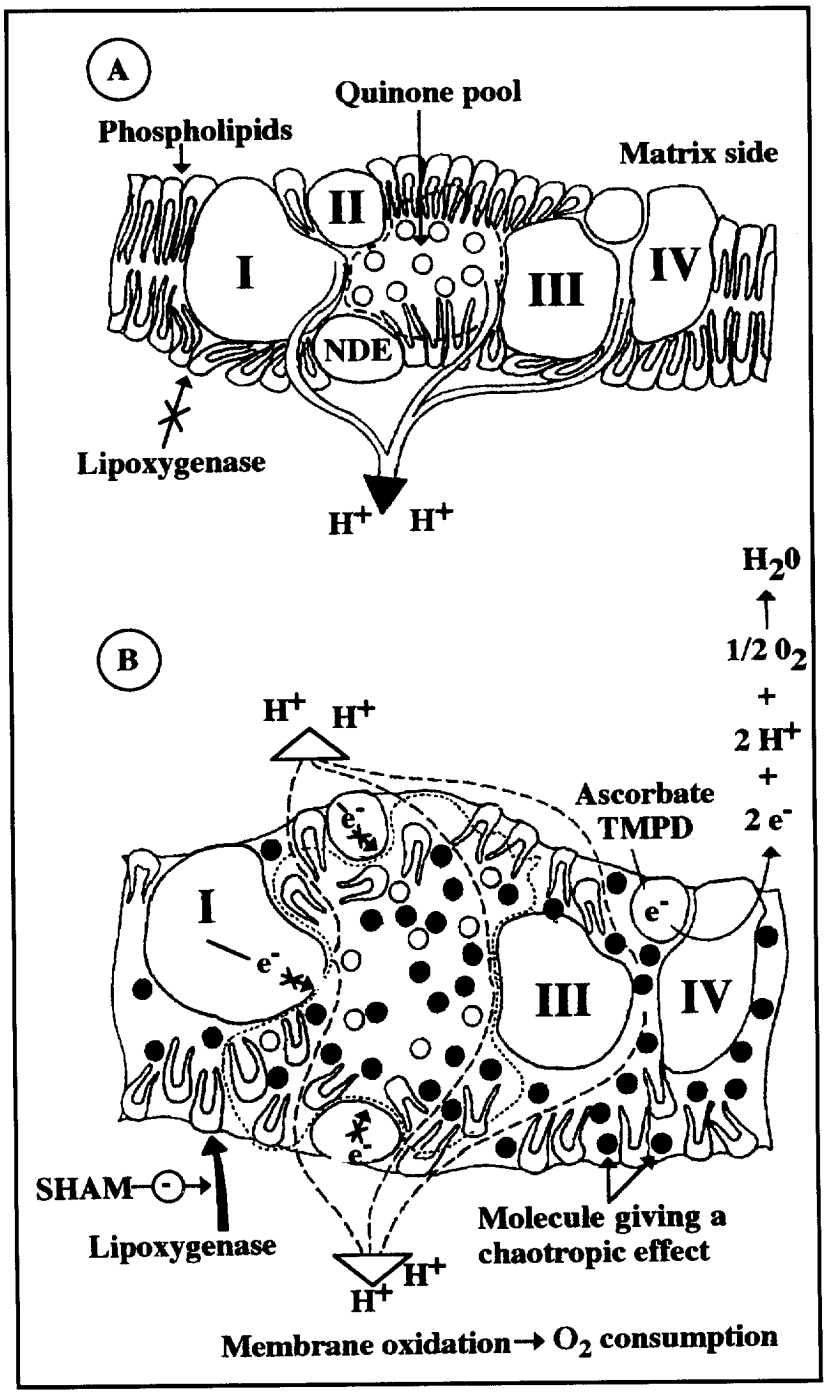

Fig. 5. Interpretative scheme presenting the chaotropic activity of a lipophilic molecule in the inner mitochondrial membrane: A, intact membrane; B, effects induced through a chaotropic activity.

c on the membrane. The third symptom corresponds to the fact that the membrane polar lipids become freely accessible to water soluble enzymes such as acylhydrolases and lipoxygenases. As these enzymes were present in the 'washed mitochondria' pellets, this symptom was easily detected as an O_2 consumption without respiratory substrate.

These three symptoms were typical of the ML action (Fig. 5) and were clearly different from the effects of tensio-active compounds [14], except for uncoupling. It seems that the effect of ML on the membrane could be named a 'chaotropic effect'.

The most typical and simple test for investigating such a chaotropic effect with unknown compounds is certainly the O_2 consumption without substrates in the presence of lipoxygenase. In the series studied here, the induction of a powerful chaotropic effect seemed to be bound to log P, the most lipophilic

compounds being the most effective. This could be explained by the fact that a threshold concentration inside the membrane could be readily obtained.

EXPERIMENTAL

Preparation of mitochondria. Mitochondria from potato tubers (*Solanum tuberosum* L.) were prepd by methods previously described [15], and further purified if necessary in Percoll gradient [12].

O_2 exchange measurements. O_2 exchanges were followed polarographically at 25° using a Clark-type electrode system. The reaction medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM Pi buffer and 0.1% bovine serum albumin (BSA). All incubations were carried out at pH 7.2 except when malate was the substrate (pH 6.5 or 7.5).

Mitochondrial swelling. Mitochondrial swelling was measured by the apparent absorbance decrease at 540 nm with the use of an Uvikon 810 spectrophotometer (Kontron). Iso-osmotic medium contained 100 mM NH_4Cl or NH_4NO_3 or KCl, 15 mM Tris–HCl, 0.1% BSA, pH 7.2. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 5 μM) or valinomycine (0.5 mM) were used as reference uncoupler or ionophore.

Log P *measurements.* Experimental values were obtained as previously described, by spectrophometric measurements [17]. These values concern platanetin and quercetin. The log *P* values for the other compounds were estimated using a reversal phase TLC system (silica-C 18, MeOH–H_2O, 4:1). The *RM* values used to determine log *P* were obtained when using the equation: $RM = \log P\,(1/R_f - 1)$ [10].

Protein measurements. Protein contents were determined according to ref. [16].

Chemicals. The studied terpenoids were synthesized by Prof. Kalk's group. All products were dissolved in EtOH, the concn of which in the reaction media never exceeded 3%.

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