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3α-Hydroxymasticadienonic Acid As an Antiproliferative Agent That Impairs Mitochondrial Functions

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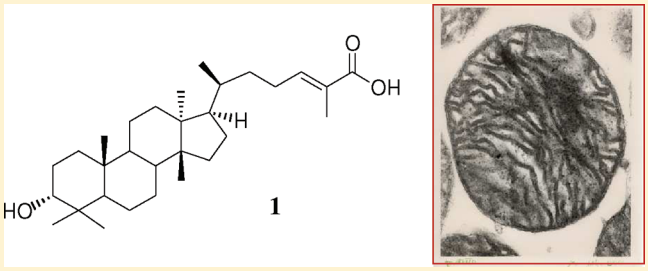
Supporting Information

ABSTRACT: Previous investigations on the biological effects of 3α-hydroxymasticadienonic acid (**1**) have demonstrated both anti-inflammatory and cytotoxic activities. However, neither the molecular mechanism of cytotoxic action nor the possible intracellular target(s) have been reported so far for this compound. The crucial role played by mitochondria on both cell survival and death, due to production of ATP and intrinsic apoptosis, respectively, prompted a study of the effect of **1** on isolated rat liver mitochondria. It was found that **1** causes a dose-dependent impairment of mitochondrial bioenergetic parameters, such as the respiratory control index and transmembrane electrical potential. Moreover, in the presence of Ca^{2+}, at a 10 μM concentration, **1** resulted in the induction of membrane permeability transition by oxidative stress, leading to the release of pro-apoptotic factors. At a 100 μM concentration, compound **1** affected mitochondrial Ca^{2+} transport by inhibiting the accumulation of the cation in the mitochondrial matrix. Altogether, it was demonstrated that **1** induces an impairment of mitochondrial functions that may account for the cytotoxicity exhibited by this compound.



3α-Hydroxymasticadienonic acid (**1**) is one of the main tirucallane-type triterpenes isolated from the organic solvent-soluble extract of the bark of *Amphipterygium adstringens* Schiede ex Schlecht (Anacardiaceae), an endemic tree in Mexico and Central America.[1] This plant, known in México as "cuachalalate" or "cuachalala", has been used widely in traditional medicine, and its ethnopharmacological importance is apparent by the fact that more than 40 traditional uses have been reported for this species. In particular, a decoction of the bark is used commonly for the treatment of gastric ulcers, gastrointestinal cancer, and various inflammatory conditions.[2−6]

The above-mentioned properties have stimulated a number of investigations concerning the biological effects of an organic solvent extract of the bark and its main isolated constituents, masticadienonic acid and **1**. These studies were devoted mainly to the evaluation of anti-inflammatory properties, showing the capability of both masticadienonic acid and **1** to inhibit the occurrence of edema in rats and to induce the production of nitric oxide in macrophages.[5,7,8] Moreover, the synthesis of two derivatives, 3β-hydroxymasticadienonic acid and 24,25-dihydromasticadienonic acid, allowed the requirement of a sp^2 bond at C-24/C-25 for nitric oxide up-release from resting macrophages to be demonstrated.[8] The above studies also reported the ability of these tirucallane-type triterpenes to show comparable antiproliferative activities on tumor cell lines,[5,6,8] thus suggesting that the configuration of the hydroxy group at C-3 is not crucial for this effect.[8] Nevertheless, despite the reported cytotoxicity, no investigation on the mechanism of action responsible for this biological effect has been carried out so far.

Mitochondria play a central role in many pathways essential to both cell life and death.[9,10] Mitochondrial-mediated apoptosis constitutes a crucial pathway in mediating cell death, and some mitochondrial deregulations have been described as hallmarks of apoptosis. Among them, a prominent role is played by the induction of the membrane permeability transition (MPT). This phenomenon leads to an increase in the inner membrane permeability to solutes with a molecular mass up to 1500 Da as a consequence of the opening of a large conductance nonselective pore, known as the permeability transition pore. The MPT causes a dissipation of the electrical transmembrane potential (ΔΨ), matrix swelling, and outer membrane disruption that lead to the release of caspase activators, such as cytochrome *c* (cyt *c*), and apoptosis-inducing factor (AIF). The consequences are the activation of the caspase-dependent and caspase-independent pathways that lead to DNA fragmentation and cell death.[11]

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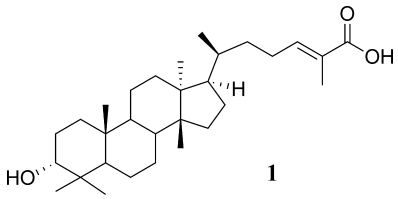
Interestingly, some clinically used anticancer drugs have shown a direct and, in some cases, specific action on mitochondria, in addition to cytosolic or nuclear effects.[12−14] Notably, etoposide, a semisynthetic derivative of the natural product podophyllotoxin, has demonstrated a capacity to interact with mitochondrial functions, as well as having a mechanism related to the permeability transition pore opening.[15] Our group has demonstrated that mitochondria are the molecular targets responsible for the cytotoxic activity of pyridothiopyranopyrimidine and 1,4-dihydrobenzothiopyrano-[4,3-*c*]pyrazole derivatives. Indeed, these compounds act on the mitochondria by inducing the MPT phenomenon and thus provoke the release of pro-apoptotic factors, which trigger the apoptotic pathway leading to cell death.[16,17]

On the basis of the above considerations, the aim of the present study was to investigate the effect of **1** on both bioenergetic functions and the MPT process in isolated rat liver mitochondria, in order to elucidate the possible mechanism of cytotoxicity exerted. The cytotoxic activity of **1** was evaluated on three human tumor cell lines, and effects on mitochondrial functional parameters were determined. The induction of MPT via oxidative stress and release from mitochondria of pro-apoptotic factors, such as cyt *c* and AIF, were also investigated.



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RESULTS AND DISCUSSION

The antiproliferative ability of **1** was evaluated against three human tumor cell lines, namely, HeLa (human cervix adenocarcinoma cells), HepG2 (hepatocellular carcinoma), and A-431 (epidermoid carcinoma). The results, expressed as IC_{50} values, are shown in Table 1.

Table 1. Antiproliferative Activity of 3α-Hydroxymasticadienonic Acid (1) against Three Human Cancer Cell Lines

cytotoxicity (IC_{50} μM)[a]		
HeLa[b]	HepG2[c]	A-431[d]
28.9 ± 3.5	20.1 ± 2.6	40.0 ± 5.2

[a]Mean values ± SD of at least four experiments are reported. [b]Human cervix adenocarcinoma. [c]Human hepatocellular carcinoma. [d]Human epidermoid carcinoma.

Compound **1** exhibited a cytotoxic effect for all cell lines used, with IC_{50} values in the micromolar range. Interestingly, the compound appeared more active toward the hepatocellular carcinoma cells (HepG2) than the cervix adenocarcinoma (HeLa) and epidermoid carcinoma (A-431) cell lines. The observed cytotoxic effects appear to be in agreement with previous studies, where both **1** and some related compounds were investigated.[6,8] In particular, a comparison between several tirucallane-type triterpenes showed that neither the configuration of the hydroxy group at C-3 nor a double bond at C-24/C-25 is essential for the resultant cytotoxicity.[8]

The cytotoxicity of **1** prompted an investigation of the mechanism responsible for cell death. The effect of this compound was examined on bioenergetic functions of isolated rat liver mitochondria along with the induction of MPT, by considering the crucial role played by mitochondria in energy production and in triggering the process of apoptosis, as well as the more pronounced cytotoxicity of **1** on hepatocellular carcinoma.

The respiratory control index (RCI) is a typical mitochondrial parameter relating coupling of the respiratory chain activity with the synthesis of ATP. High values of RCI (>4) reflect the effectiveness of this coupling and the quality of mitochondrial preparations. The results shown in Figure 1

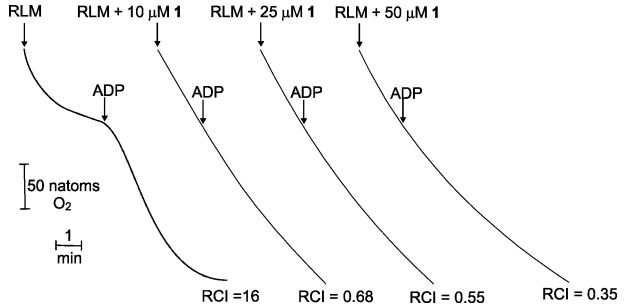

Figure 1. Effect of **1** on respiratory control index (RCI) of rat liver mitochondria energized by succinate in the presence of rotenone. All incubations were carried out as indicated in the Experimental Section. Where indicated, 300 μM ADP was added for RCI evaluation. Compound **1** was present at the concentrations indicated in the figure. Data are representative of five similar experiments.

demonstrate that **1** at 10, 25, and 50 μM concentrations not only completely abolished the normal value of RCI but also caused an inhibition of oxygen consumption after ADP addition (when compared with a control). This effect reduces RCI values appreciably (RCI < 1), suggesting an impairment of mitochondrial bioenergetic functions by **1**.

Subsequent experiments were performed to gain further information about this damaging effect and to investigate the possible induction of the MPT by **1**. MPT is detectable routinely by measuring a decrease of the apparent absorbance of the mitochondrial suspension, which is indicative of a colloid-osmotic swelling of the matrix. This swelling is the result of the opening of the large conductance nonselective permeability transition pore. The results reported in Figure 2 show the effects of 10 and 100 μM **1** on MPT induced by 50 and 90 μM Ca^{2+}, in the presence of phosphate. These two experimental conditions provoked a small or large effect, respectively. As can be seen in this figure, swelling to a smaller extent, induced by 50 μM Ca^{2+}, was increased significantly by 10 μM **1** and inhibited completely by 100 μM **1**. Similarly, swelling to a larger extent, induced by 90 μM Ca^{2+}, was further increased by 10 μM **1** and inhibited strongly by 100 μM **1**. In the absence of Ca^{2+}, compound **1** in the range 10−100 μM was completely ineffective. The two Ca^{2+} concentrations used, which were able to induce either a large or a small swelling, exhibited an unexpected behavior by **1**.

Another parameter generally utilized to evaluate MPT induction is the ΔΨ value. Rat liver mitochondria, under the standard experimental conditions used, exhibited a ΔΨ value of about 170 mV. In the presence of Ca^{2+}, the ΔΨ underwent a time-dependent, gradual drop. A complete and more rapid

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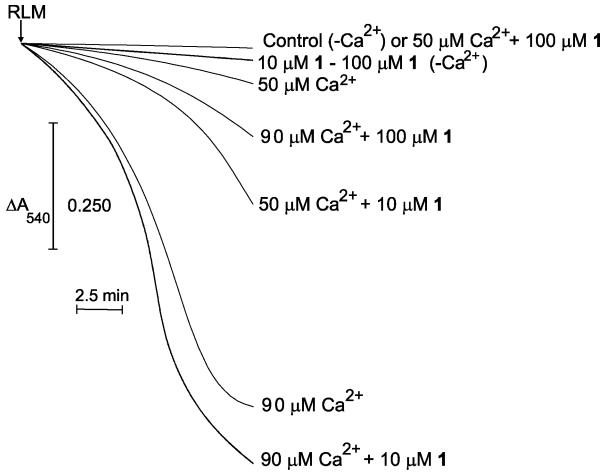

Figure 2. Effects of **1** on mitochondrial swelling induced by Ca^{2+}. All incubations were carried out as reported in the Experimental Section in the presence of 50 or 90 μM Ca^{2+}, as shown in the figure, except where indicated ($-Ca^{2+}$). Compound **1** was present at 10 or 100 μM, as indicated. The assays were performed at least four times with similar results. A downward deflection indicates absorbance decrease, demonstrative of the occurrence of swelling.

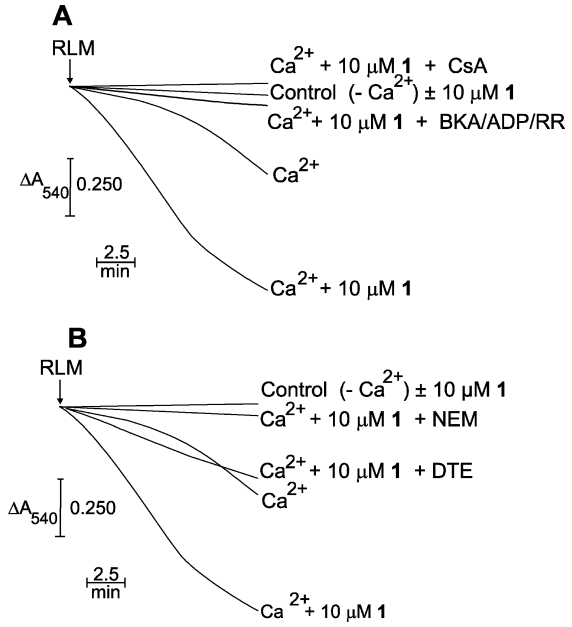

Figure 3. Protection by MPT inhibitors (A) and antioxidant agents (B) on mitochondrial swelling induced by **1** plus Ca^{2+}. Incubation conditions as in Figure 2 in the presence of 50 μM Ca^{2+}. When present: 10 μM **1**, 1 μM CsA, 5 μM BKA, 1 mM ADP, 2 μM RR, 10 μM NEM, 1 mM DTE. The assays were performed at least five times with similar results.

collapse of $\Delta\Psi$ was obtained when 1, 5, or 10 μM **1** was also present (Figure S1, Supporting Information). It should be noted that 1 and 5 μM **1** also increased mitochondrial swelling induced by 50 μM Ca^{2+}, although to a lower extent if compared with 10 μM **1** (results not reported). Instead, in the presence of 100 μM **1**, $\Delta\Psi$ exhibited a value of about 130 mV that was maintained as a constant during this time.

Thus, compound **1**, in the presence of Ca^{2+}, exhibited a particular action, with a dose-dependent relationship opposite that observed in Figure 1, where the increase of the concentration of **1** caused a rise in the damaging effect on the mitochondria. In fact, at the lower concentration (10 μM), this compound amplified swelling and increased the $\Delta\Psi$ collapse induced by Ca^{2+}, while at the higher concentration (100 μM), it protected against both these effects (Figures 2 and S1 Supporting Information).

In the absence of Ca^{2+}, the addition of **1**, in the range of 10–100 μM, induced a dose-dependent drop of $\Delta\Psi$ that did not completely collapse, but reached lower stable levels (Figure S2, Supporting Information). This result demonstrated that **1** exhibited an effect similar to a protonophore, most likely due to the opening of proton leaks. This observation is in agreement with the results shown in Figure 1, in which stimulation of respiration in state 4 and inhibition in state 3 (addition of ADP) strongly suggested that **1** behaved as an uncoupler of oxidative phosphorylation. Interestingly, these alterations on the inner mitochondrial membrane functions cannot be attributable to the induction of the MPT, as the compound by itself was not able to cause mitochondrial swelling (Figure 2). In addition, the immunosuppressant cyclosporin A (CsA), a typical MPT inhibitor, did not exhibit any protection on the $\Delta\Psi$ drop induced by 100 μM **1** (Figure S2, Supporting Information).

The results of Figure 3A show that the swelling induced by 50 μM Ca^{2+} and amplified by 10 μM **1** is almost completely prevented by several agents, including CsA, the adenine nucleotide translocase (AdNT) inhibitor bongkrekic acid (BKA), the Ca^{2+} transport inhibitor ruthenium red (RR), and ADP. Moreover, the reductant dithiothreitol (DTE) and the alkylating agent *N*-ethylmaleimide (NEM) exhibited a partial or complete protective effect, respectively (Figure 3B). The partial inhibition by DTE is attributable to a known lack of ability to cross the mitochondrial membrane. Taking into account that CsA, ADP, and BKA are typical MPT inhibitors[18] and that NEM and DTE are protective agents against thiol oxidation, these results supported a hypothesis that the amplification of swelling by a low concentration of **1** was due to a further increase of the Ca^{2+}-dependent transition pore opening and that this amplification was related to the induction of oxidative stress.

The redox state of mitochondrial thiol groups was determined in the presence of Ca^{2+} and **1** to evaluate this latter hypothesis. A histogram shown in Figure 4A shows that 50 μM Ca^{2+} and 10 μM **1** induced a decrease of reduced thiols of about 25% and 20%, respectively. When rat liver mitochondria were incubated with both Ca^{2+} and **1**, the decrease of reduced thiols reached 50%. This latter strong thiol oxidation was inhibited significantly by RR, DTE, NEM, and CsA, while BKA and ADP exhibited only a slight protection (Figure 4B). Obviously, a reduction in the number of SH groups corresponds to a parallel increase of their oxidation with the formation of disulfide bridges. In the presence of 90 μM Ca^{2+} or 100 μM **1**, the decrease of SH groups was about 80% and 25%, respectively. Interestingly, the presence of both compounds maintained a 25% drop in the reduced thiols (Figure 4A).

The pyridine nucleotide redox state was analyzed along with sulfhydryl groups, and the results showed a behavior similar to that observed in Figure 4A (Figure S3, Supporting Information). The oxidation of thiol groups and pyridine nucleotides by **1** alone demonstrated that it was able to induce oxidative stress. The oxidation of about 50%, observed with Ca^{2+} and 10 μM **1**, was the result of the opening of the

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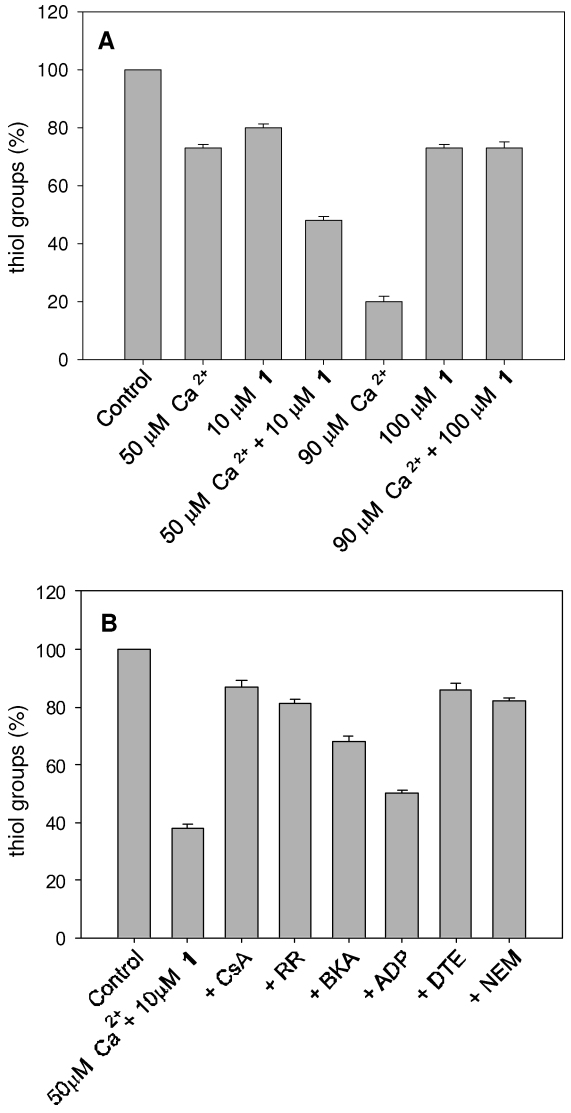

Figure 4. Redox level of mitochondrial sulfhydryl groups in the presence of **1** and Ca^{2+} (A). Effect of MPT inhibitors and antioxidant agents (B). Experimental conditions and compound concentrations as in Figure 3. Incubation time: 15 min. The data are expressed as a percentage of the reduced thiols and represent the average ± mean SD from four independent experiments.

transition pore, which further increased oxidative stress. It should be pointed out that the oxidation of critical SH groups belonging to cysteines located on AdNT is, together with the interaction of Ca^{2+} with specific site(s) of AdNT, the specific requirement for MPT induction.[19]

All these observations, however, do not explain why **1** at 10 μM concentration strongly amplified mitochondrial swelling, while a 100 μM concentration inhibited it, which is an unusual opposite dose-dependent effect observed on MPT. The Ca^{2+} movements in mitochondria were measured during the induction of MPT with an intermediate Ca^{2+} concentration (70 μM) to investigate the molecular mechanism responsible for this behavior (Figure 5). The concentration selected gave a particular extent of swelling that permitted both the amplification and the protection induced by 10 or 100 μM **1**, respectively, to be better evaluated. The results in Figure 5A show, as expected, that 100 μM **1** inhibited completely mitochondrial swelling induced by 70 μM Ca^{2+}, while 10 μM **1** strongly amplified it. The inset in Figure 5A shows a dose-dependent effect of **1** on the swelling induced by 70 μM Ca^{2+}, allowing the threshold concentration of about 20 μM **1** to be elucidated. Below this value, compound **1** amplified swelling, while inhibition occurred above this value.

The corresponding movements of Ca^{2+} across the mitochondrial membrane detected in parallel with the swelling experiment of Figure 5A are shown in Figure 5B. Thus, Ca^{2+} was rapidly and completely accumulated by rat liver mitochondria, and it began to exit after 5 min with the opening of the transition permeability pore. Equally, in the presence of 10 μM **1**, Ca^{2+} is rapidly accumulated, but since under these conditions the opening of the pores was more rapid and of larger extent than under the previous conditions, the cation is suddenly (1−2 min) released before its complete accumulation. Instead, in the presence of 100 μM **1**, Ca^{2+} accumulation, apart from an initial uptake of about 20 nmol/mg protein, is completely blocked such that the cation is not able to induce the pore opening (see Figure 5A).

The results shown in Figure 5 explain the effect of **1** on MPT, from the ability of the compound at 100 μM concentration to inhibit the entry of a large amount of Ca^{2+} in the mitochondrial matrix. This entry constitutes an essential requirement for the occurrence of MPT.[19]

The results obtained did not permit a clear explanation to be given about the redox mechanisms induced by **1**. However, a proposal could be that the compound at 10 μM concentration may exhibit a synergistic effect with Ca^{2+} in affecting the electron flux between complex II and ubiquinone by favoring an increased concentration of the semiquinone radical. This radical, by interacting with oxygen, generates the superoxide anion ($O_2^{\bullet-}$), which is then transformed by superoxide dismutase in hydrogen peroxide. This latter compound, in turn, could generate the highly damaging hydroxy radical ($OH^{\bullet}$) by the Fenton reaction with the FeS groups of the respiratory chain. This radical is most likely responsible for the observed oxidative stress, and such a mechanism has also been proposed for other cytotoxic agents.[20] At a 100 μM concentration, **1** exhibited an opposite behavior. In fact, it prevented the large amplitude swelling induced by high Ca^{2+} concentrations (Figure 2) and protected sulfhydryl groups (Figure 4) and pyridine nucleotides (Figure S3, Supporting Information) against the oxidative stress promoted by Ca^{2+}. As demonstrated by the results of Figure 5B, the mechanism of this protection is attributable to the strong inhibitory effect exhibited by **1** at a high concentration on the Ca^{2+} uniporter. In fact, this action hampered the interaction of Ca^{2+} with its specific site(s) on AdNT and, thus, prevented MPT induction.

MPT provokes the release from mitochondria of some pro-apoptotic factors, such as cyt *c* and AIF. These factors trigger the caspase-dependent and the caspase-independent cascade, respectively, which results in the apoptotic phenotype with DNA fragmentation and cell death. The results demonstrated that **1**, at 10 μM, in the presence of Ca^{2+}, further increased the loss of these pro-apoptotic factors, while at 100 μM the compound was ineffective. It is important to note that in the absence of Ca^{2+} both concentrations of **1** did not exhibit any effect (Figure S4, Supporting Information).

In conclusion, 3α-hydroxymasticadienonic acid (**1**) acts at the mitochondrial level by affecting energy production in the absence of Ca^{2+} and Ca^{2+} homeostasis in the presence of this cation. Indeed, **1** amplifies MPT induced by Ca^{2+}. These observations allow the mitochondria to be singled out as a

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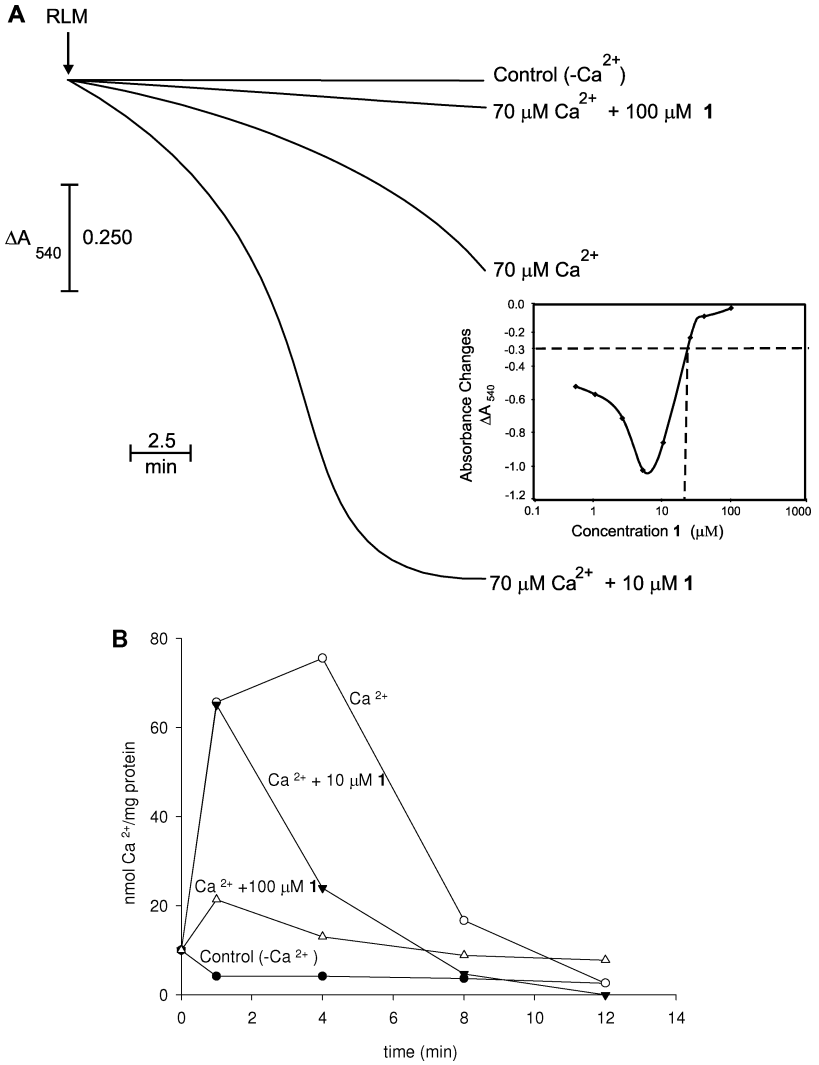

Figure 5. (A) Effect of different concentrations of **1** on mitochondrial swelling induced by Ca^{2+}. (B) Ca^{2+} movements across the mitochondrial membrane in the presence of different concentrations of **1**. All incubations were carried out as reported in the Experimental Section in the presence of 70 μM Ca^{2+}. Concentrations of **1** as indicated in the figure. The inset shows the dose-dependent absorbance changes induced by **1** on the absorbance decrease caused by **1** plus Ca^{2+}. The horizontal line indicates the absorbance decrease of about 0.300 unit induced by Ca^{2+} alone. The curve obtained elucidates the threshold concentration (~20 μM) (dashed line) between the amplification and inhibition of the MPT. The abscissa is in a log scale.

possible cellular target for the antiproliferative effect of **1**. Interestingly, the opposite effects observed with different concentrations of **1** on pore opening raise the hypothesis that this triterpenoid may act through two different mechanisms. At a 10 μM concentration, **1** behaves as an intrinsic pro-apoptotic agent, promoting the MPT process and the consequent release of pro-apoptotic factors, such as cyt *c* and AIF. In contrast, at a 100 μM concentration, **1** protects rat liver mitochondria against MPT by blocking Ca^{2+} entry. In the latter condition, cell death could be mediated by the impairment of bioenergetic and Ca^{2+}-dependent mitochondrial functions.

Isolated mitochondria can be considered an important model to study the activity of cytotoxic agents; this is true in particular at the level of tumor cells, as also reported by previous observations.[16,17,21] In general, mitochondria in cancer cells exhibit the overexpression of some proteins. These proteins facilitate the opening of the transition pore and favor the action of the MPT inducers at lower concentrations than in mitochondria in normal cells.[22] In this regard, the effect of **1** in isolated rat liver mitochondria can assume great importance in vivo, because the compound, at appropriately lower concentrations, could act at the level of the cells that are more susceptible to MPT without affecting normal cells.

EXPERIMENTAL SECTION

General Experimental Procedures. 3α-Hydroxymasticadienonic acid (3-epimasticadienolic acid or schinol) (**1**) was isolated from the hexane extract of *Amphipterygium adstringens*, as previously described.[5] The purity determined by HPLC was 96.3% for this compound.

Inhibition Growth Assay. HeLa (human cervix adenocarcinoma cells) were grown in Nutrient Mixture F-12 [HAM] (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries); A-431 (human epidermoid carcinoma) and HepG2 (human hepatocellular carcinoma) were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. Then, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma Chemical Co.) were added to the medium. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

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Cells (4×10^4) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of fresh medium, and various concentrations of the test agent were added. The cells were then incubated in standard conditions for a further 72 h.

A trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, i.e., the concentration of the test agent resulting in 50% reduction in cell number compared with control cultures.

Mitochondrial Isolation and Standard Incubation Procedures. Rat liver mitochondria were isolated by conventional differential centrifugation in a buffer containing 250 mM sucrose, 5 mM Hepes (pH 7.4), and 1 mM EGTA.[23] EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with BSA as a standard.[24]

Mitochondria (1 mg protein/mL) were incubated in a water-jacketed cell at 20 °C. The standard medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 1.25 μM rotenone (Sigma-Aldrich), and 1 mM sodium phosphate. Variations and/or other additions are given with each experiment.

Determination of Mitochondrial Functions. Oxygen uptake was measured by a Clark electrode. This measurement allowed calculation of the rate of state 3 (ADP-stimulated) respiration (V3), the rate of state 4 (absence of ADP) respiration (V4), and the respiratory control index (RCI = V3/V4). Utilizing the values of ADP concentration and V3, ADP/O values were calculated. Mitochondrial swelling was determined by measuring the apparent absorbance change of mitochondrial suspensions at 540 nm in a Kontron Uvikon model 922 spectrophotometer equipped with thermostatic control. $\Delta\Psi$ was calculated on the basis of distribution of the lipid-soluble cation tetraphenylphosphonium (TPP^+) measured across the inner membrane using a TPP^+-selective electrode.[25] The redox state of endogenous pyridine nucleotides was followed fluorometrically in a Shimadzu RF-5000 spectrofluorometer, with excitation at 354 nm and emission at 462 nm. The protein sulfydryl oxidation assay was performed as in Santos et al.[26] Ca^{2+} content in mitochondrial pellets was estimated by atomic absorption spectroscopy, using a Perkin-Elmer 1100B spectrometer.[27]

Detection of Cyt *c* and AIF Release. The mitochondria (1 mg protein/mL) were incubated for 15 min at 20 °C in standard medium with the appropriate additions. The reaction mixtures were then centrifuged at 13000*g* for 10 min at 4 °C to obtain mitochondrial pellets. The supernatant fractions were concentrated using a PAGEprep protein cleanup and enrichment kit (Pierce, Rockford, IL, USA). Aliquots of 20 μL of the concentrated supernatants were subjected to 15% and 10% SDS−PAGE for cyt *c* and AIF, respectively, and analyzed by Western blotting using mouse anti-cyt *c* and rabbit anti-AIF antibodies (Pharmingen, San Diego, CA, USA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S4 and a list of acronyms. These materials are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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