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SPECIFIC COMPETITIVE INHIBITOR OF SECRETED PHOSPHOLIPASE A_2 FROM BERRIES OF *SCHINUS TEREBINTHIFOLIUS*

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Key Word Index—*Schinus terebinthifolius*; Anacardiaceae; pink peppercorn; triterpenoids; masticadienoic acid; masticadienolic acid; schinol; ganoderic acids; phospholipase A2; inhibitors.

Abstract—Two structurally related triterpenoids **1** and **2** from pink peppercorn (berries of *Schinus terebinthifolius*) are identified and characterized as active site-directed specific competitive inhibitors of the three classes of secreted 14 kDa phospholipase A_2. The inhibitors not only protect the active site histidine from alkylation but also inhibit the action of secreted phospholipase A_2 from pig pancreas, human synovial fluid, and bee venom. Detailed X-ray crystallographic results on the structures of the inhibitors are provided. By physical methods and X-ray crystallography the triterpenoids were identified as masticadienoic acid and masticadienolic acid (schinol). Several other triterpenoids were ineffective as inhibitors of phospholipase A_2; however certain ganoderic acid derivatives showed noticeable inhibition. Results show that the side chain of these acidic tetracyclic terpenoids can access the catalytic-site region of phospholipase A_2, whereas the acyclic nucleus is at the interfacial recognition region. The selectivity of the assay protocol used here is demonstrated by the fact that the original screen of ethyl acetate extracts of 60 commercially available spices and herbs was carried out with phospholipase A_2 from pig pancreas, and only one extract showed inhibitory action on the hydrolytic activity in the scooting mode. Under such assay conditions the enzyme remains tightly bound to the surface of the substrate vesicles. In this way, nonspecific effects of additives that promote desorption of the enzyme from the substrate vesicle surface, under conditions in which the binding of the enzyme to the vesicle is weak, are avoided. The assay protocol is useful for the kinetic characterization of the inhibitors of phospholipase A_2, and it does not give false positive results with amphiphilic and hydrophobic compounds, as is the case with virtually all assay systems in use.

INTRODUCTION

Specific inhibitors of phospholipase A_2 have been sought for a variety of purposes. The active site-directed inhibitors are useful for kinetic and mechanistic studies of interfacial catalysis. Competitive inhibitors with a range of specificities, solubility and permeability properties are useful for elaborating the metabolic role of enzymes in organisms. Since phospholipase A_2 is believed to control a variety of processes ranging from mobilization of eicosanoids to metabolism of phospholipids, these inhibitors of phospholipase A_2 could be useful for controlling inflammatory processes such as rheumatoid arthritis, asthma and psoriasis [1, 2]. Until recently the progress in the assay and characterization of the inhibitors of phospholipase A_2 has been slow largely because of the inadequate characterization of most assay protocols which do no explicitly take into consideration the contribution of the binding of the enzyme to the interface [3]. Synthetic substrate analogues with an ether [4], phosphonate [5, 6], phosphate [7], or amide [8] substituent for the ester group in the *sn*-2 position have been useful for biochemical and crystallographic studies with the enzyme. Searches for specific inhibitors of phospholipase A_2 with suitable pharmacodynamic properties have also met with limited success. In this search, a series of phospholipase A_2 inhibitors were developed which lack the *sn*-3-phosphodiester group of phospholipids but contain a *sn*-2-phosphate or phosphonate group as a tetrahedral transition-state mimic. Such inhibitors were synthesized to satisfy a range of biophysical requirements and they are virtually as effective as the *sn*-3-phospholipid analogues [7, 9]. Recently we also reported isolation and identification of inhibitors of secreted phospholipase A_2 from the culture filtrates of an actinomycete [10]. These primary amides of fatty acids have the pharmacodynamic advantage of readily crossing the cell membrane, however design of 'better' inhibitors by structural modifications on such simple structures is relatively difficult. In this paper we report a class of phospholipase A_2 inhibitors which cannot readily cross cell membranes, but at

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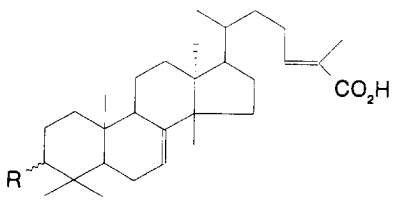
the same time contain a novel pharmacophore that interacts with the catalytic region of the enzyme. Such inhibitors could not only be useful as pharmacological leads, but as such these triterpenoids could be used for the characterization of the physiological functions of secreted phospholipase A_2 such as the ones from pancreas and synovial fluid.

Pancreatic phospholipase A_2 is an important component of the digestive system. In searching for a natural product that might inhibit phospholipase A_2, we reasoned that some dietary components could modulate the action of pancreatic phospholipase A_2. Through empirical experience, spices are often associated with putative effects on digestive processes. Therefore, it could be reasoned that a search for an inhibitor of pancreatic phospholipase A_2 in commonly available spices may have a reasonable expectation for success. Sixty samples of commercially available spices and herbs were tested. The crude ethyl acetate extract of only one of these plant materials showed significant phospholipase A_2-inhibitory activity. Two closely related active components isolated from the ethyl acetate extract of the berries of pink peppercorn account for more than 70% of the inhibitory activity.

RESULTS

The X-ray crystallographic results confirm the earlier report [11] that pink peppercorn contains masticadienoic acid (**2**) and the corresponding alcohol schinol or 3β-masticadienolic acid (**1**). The molecular structures of **1** and **2** are shown in Fig. 1. All intramolecular bond lengths and angles in the refinements of both **1** and **2** were normal. Considering the similarity of **1** and **2** (see structures), and the fact that **2** crystallizes with two molecules in the asymmetric unit, there is a possibility of several conformations of the carboxylic acid chain.

The methyl esters of **1** and **2** were not effective as inhibitors, whereas the potency of the corresponding amides was comparable to those of the acids. Coupled with the fact that the free fatty acids are not effective inhibitors of secreted phospholipase A_2 [7], it appears that the molecular characteristics important to the binding of these terpenoids to phospholipase A_2 include the conformation of the carboxylic acid chain, the hydrogen bonding occurring at the carboxylic acid site, and the bulk size of the alicyclic nucleus. The crystal structures show that, as expected, there is a significant degree of rotational freedom in the chain linking the acid and the triterpenoid nucleus. A least-squares best fit of atoms C-1 to C-14 of the triterpenoid nucleus allows a comparison of the different conformations of the acid-functional group (Fig. 2). Note that the change in hybridization of C-3 from sp^3 in **1** to sp^2 in **2** has only a small effect on the conformation of triterpenoid nucleus.



1 R = α-OH, H; **2** R = (=O)

The presence of two molecules in the asymmetric unit of **2** is a special consequence of dimer formation involving strong carboxylic acid–carboxylic acid hydrogen bonds (Fig. 3). Crystal packing requires a large rotation in the connecting chain, carbon atoms C-22 to C-25, to bring the carboxylic acid group into a position which allows hydrogen bonding to occur; the rotation can be seen in Fig. 2c. The addition of another possible site for hydrogen bonding in **1**, namely the hydroxyl group on C-3, results in the formation of a head-to-tail acid-hydroxyl ring involving four molecules (Fig. 4).

In order to determine the possibility of inclusion of the tetracyclic nucleus into the active site cavity of phospholipase A_2, measurements were made to determine the bulk size of the triterpenoid nucleus. The dimensions were determined by calculating planes which would just touch opposing van der Waals surfaces. The results show a width of 6.7 Å (roughly from C-1 across to C-6), a depth of 6.5 Å (roughly from C-18 to C-30), and a length of 11.8 Å. The length of the entire molecule is 19.1 Å. These dimensions are suitable for the incorporation of the side chain to the active site cavity of phospholipase A_2 as suggested by the fact that both **1** and **2** are active site-directed competitive inhibitors, and that their methyl esters are not inhibitory.

Inhibition of phospholipase A_2

More than 80% of the phospholipase A_2 inhibitory activity of the ethyl acetate extract of pink peppercorn was due to the two triterpenoids. Both **1** and **2** showed significant inhibition of the interfacial catalytic activity of phospholipase A_2 in the scooting mode [5, 7]. Under these conditions the enzyme binds tightly to the substrate interface and the bound enzyme doe not desorb from the interface even in the presence of lipophilic additives [12]. Therefore, the assay system used here is not susceptible to the changes in the organization and dynamics of the substrate interface, which is not the case with other assays. This conclusion, that the assay of phospholipase A_2 inhibitors in the scooting mode does not give false-positive results, is further strengthened by the observations reported here that out of the 60 plant extracts only one showed inhibition. Thus out of the several thousand compounds present in these extracts, only two compounds with inhibitory activity were identified. Such observations underscore the integrity, fidelity and reliability of the assay in the scooting mode for the inhibitors of interfacial catalysis.

The extent of inhibition depended on the mole fraction (X_I^*) of the inhibitor in the substrate vesicles. This mole

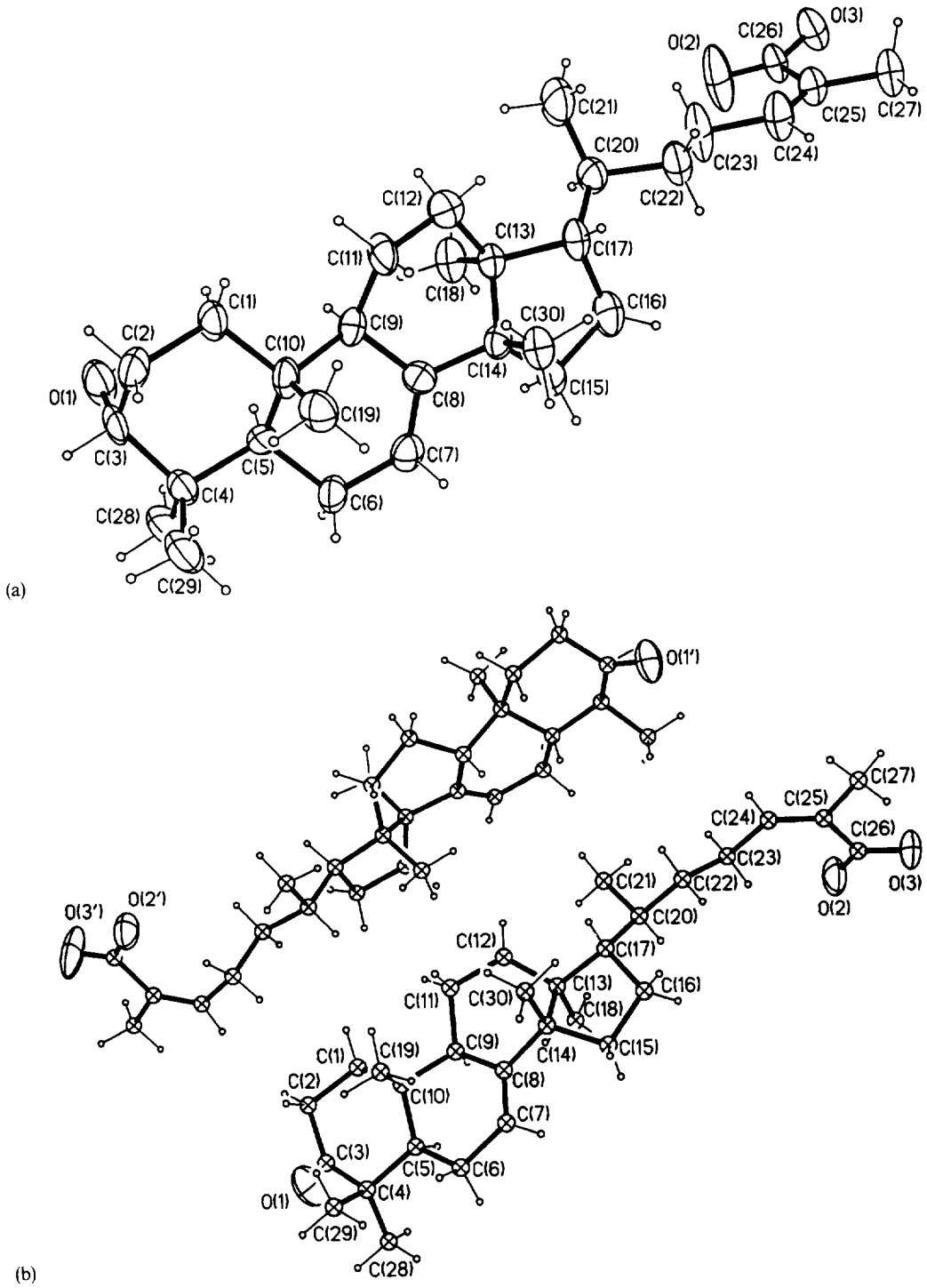


Fig. 1. Molecular structures of **1** (a) and **2** (b) drawn with 35% probability ellipsoids.

fraction dependence of the relative rate obeyed the relationship [12, 13]:

$$\frac{V_0}{V_I} = 1 + \left[\frac{1 + \frac{1}{K_I^*}}{1 + \frac{1}{K_M^*}}\right]\left[\frac{X_I^*}{1 - X_I^*}\right] \quad (1)$$

Here V_0 is the initial rate at mole fraction 1 of the substrate, V_I is the rate in the presence of the inhibitor at mole fraction X_I^*. K_I^* is the dissociation constant for the enzyme–inhibitor complex at the interface, and K_M^* is the interfacial Michaelis–Menten parameter for the substrate. From such plots the $X_I(50)$ values were obtained, i.e. the mole fraction of the inhibitor at $V_0/V_I = 2$. As summarized in Table 1, of the several triterpenoids tested

1 and **2** showed significant inhibitory effect at less than 0.05 mole fraction on the activity of phospholipase A_2 from three different sources. Compounds **1** and **2** were inhibitory and they also showed a significant difference in their inhibitory potencies with phospholipase A_2 from the various sources. It is also interesting that fusidic acid at 0.04 mole fraction showed a 50% inhibition of the pig pancreatic enzyme without any significant effect on the other two enzymes. Structure activity correlation based on the results summarized in Table 2 suggests that the aliphatic acidic side chain as well as the terpenoid nucleus are important for the inhibitory activity, however

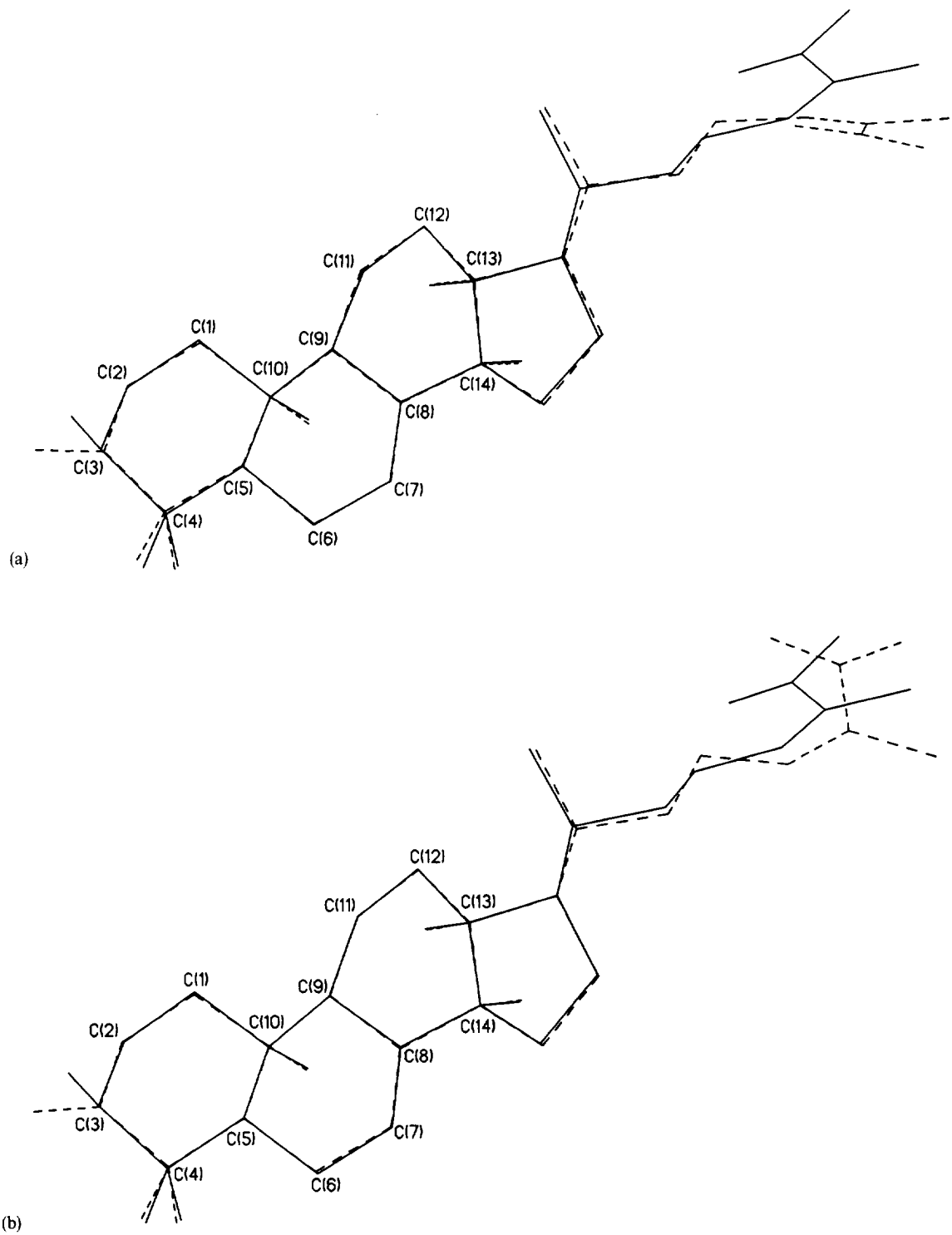
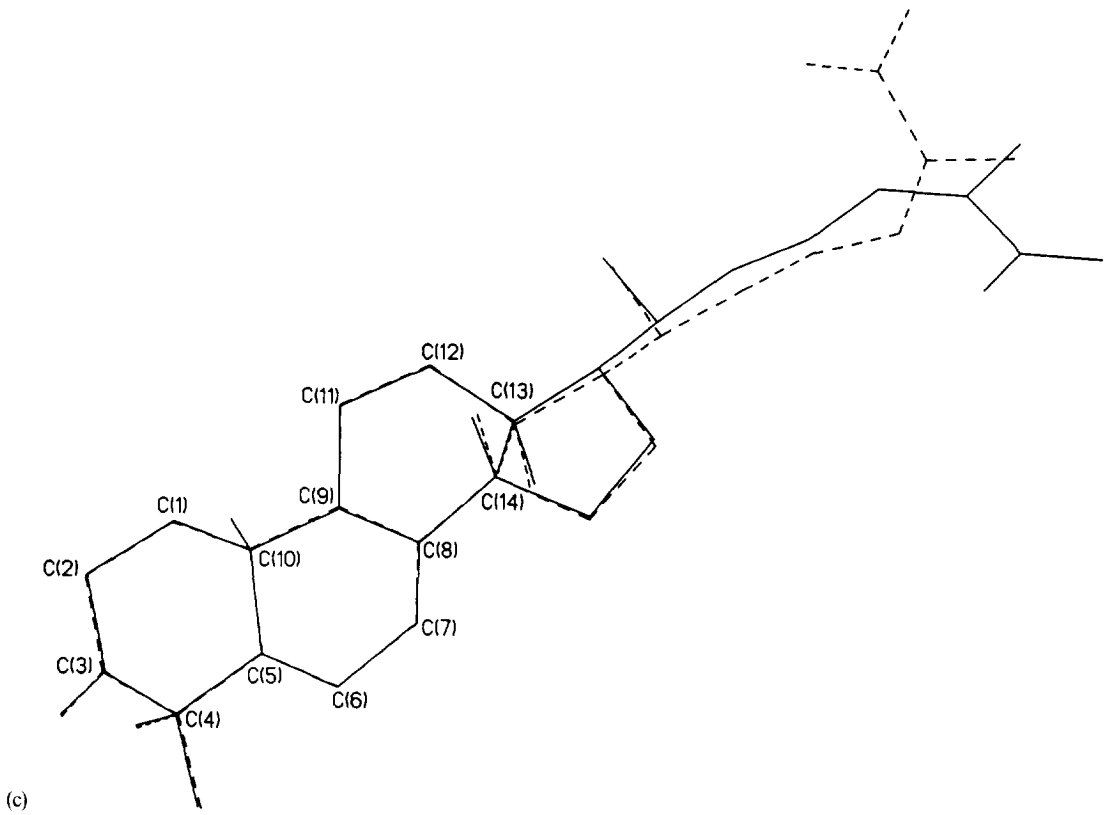


Fig. 2. Least-squares fit of (a) **1** (full) to molecule 1 of **2** (dashed), (b) **1** (full) to molecule 2 of **2** (dashed), and (c) molecules 1 (full) and 2 (dashed) of **2** for comparisons of the carboxylic acid conformations.



(c)

Fig. 2. *Continued*

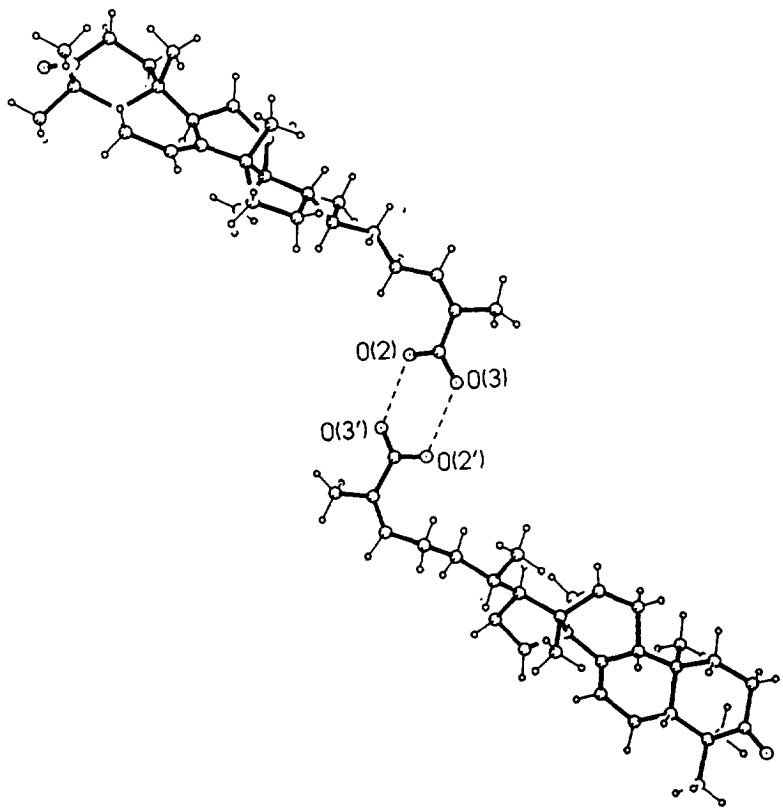


Fig. 3. Hydrogen bonding in **2**. Selected non-bonding distances and angles: O(2)···O(3′) = 2.60(2) and O(3)···O(2′) = 2.63(2) Å; C(26)-O(2)···O(3′) = 116(1)° and C(26)–O(3)···O(2′) = 122(1)°.

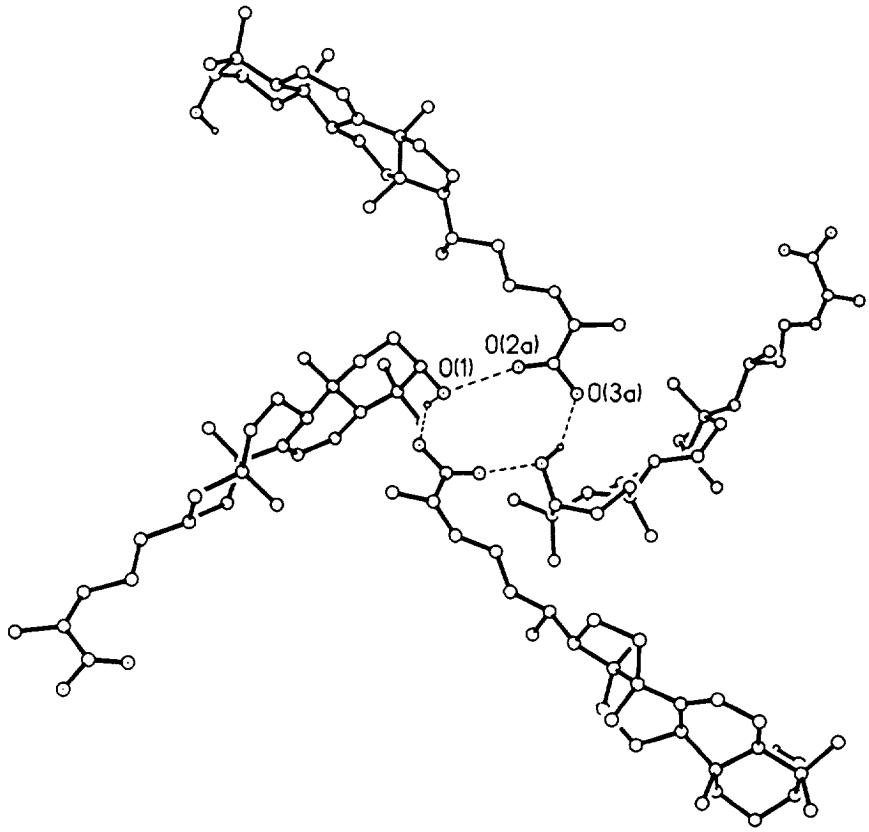


Fig. 4. Hydrogen bonding in **1**. Selected non-bonding distances and angles: O(1)···O(2a) = 2.65(2) and O(3a)···H(1ac) = 1.87(2) Å; C(26a)-O(2a)···O(1) = 166(1)° and C(26a)–O(3a)H(1ac) = 125(1)°.

Table 1. $X_1(50)$ values for inhibition of phospholipase A_2 by triterpenoids

Triterpenoid	Pig pancreas	Synovial fluid	Bee venom
1 (schinol)	0.016	0.05	0.01
2 (masticadienoic acid)	0.038	> 0.3	0.1
Helvolic acid	> 0.2	> 0.5	0.1
Oleanolic acid	> 0.5	> 0.5	> 0.5
Fusidic acid	0.04	> 0.5	> 0.5
Ursolic acid	> 0.5	> 0.5	0.1
Ganoderic acid A	NI	NI	NI
Ganoderic acid O	NI	NI	NI
Ganoderic acid R	0.03	NI	NI
Ganoderic acid S	0.08	NI	NI
Ganoderic acid T	0.02	0.03	0.05
Ganoderic acid T-OH	NI	NI	NI
Ganoderic acid T-OH-H2	NI	NI	NI

*NI, No inhibition up to 0.15 mole fraction.

a significant difference in the potency of ganoderic acids [14, 15] towards PLA2 from different sources suggests that the substitution pattern in the ring may also be critically important for the active site binding. These compounds are being investigated.

The inhibitory effect of **1** and **2** on phospholipase A_2 was examined in other assay systems. For example, hydrolysis of short chain phosphatidylcholines as well as the hydrolysis of long chain phosphatidylcholines dispersed in deoxycholate was inhibited by **1** and **2** but not by other triterpenoids summarized in Table 1. Hydrolysis of 1,2-dimyristoylglycerophosphomethanol vesicles catalyzed by secreted phospholipase A_2 from other sources was also inhibited only by **1** and **2**, but not other triter-

Table 2. Crystal data for $C_{30}H_{48}O_3$ (1) and $C_{30}H_{46}O_3$ (2)

Crystal parameters		
Formula	$C_{30}H_{48}O_3$	$C_{30}H_{46}O_3$
Formula weight	456.7*	454.7
Space group	$C222_1$	$P2_1$
a (Å)	14.580 (4)	6.907 (3)
b (Å)	22.179 (6)	19.860 (7)
c (Å)	19.810 (5)	19.768 (8)
β (deg)		94.98 (3)
V (Å^3)	6406.4 (28)	2701.4 (20)
Z	8	4
temp (K)	295	294
λ [Å (MoK_α)]	0.71073	0.71073
ρ (calc) (g cm^{-3})	0.947[a]	1.172
μ (MoK_α) (cm^{-1})	0.64[a]	0.70
R (F)†	0.0987	0.0833
RW (F)‡	0.1260	0.1114

*The solvent molecule(s) of unknown composition were removed from the formula weight, density, and absorption coefficient calculations for (1).

†$R = \sum(|F_o| - |F_c|)/\sum|F_o|$.

‡$RW = \{\sum w(||F_o| - |F_c||)^2/\sum w|F_o|^2\}^{1/2}$; $w^{-1} = \sigma^2 F_o + gF_o^2$; $g = 0.001$ (for both structures).

penoids (Table 1). These kinetic results show that **1** and **2** are specific inhibitors for secreted phospholipase A_2, and their selectivity for the three classes of secreted phospholipase A_2 is significant. The methyl ester of **1** did not show any inhibitory action. On the other hand, the $X_I(50)$ value for the amide derivative of **1** was 0.014 mole fraction.

The competitive nature of the inhibition of pig pancreatic phospholipase A_2 by **1** was also established by the kinetic protocol whose theoretical and experimental basis is described elsewhere [7, 13]. For example, the kinetics of hydrolysis can also be monitored under the substrate-limiting conditions where there is at most one enzyme molecule per enzyme-containing vesicle [12, 13]. This rate is obtained from the 'first-order' region of the progress curve where the slope decreases as the substrate is depleted. Thus $N_s k_i$ is the rate limited by the mole fraction of the substrate in the interface and by the inhibition with accumulated product. The ratio of the rate of hydrolysis ($N_s k_i$) in the absence and in the presence of an inhibitor is given by the relationship:

$$\frac{(N_s k_i)_0}{(N_s k_i)_I} = 1 + \left[\frac{1 + \frac{1}{K_I^*}}{1 + \frac{1}{K_P^*}}\right]\left[\frac{X_I^*}{1 - X_I^*}\right]. \qquad (2)$$

The mole fraction of the inhibitor required for 50% decrease in the value of $N_s K_i$, defined as $n_I(50)$ was 0.1 mole fraction for the pig pancreatic phospholipase A_2, compared to the value of $X_I(50) = 0.016$ mole fraction obtained under the zero-order kinetic conditions. The ratio of $X_I(50)/n_I(50) = 0.16$, is not only consistent with the value obtained for this enzyme with other competitive inhibitors [7, 9] but according to the following equation:

$$\frac{(N_s K_i)_0}{V_0} = \frac{\left[1 + \frac{1}{K_M^*}\right]}{\left[1 + \frac{1}{K_P^*}\right]} = \frac{\left[\frac{1}{X_I(50)} - 1\right]}{\left[\frac{1}{n_I(50)} - 1\right]}, \qquad (3)$$

this ratio is also consistent with the values of K_M^* (= 0.35 mole fraction) and K_I^* (= 0.03 mole fraction) for the pig pancreatic phospholipase A_2 obtained by independent methods [13].

A difference in the observed values of K_I^* (measured as described below) and $X_I(50)$ is consistent with equation (1). The value of K_M^* in the 0.3–0.5 mole fraction range was calculated for pig pancreatic phospholipase A_2 with DMPM, which is consistent with a value of 0.35 mole fraction obtained by other methods [13] including the use of several inhibitors [7, 12].

Compounds 1 *and* 2 *bind to the catalytic site*

An independent method for the evaluation of phospholipase A_2 inhibitors involves monitoring the inhibitor-mediated protection of the enzyme from inactivation by the active site-directed alkylating agent *p*-nitrophenacyl bromide [9, 12]. Compound **1** protects phospholipase A_2 only in the presence of calcium ion as would be expected for an active site-directed inhibitor (Fig. 5). In these studies the enzyme is bound to the aqueous dispersions of a neutral diluent such as deoxy-LPC (1-hexadecylpropanediol-3-phosphocholine) [7]. By definition, a neutral diluent is an amphiphile that forms an aggregate to which phospholipase A_2 binds via its interfacial recognition surface, but this amphiphile offers no protection of the enzyme toward alkylation because it has no detectable affinity for the active site of the enzyme. This approach yielded the K_I^* for the inhibitor. A value of $K_I^* = 0.005$ mole fraction was determined for the interaction of **1** with pig pancreatic phospholipase A_2 compared to a value of 0.015 mole fraction for **2**. Also, > 80% protection of the enzyme at saturating levels of **1** and

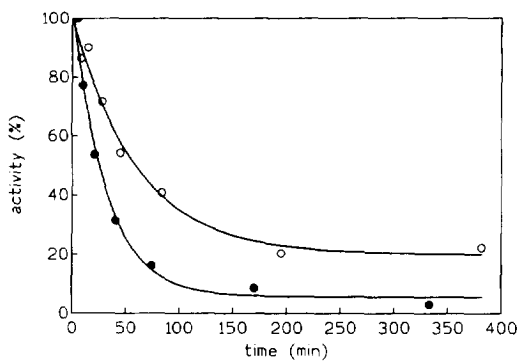


Fig. 5. The time course of inactivation of pig pancreatic phospholipase A_2 by *p*-nitrophenacylbromide in the (filled circles) absence or (open circles) presence of 0.1 mole fraction **1** at pH 7.5 in the presence of 1.6 mM deoxy-LPC (1-hexadecylpropanediol-3-phosphocholine) and 0.5 mM $CaCl_2$ [12].

2 was observed thereby establishing that the active-site histidine residue is blocked by the binding of the inhibitor. Protection from alkylation was observed only in the presence of calcium. Since the calcium-binding and catalytic sites are in the same vicinity, these results demonstrate that both 1 and 2 are active site-directed inhibitors. As already shown elsewhere [7, 10, 12, 16], the ability of organic molecules to protect His-48 from alkylation is very specific and a variety of organic solutes, including triterpenoids like ganoderic acid A and O (results not shown), do not offer any protection.

UV and fluorescence changes

Binding of phospholipase A_2 to the interface of a neutral diluent and the binding of an active site-directed ligand to the active site of the bound enzyme induce characteristic perturbations in the UV-absorbance and tryptophan fluorescence emission spectra; such changes are observed only in the presence of calcium [17, 18]. For example, 1 and 2 added to a mixture of phospholipase A_2 and the neutral diluent yields a characteristic difference spectrum for absorbance with an increase in the 250–300 nm region (Fig. 6), which is similar to the change induced by other specific competitive inhibitors. As is the case with the transition-state analogues, 1 and 2 showed the difference spectrum characteristic of twin peaks with absorbance maxima at 284 and 292 nm. The increase in the absorbance is proportional to the mole fraction of the inhibitor with the maximum change in the extinction coefficient, $\delta\varepsilon$, of about 1800 $M^{-1}\,cm^{-1}$. The concentration dependence showed a monotonic increase with the mole fraction of the inhibitor, but it could not be adequately fitted to a single dissociation constant.

As also observed with other active site-directed competitive inhibitors, 1 and 2 cause a change in the tryptophan fluorescence emission characteristics; the major change being an increase in the emission at 333 nm. The increase in the emission intensity depends on the mole fraction of the inhibitor (Fig. 7). While 1 was more effective than 2, it is not possible to calculate the apparent dissociation constant from such plots which are expected to be nonlinear. The initial linear regions shown in this figure reflect only the early part of the binding isotherm and have little significance beyond this interpretation.

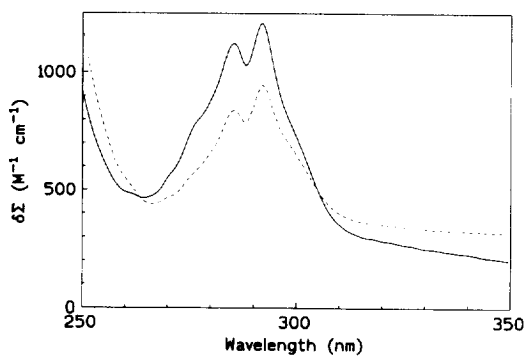


Fig. 6. The change in the ultra-violet molar extinction coefficient of pig pancreatic phospholipase A_2 bound to 3.3 mM deoxy-LPC in the presence of 0.01 mole fraction 1 (full) and 2 (dashed) in 10 mM Tris, 3 mM $CaCl_2$ at pH 8.

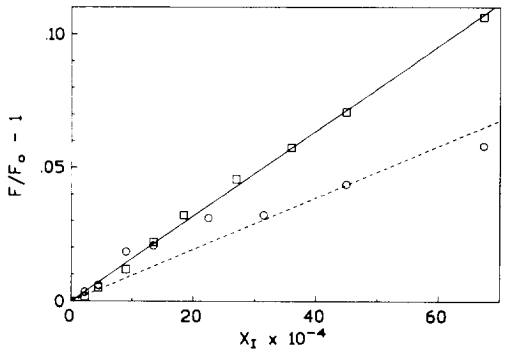


Fig. 7. The change in the fluorescence emission intensity of pig pancreatic phospholipase A_2 bound to 3.3 mM deoxy-LPC as a function of the mole fraction of (squares) 1 or (circles) 2 in 10 mM Tris, 3 mM $CaCl_2$ at pH 8.

DISCUSSION

Studies reported in this paper show that 1 and 2 are a new class of active site-directed specific inhibitors of phospholipase A_2. These compounds are structurally different than other active site-directed competitive inhibitors of phospholipase A_2 reported in the published literature, which are substrate analogues with one or two alkyl chains. The requirement for calcium ion as a cofactor for the binding to the active site, protection of His-48 from alkylation, and the characteristic spectral perturbations induced on the binding of 1 and 2 to phospholipase A_2 at the interface suggest that these triterpenoids interact with the enzyme in the active site region. The kinetic results also suggest that the mechanism of inhibition is kinetically competitive for the interfacial catalysis. Preliminary molecular modeling studies suggest that the carboxylic group of the aliphatic side chain of 1 and 2 could simultaneously coordinate with the catalytic cofactor calcium and hydrogen bond to the catalytic residue His-48. The magnitude of these interactions could account for the inhibitory potencies of these triterpenoids and this assertion is also consistent with the hydrogen-bonding tendency of these groups reflected in the crystal structure (Figs 3 and 4).

A comparison with the effect of other triterpenoids suggests that there is a strong correlation between the structural features and the inhibitory activity. Based on the shape of the molecules involved it is clear that the molecule could access the active site cavity leading to the catalytic site [6, 8] only along the long axis. The fact that the methyl esters of 1 and 2 do not exhibit any inhibition suggests that the carboxyl group containing side chain of the triterpenoid is in contact with the catalytic site. The side chain extends about 8 Å beyond the tetracyclic nucleus. The dimensions of the rigid tetracyclic triterpenoid

nucleus are such that it could possibly interact with the hydrophobic region of the collar of the cavity leading to the active site where the side chain would enter the active site. The amphipathic character of **1** and **2** would also promote their orientation in the bilayer surface such that the ionized carboxylate group would be at the surface, whereas the tetracyclic nucleus would preferentially partition into the hydrophobic region of the bilayer. Similarly the lower potency of **2** is probably due to the hydroxyl group at the end of the molecule that will be forced in the hydrophobic interior of bilayer. While **1** with its apolar cabonyl group would be suitably oriented for binding to the active site of the bound enzyme, the hydroxyl containing **2** will tend to orient in a different way in the bilayer so as to minimize contacts of the hydroxyl group with the acyl chains of the phospholipids. Such factors that determine the orientation of amphiphiles in the organized bilayer could also be responsible for differences in the potencies of triterpenoids with polar substituents in the nucleus.

The fact that masticadienoic acid inhibits action of secreted phospholipase A_2 may have pharmacological relevance. Even during the pre-Columbian period in the Mediterranean countries, gum mastic was believed to have antiinflammatory properties. Indeed, masticadienoic acid is a major component of this resin.

Finally, having demonstrated the efficacy of our assay protocols for the screening and characterization of specific competitive inhibitors of phospholipase A_2, we can make comparisons to assay protocols reported by others. A large number of compounds have been reported as inhibitors [19–27] because they reduce the activity of phospholipase A_2 under certain assay conditions. We have evaluated many of these inhibitors and found them to be ineffective as specific competitive inhibitors in our assay (data not presented, however see refs [7] and [16]). The problem with the commonly used assays is that such protocols do not consider phospholipase A_2 as an interfacial enzyme, i.e. the enzyme must bind to the interface for the catalytic turnover in the interface. Thus any perturbation of the interface that changes the binding equilibrium of the enzyme to the interface would also influence the observed rate. This problem affects virtually all the assay systems in use because the affinity of phospholipase A_2 for binding to the lipid dispersions is in the millimolar range. The problem becomes particularly acute in some assay systems when the bulk concentration of the substrate may be as low as 1 μM. Thus, depending upon the partition coefficient of the test material, many 'submicromolar' inhibitors of PLA2 have been discovered because under such conditions the effective substrate concentration that the enzyme 'sees' at the interface is reduced by 50%, even if the inhibitor concentration is 1 μM. Additional problems arise if the size of the substrate dispersions is small. In such cases the substrate replenishment on the enzyme-containing particles from the excess substrate aggregates becomes rate-limiting, rather than the chemical step of the catalytic turnover cycle [28]. This is indeed the case in the mixed micellar dispersions of phospholipid and detergents. In conclusion, the protocols described here provide one of the most effective methods for the assay and unequivocal characterization of specific competitive inhibitors of interfacial enzymes.

EXPERIMENTAL

All herbs and spices were obtained from a local shop (Pequea Trading, Strausberg, PA). All solvents (Aldrich), TLC plates (Merck), chromatography-grade silica gel (Aldrich), several triterpenoids (Aldrich) and buffers (Sigma) were used as supplied. Compounds on TLC plates were visualized by charring the glass plates after spraying with ammonium molybdate in 30% H_2SO_4. Phospholipase A_2 from the various sources were obtained as described [16, 29].

Extraction and isolation. The dried pulverized plant materials obtained from the supplier were used for extraction without further processing. For the initial screen, samples were prepared by extraction of 30 g of plant material for 6–10 hr in a Soxhlet charged with 300 ml EtOAc under reflux. More than 30 cycles of extraction for each sample took place during this period and very little, if any, additional solid extract was obtained in the last extraction cycle. The extract was evapd, then dissolved in dimethylformamide (0.2 mg ml^{-1}); this stock solution was used for the initial inhibition screen. Berries of pink peppercorn yielded 2.4% solid material in the EtOAc extract.

EtOAC extracts of the following spices were prepared and tested for the inhibitory action: allspice ground and solid, angelica, anise, star anise, basil, bay leaves, calendula, caraway seeds, cardamon pods, catnip, celery seed, chervil, chili pepper (several varieties), chives, cilantro, cinnamon bark, cloves, coriander, dill seed, dill weed, fennel, dried ginger root, hibiscus, juniper berries, lavender, lemon verbena, lemon balm, lemon peel, lemon grass, lemon thyme, mace, marjoram, mustard seeds, myrrh, oak moss, orange peel, onion flakes, oregano, orris root, patchouli, parsley, pennyroyal, green peppercorns, lampong peppercorns, pink peppercorns, Szechuan peppercorns, Tellicherry peppercorns, white peppercorns, peppermint, poppy seed, rosehip, rosemary, sage, sandalwood, sesame seed, spearmint, sumac berries, tarragon and tansy.

Assay and kinetics of inhibition. Difficulties encountered with most of the assay protocols for inhibitors of phospholipase A_2 are well known [3, 5, 7, 12]. Such problems are obviated by the assay of phopholipase A_2 activity in the scooting mode, i.e. with kinetic analysis of the hydrolytic action of phospholipase A_2 on vesicles of DMPM (1,2-dimyristoyl-glycero-*sn*-3-phosphomethanol) in the highly processive mode where the enzyme does not leave the interface between the catalytic turnover cycles. As described earlier, such studies are best carried out by the pH-stat method [13, 30–32]. Typically the reaction mixture contained 0.6 mg DMPM in 4 ml of 1 mM NaCl and 1 mM $CaCl_2$ at pH 8.0 and 23° and the reaction is initiated by adding the enzyme (typically

0.05 g or less of pig pancreatic phospholipase A_2). The test sample or inhibitor was always added to this mixture containing the substrate vesicles before the addition of the enzyme. To promote fusion of vesicles in order to extend the period for the zero-order portion of the progress curve, polymyxin B sulphate (0.02 mg) was added to the reaction mixture just before the addition of the enzyme [7, 32]. Progress of the reaction was recorded for 3–5 min. The slope, v_0, is defined as the initial rate of catalytic turnover (per enzyme per sec) in the absence of the inhibitor, and v_i is the initial rate in the presence of the inhibitor at the indicated concentration or mole fraction in the substrate. The initial rates had an uncertainty of less than 10%. By this protocol, the inhibitory effect of up to 0.4 mg crude extract in the reaction mixture could be tested readily. Due to possible difficulties (solubility, pH imbalance, covalent modification of the enzyme) arising from the unknown components present in a test sample, this protocol was strictly followed to test the inhibitory action of not only the crude extract, but also of the chromatographic fractions containing the organic solvents. We did not encounter any 'false positives' although other assays for phospholipase A_2 inhibitors suffer from nonspecific inhibition due to modification of the substrate interface.

For the kinetic characterization of inhibitors under first-order reaction progress, the conditions were essentially the same as above, except that the population of DMPM vesicles was reasonably monodisperse and the calcium concentration was kept at 0.6 mM and polymyxin was not added [12, 30, 31].

Isolation of inhibitors. Of the 60 crude plant extracts tested, only the extract of pink peppercorn showed a reasonable inhibition, i.e. 50% inhibition at about 0.2 mg in 4 ml reaction mixture containing 0.4 mg DMPM vesicles as substrate. The crude extract left after the evaporation of EtOAc was triturated with hexane and most of the inhibitory activity was found in the gummy solid that separated from the mixture after storage at room temperature. For further fractionation, the hexane insoluble solid (10 g) was applied in 100 ml EtOAc to a column of silica gel (300 g) packed in hexane. The column was successively eluted with increasing proportion of EtOAc in hexane in increments of 10% v/v. The eluted fractions were assayed for the inhibition of pig pancreatic phospholipase A_2 activity, usually without removal of the solvent. Typically up to 0.2 ml of the solvent could be added without any noticeable anomalous effect on the initial steady-state rate. Most of the phospholipase A_2-inhibitory activity of the pink peppercorn extract was eluted with 30–60% EtOAc in hexane. It showed the presence of at least three major components, two of which could be separated by fractional crystallization from a mixture of hexane and $CHCl_3$ or a mixture of hexane and Et_2O. Two active crystalline components, **1** and **2**, were homogeneous by TLC and by HPLC on the ODS (octadecyl-chain bonded) reversed phase column eluted with MeOH. The isolated compounds did not show a further increase in the phospholipase A_2 inhibitory activity on further column chromatography or crystallization. It appears that **1** and **2** are responsible for more than 70% of the phospholipase A_2-inhibitory activity of the crude extract. The eluted fractions in which **1** or **2** were not present did not show any significant inhibitory activity.

The methyl esters of **1** and **2** were prepared by treatment of the acid in Et_2O with CH_2N_2. The amide of **1** was prepared by treatment of the acid with oxaloyl chloride followed by treatment with ammonia. The product was purified by chromatography on silica gel.

Characterization of compounds **1** *and* **2**. The molecular formula of the alcohol **1** was $C_{30}H_{48}O_3$ (*m/z* 456) and $C_{30}H_{46}O_3$ (*m/z* 454) for **2**, a ketone. Both gave crystals of good quality, with further characterization of their structure (Fig. 1) carried out by X-ray diffraction analysis on single crystals.

Crystallographic analysis. Crystals of **1** and **2** were obtained from slow evapn of an Et_2O–hexane mixture. The crystallographic data are summarized in Table 2. Preliminary photographic characterization showed that **1** possessed *mmm* Laue symmetry, while **2** possessed 2/*m* symmetry. The systematic absences in the diffraction data uniquely established the space group as $C222_1$ for **1**, while the combination of the systematic absences and optical activity of **2** indicated $P2_1$ as an appropriate choice. Because the absorption coefficients for **1** and **2** (0.64 and 0.70 cm^{-1}, respectively) were low, absorption corrections were considered unnecessary.

Both structures were solved by direct methods and completed by difference Fourier synthesis. For **1**, all non-hydrogen atoms were refined with anisotropic thermal parameters and all hydrogen atoms were treated as idealized, updated isotropic contributions. The structure of **1** contains five non-hydrogen atoms which could not be modeled adequately to allow chemical identification. These five atoms occupy a position in the lattice far enough removed from the triterpenoid molecules to preclude hydrogen bonding and were assumed to be disordered solvent. Compound **2** contains two crystallographically independent molecules in the asymmetric unit. Due to the small number of observed reflections, only the oxygen atoms in **2** were refined anisotropically. The hydrogen atoms were again treated as idealized, updated isotropic contributions. All computations used the SHELXTL PLUS (4.27) program library (G. Sheldrick, Siemens, Madison, WI).

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