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Inactivation of Human Pleural Fluid Phospholipase A_2 by Dioscin

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The natural product, spirostanol glycoside dioscin, was shown to directly inactivate human pleural fluid phospholipase A_2 (PLA_2). Inactivation was dose, and time dependent. The IC_{50} was estimated at 18 μM and virtually complete inactivation of the enzyme occurred at 50 μM. Using Michaelis-Menten kinetics, dioscin inactivated the enzyme by a competitive inhibitory manner, the apparent *Ki* value was 6.9×10^{-4}M. Reversibility was studied directly by dialysis method, the inhibition was reversible. Addition of excess Ca^{2+} concentration up to 8 mM did not antagonize the inhibitory activity of dioscin. Inactivation of several kinds of PLA_2 by dioscin, showed a broad range of PLA_2 specificity. These data suggest that inactivation of PLA_2 by dioscin is due to interaction with the active site of PLA_2 and may be a useful adjunct in the theraphy of inflammatory diseases.

Key words: Phospholipase A_2 (PLA_2), Dioscin, Human pleural fluid (HPF)

INTRODUCTION

Phospholipase A_2 (PLA_2) is lipolytic enzyme that specifically hydrolyzes the *sn-2* position of glycerophospholipid. It exits in both extracellular and intracellular forms. The former can be classified into two types, group I and group II, based on their primary structure (Heinrikson *et al.*, 1977). Mammalian PLA_2s of group II have a molecular weight of 14 KDa and are activated and secreted as soluble proteins from a variety of physiologically stimulated cells, such as platelets (Horigome *et al.*, 1987), neutrophils (Lanni *et al.*, 1983), and renal mesangial cells (Pfeilschifter *et al.*, 1989).

Especially, mammalian extracellular group II PLA_2s are found in inflammatory regions, such as glycogen-induced ascitic fluid in rats (Franson *et al.*, 1987), casein-induced peritoneal fluid in rats (Chang *et al.*, 1987), synovial fluid of patients with rheumatoid arthritis (Vadas *et al.*, 1985), and pleural fluid of patients with tuberculosis (Baek *et al.*, 1991, 1992). Recently, Bomalaski *et al.* (1991) reported that the recombinant enzyme of human group II PLA_2 elicits a dramatic inflammatory arthritogenic response when injected into the joint space of healthy rabbits. Thus, these findings strongly implicate the importance of group II PLA_2 in the development and possibly in the propagation of inflammatory processes. If such is the case, one might anticipate that inhibition of group II PLA_2 would attenuate the severity of inflammation. Therefore, several institutions are screening and developing PLA_2 inhibitors, such as marine products (manolide;Marshal *et al.*, 1990, scalaradial;marianne *et al.*, 1991), synthetic chemicals (Gelb, 1986), endogenous proteins (lipocortin; Marshall *et al.*, 1990, C3dg; Suwa *et al.*, 1990), and many kind of natural products (Rosenthal *et al.*, 1989).

With the use of group II PLA_2 purified from pleural fluid patients with tuberculosis, we successfully isolated a new type of compound, dioscin, a spirostanol glycosides from the rhizoma of *Smilax china* L. which has a strong PLA_2 inhibitory effect. The present investigation describes the inactivation mechanism of group II PLA_2 by dioscin.

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EXPERIMENTAL METHODS

Materials

[1-^{14}C]Linoleic acid was purchased from Amersham, U.K. Sepharose CL-4B and heparin-Sepharose CL-6B were purchased from Pharmacia Fine Chemicals, Sweden. Butyl-Toyopearl 650 M was purchased from Tosoh, Japan. *Naja naja naja* PLA_2, *Crotalus durissus terri-*

ficus PLA_2 and porcine pancreatic PLA_2 were purchased from Boeringer Mannheim Biochemica, Germany. Human synovial fluid PLA_2 and rat platelet PLA_2 were purified as described previously (Hara *et al.*, 1989, Horigome *et al.*, 1987). Dioscin also was isolated from *Smilax china* L. (Kim *et al.*, 1989).

Preparation of Labeled Substrates

1-Acyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine and 1-acyl-2-[1-^{14}C]-linoleoyl-*sn*-glycero-3-phosphoethanolamine were prepared as described previously (Arai *et al.*, 1985). Their specific activities were adjusted to 1000 cpm/nmol by dilution with unlabeled egg yolk phosphatidylcholine, and egg yolk phosphatidylethanolamine prepared from egg yolk phosphatidylcholine by transphosphatidylation, respectively (Kokusho *et al.*, 1987).

Purification of Pleural Fluid PLA_2

Pleural fluids were obtained from patients with tuberculosis. Cells and debris were removed from pleural fluids of the patients by centrifugation immediately at 4℃, and then pooled and stored −20℃ until used. During storage, no significant decrease in PLA_2 activity was observed. Human pleural fluid PLA_2 was purified by sequential use of chromatography on heparin-Sepharose CL-6B, butyl-Toyoperal 650 M and Protein PAK-125 HPLC (Waters) (Baek *et al.*, 1992).

Assay of PLA_2

The standard incubation mixture (0.2 ml) for the assay of PLA_2 contained 100 mM Tris-HCl buffer, 6 mM $CaCl_2$, 10 ng of PLA_2 and 20 nmol of 1-acyl-2-[1-^{14}C]linoleoyl-phosphatidylethanolamine. The reaction was carried out at 37℃ for 10 min, then stopped by adding 1.25 ml of Dole's reagent (Dole *et al.*, 1960) and released free fatty acid was extracted and subjected to liquid scintillation counting (Packard) by the method of Natori (Natori *et al.*, 1983).

Treatment of PLA_2 with Dioscin

HPF-PLA_2 and dioscin were preincubated at 8X the desired final concentration at 37℃ for 10 min. In control assay tubes, identical volumes of vehicle were incubated with the enzyme. Following preincubation, aliquots of the dioscin/enzyme mixture were added to equilibrated substrate, and the reaction was allowed to proceed as assay of PLA_2. Vehicle effects of dimethylsulfoxide (DMSO) on the enzyme were examined similarly in control assay tubes. The reactions were started by the addition of substrate to the preincubation mixtures. In both systems, percent inhibition relative to control samples was calculated.

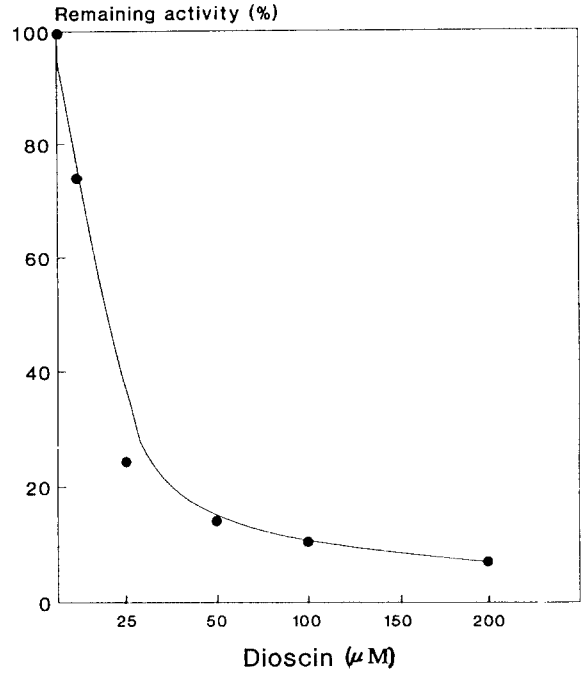

Fig. 1. concentration-dependent inactivation of human pleural fluid PLA_2 by dioscin. PLA_2 (10 ng) was incubated for 20 min. at 37℃ in 0.1 M Tris-HCl buffer, pH 9.0, 6 mM $CaCl_2$, 20 nmol substrate in the presence of various concentrations of dioscin. Each value is the mean of duplicate determinations.

Dialysis Studies

HPF-PLA_2 was incubated with various concentrations of dioscin as mentioned above for 30 min. Equal aliquots were placed in two separate dialysis bags with a molecular weight cut off of 6000-8000 Da. One dialysis bag was dialyzed with a 1 : 1000 ratio of enzyme mixture to buffer at 4℃ with two buffer changes during 4 hours and the other bag was kept at 4℃ for 4 hours, after which a post-dialysis sample was assayed for PLA_2 activity. The hydrolytic activity of the inactivated enzyme was compared between dialyzed and non-dialyzed samples. Controls were analyzed in a similar manner.

RESULTS AND DISCUSSIONS

Phospholipase A_2 (PLA_2) specifically hydrolyzes the *sn*-2 ester bond of glycerophospholipids to liberate arachidonate which profoundly influences inflammatory reactions. The regulatory molecules of this enzyme activity could be implicated in the control of a wide range of physiological and pathological states such as inflammation, asthma, ischemia, pancreatitis and rheumatoid arthritis. Thus the relationship of PLA_2 activation to the inflammatory response has assumed an increasing role in the development of pharmacolo-

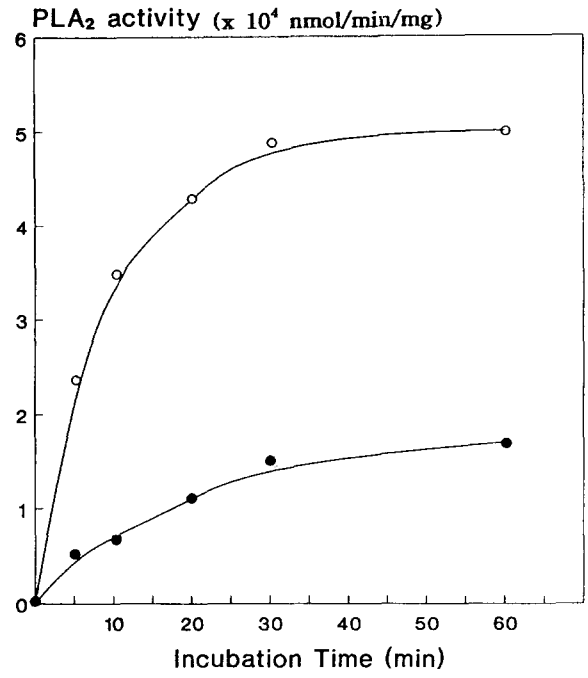

Fig. 2. Time course of the effect of dioscin on human pleural fluid PLA_2. PLA_2 was incubated for the periods indicated with dioscin. For each time point, released radioactivity is expressed as a percent activity remaining of that obtained in controls (DMSO). Each value is the mean of duplicate determinations.

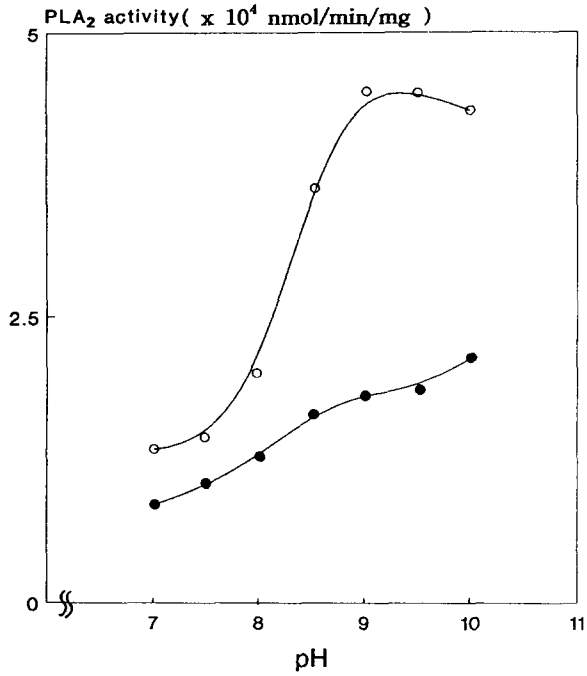

Fig. 3. Effect of pH on the inactivation of human pleural fluid PLA_2 by dioscin. PLA_2 was incubated in the presence (●) or absence (○) of dioscin. The buffers used were 100 mM Tris-HCl, pH 7.0-9.0, 100 mM glycine-NaOH, pH 9.5-10.0. Each value is the mean of duplicate determinations.

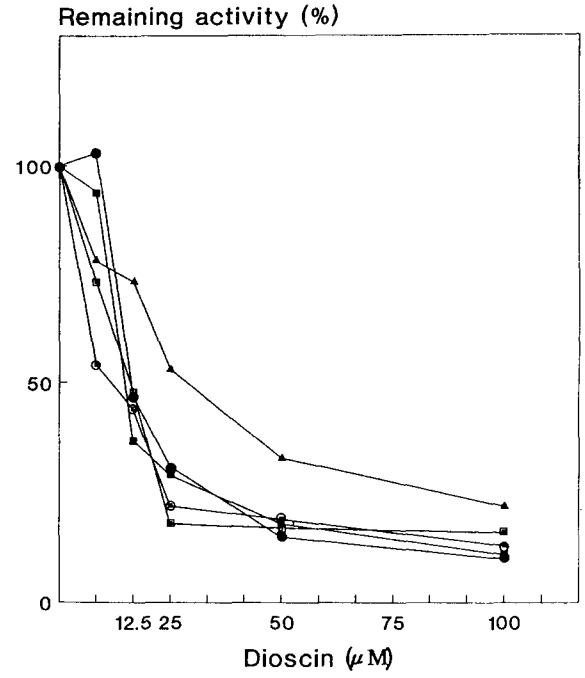

Fig. 4. Inactivation of various PLA_2s by dioscin. Standard reaction mixtures contained 10 ng of various PLA_2s, and the indicated concentrations of dioscin. Inhibition is expressed as a percent activity remaining of that obtained in controls (0 μM inhibitor). PLA_2s used were *naja naja naja* PLA_2 (□), procine pancreatic PLA_2 (▲), Crotalus durissus temificus PLA_2 (■), rat platelet PLA_2 (○) and synovial fluid PLA_2 (●). Each value is the mean of duplicate determinations.

gical agents which mitigate reversal of disease. Recently, many kinds of PLA_2 inhibitors have been developed: Irreversible inhibitors, including *p*-bromophenacyl bromide (*p*-BPB), mepacrine (Wallach *et al.*, 1981), manoalide (Marshall *et al.*, 1990) and thielocin from Ascomycetes (Yoshida *et al.*, 1991) as well as amide ether analogues of phospholipids (Gelb, 1986). Proteinous inhibitors such as lipocortin (Davidson *et al.*, 1987) and C_3dg fragment (Suwa *et al.*, 1990) were produced from peritoneal exudate of rats in response to the glucocorticoid.

In the course of screening for PLA_2 inhibitors from natural products, we found that the spirostanol glycoside, dioscin from the rhizome of *Smilax china* L. has a strong PLA_2 inhibitory effect. We found that dioscin inhibits group II PLA_2 in a dose dependent manner. The purified PLA_2 used in this stady was obtained from the pleural fluid patients with tubereulosis.

The IC_{50} was estimatedat 18 μM and virtually complete inactivation of the enzyme occurred at 50 μM (Fig. 1). The degree of inactivation of PLA_2 was gradually increased and maximum inhibitory effect emerged about 10 min later. The loss of enzyme activity was non-linear with respect to time on a logarithmic plot (Fig. 2). When the IC_{50} concentration of dioscin and

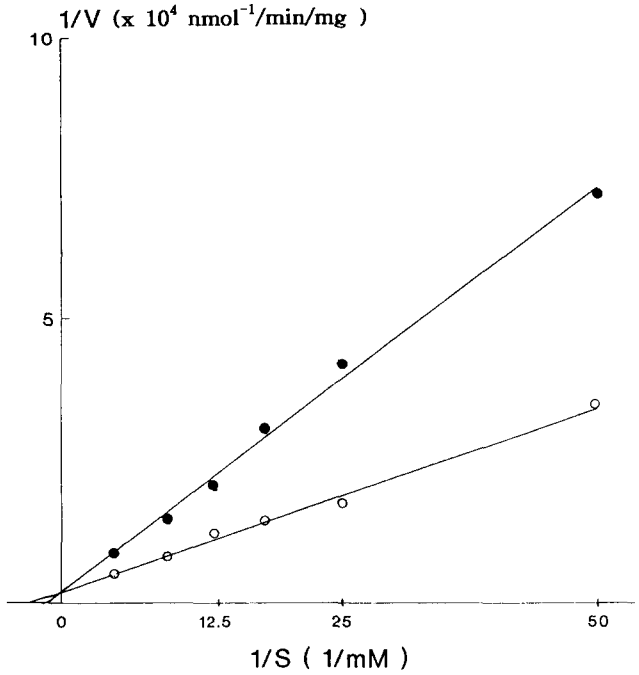

Fig. 5. Effect of substrate concentration on the inhibition of human pleural fluid PLA_2 by dioscin. Double-reciprocal plot of human pleural fluid PLA_2 activity toward phosphatidylethanolamine in the presence (●) or absence (○) of dioscin. Standard assay conditions were employed and the lines drown on the basis of regression analysis.

Table I. Reversibility of the dioscin-human pleural fluid PLA_2 complex. Dioscin-PLA_2 mixtures were preincubated at 37℃ for 30 min prior to pre-dialysis sampling (Before dialysis); the remainder of the dioscin-PLA_2 mixture was dialyzed in cellulose tubing at 4℃ for 4 hours with two buffer changes during the 4 hours, after which post-dialysis samples were radioassayed (After dialysis)

	Dialysis following 30-min preincubation % Reduction of enzyme activity	
	Before dialysis	After dialysis
Dioscin	90.6	26.6

PLA_2 were incubated at varied pH, the inhibitory effect of dioscin was 35% at pH 7 and 50% at pH 10.0. Jacobs *et al.* (1987) have shown that manoalide from marine sponges inhibits bee venom PLA_2 pH dependent, they argued that the ionization of specific residues of manoalide is essential for maximum inactivation. However, dioscin didn't show such an effect, because it dosen't have any ionizable functional group in the molecule at this pH range.

Hydrolytic activity of PLA_2 was also dependent on the concentration of Ca^{2+} which has a specific binding site on the enzyme. Ca^{2+} concentrations up to 8 mM afford no protection against inactivation of PLA_2 by dioscin (data not shown). This result demonstrates that dioscin did not change the PLA_2 activity by antagonism of the Ca^{2+} binding site as does manoalide.

A comparative test was done to find out how dioscin affects of PLA_2s from other sources. We used *naja naja naja* PLA_2, porcine pancreatic PLA_2 (group I type), *Crotalus durrissus terrificus* PLA_2, rat platelet PLA_2, and human synovial fluid PLA_2 (Group II type). As a reult, dioscin inhibited all these enzymes in a dose dependent manner. The IC_{50} for these enzymes was 12 μM, 29 μM, 11 μM, 9 μM and 14 μM, respectively. The IC_{50} for pancreatic PLA_2 was considerably higher than that of the other enzymes as shown in Fig. 4. To date, we have examined dioscin inactivation of various types of PLA_2; we have not found any specificity for PLA_2 such as in the case of *p*-BPB. To confirm the reversibility of the dioscin-PLA_2 complex, we investigated directly by dialysis method. As shown in Table I, the dioscin-PLA_2 complex was preincubated for 30 min at 37℃ and stored at 4℃ for 4 hours. The remaining PLA_2 activity was barely detectable. However, when the diosin-PLA_2 complex was preincubated in the same condition and then dialyzed at 4℃, about 80% of the enzyme activity was recovered. Our data indicate that the inactivation is apparently reversible.

As shown in Fig. 5, inactivation of PLA_2 by dioscin showed a competitive inhibitory profile using [^{14}C]linoleoyl PE as substrate. The *Ki* value was about 6.9×10^{-4} M. This result suggests that inactivation of PLA_2 might be caused by binding of dioscin with the active site of PLA_2. Kinetic studies with PLA_2 are very complicated by the fact that the binding of the enzyme to the lipid-water interface must precede catalytic tumover. Many nospecific inhibitors have been thought to affect the quality of the interace by modifying phospholipid bilayer properties that render phopholipid inaccessible to the enzyme (Fawzy *et al.*, 1988). In this point, the possibility that dioscin does bind at the substrate binding site of PLA_2 cannot be completely exclued.

To our knowledge, the PLA_2 inhibitory effect of dioscin has not been described elsewhere. Although we do not know whether the mechanism of PLA_2 inhibition by dosicin and prosapogenin A of dioscin is the same (data not shown), it will be of great interest to monitor the relationship between the inhibitory effect and the structure of the sugar moieties of dioscin. Further studies should be carried out.

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REFERENCES CITED

Arai, H., Inoue, K., Natori, Y., Banno, Y., Nozawa, Y. and Nojima, S., Intracellular phospholipase activities of *Tetrahymena pyriformis*. *J. Biochem.*, 97, 1525-1532 (1985).

Baek, S. H., Takayama, K., Kudo, I., Inoue, K., Lee, H. W. and Chang, H. W., Detection and characterization of extracellular phospholipase A_2 in pleural effusion of patients with tuberculosis. *Life Sci.*, 49, 1095-1102 (1991).

Baek, S. H., and Chang, H. W., Purification and characterization of extracellular phospholipase A_2 in pleural fluid of patients with tuberculosis. *Kor. Biochem. J.*, 25, 647-652 (1992).

Chang, H. W., Kudo, I., Tomita, M. and Inoue, K., Purification and characterization of extracellular phospholipase A_2 from peritoneal cavity of caseinate-treated rat. *J. Biochem.*, 102, 147-154 (1987).

Davidson, F. F., Dennis, E. A., Powell, M. and Glenney, J., Inhibition of phospholipase A_2 by lipocortin: an effect of binding to phospholipids. *J. Biol. Chem.*, 262, 1698-1705 (1987).

Dole, V. P. and Meinertz, H., Microdetermination of long-chain fatty acids in plasma and tissues. *J. Biol. Chem.*, 235, 2595-2599 (1960).

Fawzy, A. A., Vishwanath, B. S. and Frason, R. C., Inhibition of human non-pancreatic phospholipase A_2 by retinoids and flavonoids. *Agents and Actions.*, 25, 394-400 (1988).

Franson, R., Dobrow, R. and Weiss, J., Isolation and characterization of phospholipase A_2 from an inflammatory exudate. *J. Lipid Res.*, 19, 18-23 (1987).

Gelb, M. H., Fluoro ketone phospholipid analogues: new inhibitor of phospholipase A_2. *J. Am. Chem. Soc.*, 108, 3146-3147 (1986).

Glaser, K. B. and Jacobs, R. S., Inactivation of bee venom phospholipase A_2 by manoalide: a model based on the reactivity of manoalide with amino acids and peptide sequences. *Biochem. Pharmacol.*, 36, 2079-2086 (1987).

Hara, S., Chang, H. W., Kudo, I. and Inoue, K., Purification and charaterization of extracellular phospholipase A_2 from human synovial fluid in rhuematoid arthritis. *J. Biochem.*, 105, 395-399 (1989).

Horigome, K., Hayakawa, M., Inoue, K. and Nojima, S., Purification and characterization of phospholipase A_2 from rat platelets. *J. Biochem.*, 101, 53-61 (1987).

Kim, S. H., Chung, K. C., Son, K. H. and Kang, S. S., Steroidal saponins from the rhizomes of *Smilax china*. *Kor. J. of Phamacognogy*, 20, 76-82 (1989).

Kokusho, Y., Kato, S. Machida, H. and Iwasaki, S., Purification and properties of phospholipase from *Actinomadure* sp. strain No. 362. *Argic. Biol. Chem.*, 51, 2515-2524 (1987).

Marianne, S., De Calvalho and Jacobs, R. S., Two step inactivation of bee venom phospholipase A_2 by *Scalaradial*. *Biochem. Pharmacol.*, 42, 1621-1626 (1991).

Marshall, L. A., Sung, A. and Jacobs, R. S., Inactivation of human synovial phospholipase A_2 by the marine natural product, manoalide. *Biochem. Pharmacol.*, 39, 1557-1564 (1990).

Natori, Y., Karasawa, K., Arai, H., Tamori-Natori, Y. and Nojima, S., Partial purification and properties of phospholiase A_2 from rat liver mitochondria. *J. Biochem.*, 93, 631-637 (1983).

Rosenthal, M. D., Viswanath, B. S. and Franson, R. C., Effects of aristolochic acid on phospholipase A_2 activity and arachidonate metabolism of human neutrophils. *Biochem. Biophys. Acta.*, 1001, 1-8 (1989).

Suwa, Y., Kudo, I., Imaizumi, A., Okuda, M., Chang, H. W., Hara, S. and Inoue, K., Proteinous inhibitors of phospholipase A_2 purified from inflammatory sites in rats. *Proc. Natl. Acad. Sic. USA*, 87, 2395-2399 (1990).

Vadas, P., Stefanski, E. and Pruzanski, W., Characterization of extracellular phospholipase A_2 in rheumatoid synovial fluid. *Life Sci.*, 36, 579-583 (1985).

Verheij, H. M., Slotboom, A. J. and de Haas, G. H., Structure and function of phospholipase A_2. *Rev. Physiol. biochem. Pharmacol.*, 91, 91-103 (1981).

Wallach, D. P. and Brown, V. J. R., Studies on the arachidonic acid cascade-I: inhibition of phospholipase A_2 *in vitro* and *in vivo* by several novel series of inhibitor compounds. *Biochem. Pharmacol.*, 30, 1315-1424 (1981).

Yoshida, T., Nakamoto, S., Sakazaki, R. Matsumoto, K., Terui, Y., Sato, T., Arita, H. and Matsutani, S., Thielocins A1α and A1β, novel phospholipase A_2 inhibitors from *Ascomycetes*. *J. Antiboitics*, 44, 1467-1470 (1991).