Two Fungal Lanostane Derivatives as Phospholipase A_2 Inhibitors

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The hydroalcoholic extract of *Poria cocos* and two lanostane derivatives isolated from it, pachymic acid (**1**) and dehydrotumulosic acid (**2**), were active as inhibitors of phospholipase A_2 from snake venom when a polarographic method was used. Dehydrotumulosic acid exhibited an IC_{50} of 0.845 mM. These two compounds are structurally related to certain triterpenoids from *Ganoderma* and *Schinus* that have previously been described as competitive inhibitors of phospholipase A_2. These comprise a new group of natural potential antiinflammatory agents due to their interaction with that enzyme.

Poria cocos Wolf (Polyporaceae) is a well-known Chinese traditional medicine used for its diuretic, sedative, and tonic effects. Our research on this fungus has demonstrated marked antiinflammatory activity in different topical acute and chronic models. Topical application of its hydroalcoholic extract inhibited the TPA- and AA-induced ear edemas. It was also active against chronic inflammation induced by repeated applications of TPA (unpublished data).

Here we report on this fungus as a source of inhibitors of phospholipase A_2 (PLA_2) since such compounds could prove very useful in controlling many inflammatory processes, due to the effect derived from this enzyme activation. Extracellular PLA_2 plays a pathogenic role of two main pathways: direct damage to cellular membrane due to phospholipid hydrolysis and liberation of arachidonic acid, which is the precursor of many eicosanoids responsible for inflammatory responses. Consequently, it can have a pivotal influence on certain diseases such as rheumatoid arthritis, psoriasis, autoimmune uveitis, septic shock, and possibly bronchial asthma.[1,2]

In the polarographic assay protocol that was carried out with PLA_2 from *Naja naja* venom, phospholipid hydrolysis is measured as soybean lipoxidase-catalyzed oxygen incorporation into the released unsaturated fatty acids. To measure this process, we applied the conditions established by Wallach *et al.*[3] with a few modifications. Optimal conditions were determined by varying temperature, cell volume, and the concentrations of substrate and PLA_2. For a given level of lipoxygenase (1.500 mg/mL), the maximum activity of PLA_2 was observed for 100 μL of the phosphatidyl choline solution in a cell volume of 1.5 mL at 37 °C. The reactions were then run in the presence of different concentrations of PLA_2 (0.031 → 6.000 μg/mL). A linear relationship between the PLA_2 concentration and total oxygen incorporation was obtained over the range tested (up to 3.000 μg/mL), with a maximum rate of oxygen uptake within this linear portion when PLA_2 concentration was 2.900 μg/mL (Figure 1).

Inhibition of the reaction was observed when different concentrations of the hydroalcoholic extract of *P. cocos* were added to the incubation mixture. It exhibited an IC_{50} of 0.813 mg/mL. The two lanostane-type triterpenes, pachymic acid (**1**) and dehydrotumulosic acid (**2**), isolated from this extract showed significant PLA_2 inhibitory activity. Dehydrotumulosic acid was the most active inhibitor with an IC_{50} of 0.845 mM, three times lower than that of mepacrine (IC_{50} = 2.160 mM). Pachymic acid exhibited a potency (IC_{50} = 2.897 mM) in the same range as mepacrine (Table 1).

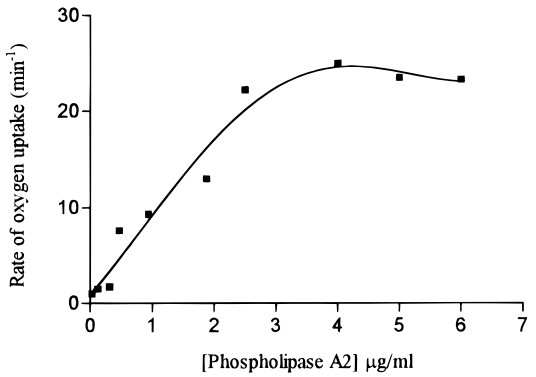
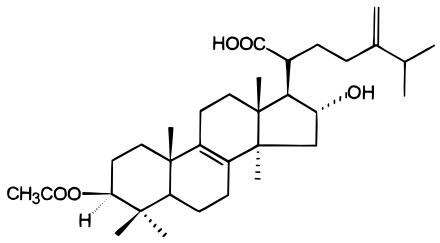


Figure 1. Initial reaction rates against PLA2 concentration.



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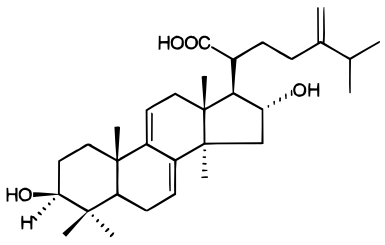

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Acidic triterpenoids seem to be emerging as a new class of PLA_2 inhibitors. After their recent thorough study, Jain *et al.*[4] reported on the behavior of two euphol derivatives, masticadienoic and masticadienolic acids,

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Table 1. Inhibitory Effect of *Poria cocos* Products on Phospholipase A_2 Activity

sample	IC_{50}[1]	I.R.[2]
hydroalcoholic extract	0.813 mg/mL (0.573−1.327 mg/mL)	60.3*[a]
pachymic acid	2.897 mM (2.617−3.267 mM)	66.0*[b]
dehydrotumulosic acid	0.845 mM (0.623−1.045 mM)	58.6**[c]
mepacrine	2.160 mM (1.905−2.581 mM)	85.5**[d]

[1] The results are expressed as IC_{50} values with their 95% confidence limits given below in parentheses. [2] I.R. = inhibition ratio (percentage of inhibition of PLA_2 activity (a) at 1.00 mg/mL, (b) at 3.700 mM, (c) 1.00 mM, and (d) at 5.800 mM). *$p < 0.05$, **$p < 0.01$ Dunnet's *t*-test.

from the pink peppercorn (*Schinus terebinthifolius*, Anacardiaceae) in the relation to the interaction with three different forms of PLA_2. The same authors have compared the activity of these derivatives with that of some of the closely related lanostane derivatives from the basidiomycete *Ganoderma lucidum*, called as ganoderic acids.[5] Until now, only few natural terpenoids, particularly certain marine diterpenoids (scalaradial) and sesterterpenoids (manoalide), have been recognized as having these inhibitory properties.[6,7]

It has been postulated that both a bulk tetracyclic structure and a side alkyl carboxylic chain are necessary for PLA_2 inactivation, although the latter part of the molecule seems to be the one that occupies and blocks the catalytic site of the enzyme. This hypothesis is supported by theoretical spatial considerations and by the fact that homologous methyl esters of masticadienoic and masticadienolic acids are inactive. Our data from pachymic and dehydrotumulosic acids suggest that, although a free carboxylic group is essential, its position on the side chain can vary (C-26 in *Schinus* triterpenoids, C-21 in *Poria* triterpenoids). Furthermore, the hybridization state of the "central" carbons (7−10) also admits some variations without losing activity; in other words, both 8,9-ene and 7,8 and 9,10-diene forms with indoubted different configurations for B/C rings have a PLA_2 inhibitory effect. Therefore, it is possible that the alicyclic structure does not exert any spatially-specific function, but only provides the lipophilicity needed to go along the hydrophobic channel leading to the active site of $PLAs_2$.[8] In a similar way, it seems that the relative position of the methyl groups at 13 and 14 is not decisive at all, although when the superior activity of euphol derivatives is considered, a 13α and 14β conformation is better than its reciprocal. With respect to this last point, it should pointed out that our compounds present the same skeleton as the above mentioned ganoderic acids that have been shown to inhibit PLA_2 activity.[4]

Experimental Section

General Experimental Procedures. Identification was carried out by ^1H (400 MHz) and ^{13}C (75 MHz) on a Bruker AMX-400 spectrometer in $CDCl_3$. High-resolution FAB-MS were recorded on a VG Analytica Fisons spectrometer. Compounds were visualized by sulfuric anisaldehyde. Silica gel 60 and silica gel 60G (Merck) were used for CC and TLC, respectively. HPLC-DAD analysis was performed using a Merck-Hitachi HPLC system (L-6200 pump) equipped with an L-3000 photodiode array detector (DAD) and a prepacked analytical column (12.5 × 0.7 mm) of Lichrospher RP-18 (5 μm). The following conditions were used: eluents: H_2O + TFA 0.05% (A), MeOH + TFA 0.05% (B). Elution profile: 0−5 min 70% A, 5−15 min 50% A, 15−29 min 30% A, 29−30 min 70% A. Flow rate was 1 mL/min, column pressure was 60−80 bar, and the UV detector was set at 214 nm. Chemicals: phosphatidyl choline substrate Type V-E (fatty acid composition: 35% palmitic, 12% stearic, 28% oleic, 15% linoleic, 1.5% palmitoleic, and 4% arachidonic acids by weight), soybean lipoxygenase, PLA_2 from *Naja naja* venom, linoleic acid, deoxycholic acid, 2-amino-2-methyl-1,3-propanediol (ammediol) buffer, mepacrine, and calcium chloride were purchased from Sigma Chemical Co., St. Louis.

Vegetal Material. Sclerotia of *P. cocos* (Schw.) Wolf (Polyporaceae) was supplied by Asia Natural Products S.L., Amposta, Spain. The crude drug was authentified by D. Carlo Llopis, pharmacist of that company.

Extraction and Isolation of the Compounds. Dried sclerotia of *P. cocos* was macerated with hot 70% aqueous MeOH. The solvent was removed under reduced pressure and the hydroalcoholic extract obtained (3.4 g) lyophilized. It was purified by precipitation of polysaccharides with cold MeOH (5 °C). Supernatant was separated by centrifugation and reprecipited with EtOAc. The second supernatant was fractionated by silica gel column chromatography eluting with $CHCl_3$/EtOAc mixtures yielding two compounds, which were identified by ^1H- and ^{13}C-NMR spectral analysis. Both of them had been previously isolated from the same source, and the comparison of spectral data has facilitated the confirmation of their structure.[9,10]

Phospholipase Assay System. Micellar solutions of phosphatidyl choline were made from 7.8 mg of the substrate, to which 0.7−0.8 mL of 0.1 M ammediol−HCl buffer (pH 8.0) containing 1% deoxycholate was added. The material was sonicated, left standing until the foam subsided, and then made up to 1 mL. The substrate was stored at −10 °C for a maximum of 4 days.

Snake venom PLA_2 was dissolved in water at a concentration of 4.7 μg/mL. Then 6 μL, equivalent to 8.3 units, was added to one of three oxygraph cells equipped with magnetic stirring bars. Ammediol−HCl buffer (pH 8.49) (1.5 mL) containing 1×10^{-4} M Ca^{2+} and 2.25 mg of soybean lipoxygenase (1.5 mg lipoxygenase/mL buffer) were then added.

The oxygen probe of the oxygraph was inserted in the cell; care was taken to exclude all air bubbles. A 3 min preincubation period with stirring was allowed for temperature equilibration at 37 °C. Then, 100 μL of phosphatidyl choline was added to the cell to initiate the reaction, which was monitored for oxygen consumption. The progress of the reaction was recorded for 3 min.

The initial rates of oxygen incorporation were determined from the linear portion of the oxygen monitor trace at the onset of the reaction. These initial rates were linear for only 1 min, during which time a maximum of 30% of the total substrate was consumed.

The extract and the test compounds were dissolved in water, methanol, or acetone and added directly to the PLA_2 to ensure contact of the inhibitor with the enzyme.

The process was repeated as described before. If an inhibition of the reaction was observed, 20 μL of linoleic acid was added to the incubation mixture to determine whether it was really an inhibition of PLA_2 or of lipoxygenase. Dilutions of inhibitor were tested to obtain partial inhibition at different concentrations. The degree of inhibition was calculated from the disminution of the slopes when compared with noninhibited controls. The IC_{50} values were calculated.

For measurements of oxygen consumption, an "oxygraph" from the Yellow Springs Instrument Co. coupled to a Merck-Hitachi recorder was used.

Statistics. The 50% inhibitory concentration (IC_{50}) was calculated from the concentration/response analysis at a range of concentrations between 0.5 and 4 mM for those compounds and between 0.250 and 1.00 mg/mL for the extract, reaching over 50% inhibition at the last concentration. The level of statistical significance was determined by Dunnet's *t*-test for unpaired samples.

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