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A NOVEL PHOSPHOLIPASE C INHIBITOR, S-PLI PRODUCED BY *STREPTOMYCES* SP. STRAIN NO. A-6288

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S-PLI, an inhibitor of phospholipase C (PLC) produced by *Streptomyces* sp. strain No. 6288, was purified from the culture filtrate by salting-out with solid ammonium sulfate, column chromatography on CM-cellulose and gel filtration on Sephadex G-75. The molecular weight of S-PLI was estimated to be 65,000 by SDS-polyacrylamide gel electrophoresis. The inhibitor was found to be a glycoprotein with a composition of 609 amino acids and 19 glucose residues having an isoelectric point at 7.8. S-PLI was stable from pH 3 to 10 at 37°C and up to 40° at pH 6.0. The inhibitory activity showed pH- and temperature-dependence with a maximum around pH 7.0 at 50°C. S-PLI inhibited phospholipase C in a competitive manner (K_i value; 9.5×10^{-6} mM), but did not inhibit S-Hemolysin, phospholipase A_2, phospholipase B, phospholipase D and phosphatases. S-PLI is the first reported example of a glycoproteinaceous inhibitor of microbial origin which is able to specifically inhibit phospholipase C.

KEY WORDS: Phospholipase C inhibitor, *Streptomyces* sp., S-PLI, glycoprotein, PLC

INTRODUCTION

In view of the substrate specificity, phospholipase C (PLC) is classified into three types, phosphatidylcholine specific phospholipase C (PC-PLC, EC 3.1.4.3), sphingomyelin specific phospholipase C (sphingomyelinase, EC 3.1.4.12) and phosphatidylinositol specific phospholipase C (PI-PLC, EC 3.1.4.10). PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). The last two compounds, in turn, serve as intracellular second messengers.[1] IP_3 increases intracellular calcium concentration, resulting in the induction of platelet thrombus,[2] inflammation[3] and various diseases, while DG activates protein kinase C. PLC is a pivotal enzyme for phosphoinositol metabolism, one of the signal transduction systems involved in various kinds of cellular responses by hormones, peptide growth factors, neurotransmitters and the other regulatory ligands.[4–6] Hence an inhibitor of PLC is expected to be an intracellular calcium regulatory drug and could be a useful tool for exploring the mechanism of the signal transduction. A number of PLC inhibitors with low molecular

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weight have been obtained,[7–12] but little have been reported about the inhibitors with high molecular weight.

We have previously reported that *Streptomyces* sp. strain No. A-6288 isolated from a soil produced a unique phospholipase C (designated as S-Hemolysin) having high specificity for sphingomyelin.[13,14] Recently, we found that the strain also produced two other phospholipase C inhibitors in the culture filtrate. One of the inhibitors, called S-PLI, is a high molecular weight substance with glycoproteinaceous composition. This report describes the purification procedure and some properties of S-PLI.

MATERIALS AND METHODS

Enzymes and Substrates

Acid phosphatase (EC 3.1.3.2) from wheat germ,[15] alkaline phosphatase (EC 3.1.3.1) from bovine intestine,[16] phosphodiesterase I (EC 3.1.4.1) from *Crotalus atrox* venom,[17] phospholipase A_2 (EC 3.1.1.4) from porcine pancreas,[18] phospholipase B (EC 3.1.1.5) from *Vibrio* sp.,[19] phospholipase C (EC 3.1.4.3) from *Bacillus cereus*[20] and *Clostridium perfringens*[21] and phosphatidylinositol specific phospholipase C (EC 3.1.4.10) from *Bacillus cereus*[22] were obtained from Sigma Chemicals. Phospholipase D (EC 3.1.4.4) from *Streptomyces chromofuscus*[23] and sphingomyelinase (EC 3.1.4.12) from *Streptomyces* sp.[24] were obtained from Asahikasei Co. Ltd. S-Hemolysin was isolated and purified from the culture filtrate of *Streptomyces* sp. strain No. A-6288 according to the procedure described in a previous paper.[14] Phosphatidylethanolamine from sheep brain, phosphatidylinositol from bovine liver, sphingomyelin from bovine brain and thymidine 5′-monophosphate *p*-nitrophenyl ester were obtained from Sigma Chemicals. Lecithin from egg yolk was obtained from Merck Chemicals. *p*-Nitrophenyl-phosphate was purchased from Nacalai Tesque, Inc. Palmitoyl-(4-nitrobenz-2-oxa-1,3-diazole)-phosphatidylcholine (palmitoyl-NBD-PC) was obtained from Avanti Chemicals.

Measurement of PLC Activity

The incubation mixture (600 μl) consisted of 11.6 mM Tris-HCl buffer (pH 8.0), 5 mM $CaCl_2$, 3.3 mM suspension of lecithin or sphingomyelin sonicated at 10 KHz for 10 min using a Branson 200 Ultrasonifier, 4.5 mM sodium deoxycholate and 10 μg of PLC. After incubation at 37°C for 15 min, 800 μl of 3.6% trichloroacetic acid and 100 μl of 5.6% bovine serum albumin solution were added to the incubation mixture followed by removal of pellets by centrifugation (14,000 rpm, 1 min). Five hundred μl of supernate containing phosphorylcholine, liberated by the enzyme, was taken into a Pyrex glass tube, following the addition of 550 μl of 60% perchloric acid and heating at 200°C for 60 min. The concentration of inorganic phosphate in the hydrolysate was determined by measuring the absorbance at 660 nm according to the colorimetric method of Fiske-SubbaRow.[25] One inhibitor unit (IC_{50}) was defined as the amount which reduced the PLC activity by 50%.[26]

Other Enzyme Reactions

Activity of phosphatidylinositol specific phospholipase C, sphingomyelinase, phospholipase B or phospholipase D was determined by measuring the concentration of inorganic

phosphate in the acid-soluble hydrolysate liberated from substrate by each enzyme reaction.[19,22–24] Activity of acid phosphatase, alkaline phosphatase or phosphodiesterase I was determined from absorbance at 400 nm of *p*-nitrophenol liberated from each substrate.[15–17] Phospholipase A_2 activity was determined fluorometrically with palmitoyl-NBD-PC.[27]

Electrophoresis

Polyacrylamide gel disc electrophoresis was performed by the method of Davis[28] using 7.5% polyacrylamide gel prepared in Tris-glycine buffer (pH 8.3). The electrophoresis was carried out for 60 min at 3 mA per tube. The gel was stained overnight with 0.2% Coomassie brilliant blue R-250 prepared in the mixture of methanol-acetic acid-water (5:1:5) and destained with 7.5% acetic acid in 5% methanol. SDS-polyacrylamide gel electrophoresis was performed according to the method of Maizel[29] using 10% polyacrylamide gel prepared in Tris-glycine buffer containing 0.1% SDS. The electrophoresis was carried out for 60 min at 8 mA per tube for the determination of the molecular weight. Isoelectric focusing was done on polyacrylamide gel using carrier ampholite (pH 3~10, Pharmalyte) as described by Wrigley.[30]

Analyses of Amino Acids and Sugar Contents

Amino acid composition was determined according to the method of Shoji *et al.*[31] and tryptophan was estimated spectrophotometrically according to Goodwin and Morton's procedure.[32] Sugar contents were determined by the phenol-sulfuric acid method.[33] After hydrolysis for 13 h with 2 N trifluoroacetic acid at 100°C,[34] sugars in the hydrolysate were identified by thin layer chromatography on a silica gel plate containing 30 mM boric acid. Spots of sugars on the plate were visualized by silver nitrate reagent[35] and anisaldehyde-sulfuric acid reagent.[36] D-Glucose, D-galactose, D-mannose, D-fucose, D-xylose, D-ribose, L-arabinose, L-sorbose and meso-inositol were used as standard sugars.

Cultural Conditions for the Production of S-PLI

Streptomyces sp. strain No. A-6288 was isolated from a soil collected at Kumamoto city, Japan. The strain was grown aerobically with 50 ml of the modified S medium in a 200 ml Erlenmeyer flask at 28°C on a rotary shaker (180 rpm) for 1 day for producing seed culture and then 2 ml of seed culture were inoculated into the 50 ml of the modified S medium and cultivated at 28°C for 1 day. The modified S medium was composed of 2% glucose, 1% starch, 1% corn steep liquor, 1% soybean flour and 3% peptone, prepared in tap water, and adjusted to pH 7 prior to sterilization.

Purification Procedure of S-PLI

After cultivation, the mycelia and residual matter were removed by centrifugation at 5000 rpm for 15 min and the culture filtrate was brought to 80% saturation by adding solid ammonium sulfate. The precipitate was collected by centrifugation at 12,000 rpm for

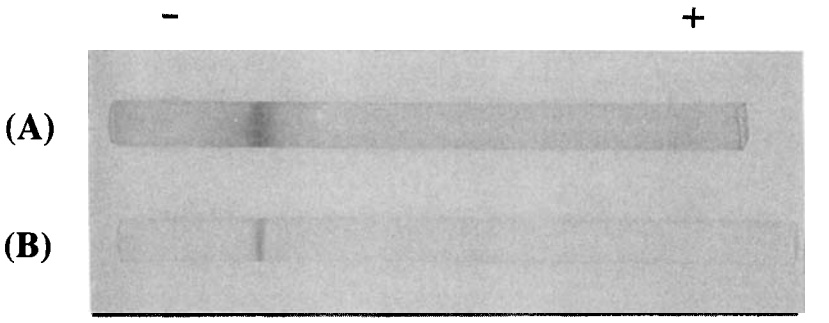

FIGURE 1 Polyacrylamide gel disc electrophoresis (PAGE) of the purified S-PLI. (A), PAGE at pH 9.4 containing 5.8 μg of S-PLI. (B), SDS-PAGE containing 2.9 μg of S-PLI.

15 min and dissolved in a minimal volume of distilled water, followed by dialysis against distilled water at 5°C for 2 days. The dialysate (crude S-PLI) was used as starting material for the purification of S-PLI by successive column chromatographies. The crude S-PLI was applied on a column (2.5 × 20.8 cm) of CM-cellulose equilibrated with 0.01 M acetate buffer (pH 6) and eluted with the same buffer containing linear concentration of NaCl from 0 to 300 mM. The fractions (active fraction I) eluted with 150 mM NaCl showed inhibitory activity against PLC. The concentrated active fraction I was applied on a column of Sephadex G-75 (1.8 × 104 cm) equilibrated with 0.01 M acetate buffer (pH 6) containing 0.1 M NaCl. The accumulated active fractions were dialyzed against distilled water, followed by lyophilization.

RESULTS AND DISCUSSION

Purification

Homogeneity of the purified S-PLI was determined by polyacrylamide gel disc electrophoresis and SDS-polyacrylamide gel electrophoresis. As shown in Figure 1, migration of S-PLI as a single, clear band, revealed its homogeneity. S-PLI was purified about 2800 fold with a yield of 28% from the culture filtrate (Table 1). About 3.1 mg of the inhibitor were finally obtained from 1000 ml of the culture filtrate.

Molecular Weight and Isoelectric Point

The molecular weight of S-PLI was estimated by SDS-polyacrylamide gel electrophoresis. As shown in Figure 2(a), the molecular weight of S-PLI was determined to be about 65,000.

Isoelectric point of S-PLI was measured by electrofocusing on 7.5% polyacrylamide gel containing carrier ampholite with pH range from 3–10. The pH gradient generated was determined by the distance of migration of marker proteins (acetylated cytochrome c's). As shown in Figure 2(b), the distance of migration was plotted against the isoelectric point of each protein, giving an approximate value of pH 7.8 for S-PLI.

TABLE 1
Summary of purification steps of S-PLI.

Step	Volume (ml)	Total activity (unit)	Yield (%)	Specific activity (unit/OD_{280})	Purification rate
Culture filtrate	1240	20,212	100	0.5	1.0
Crude S-PLI	209	15,591	77	0.7	1.4
Active fraction I	63	7573	37	126.1	252.4
Active fraction II	29	6299	31	940.3	1880.6
Purified S-PLI	18	5580	28	1402.7	2805.4

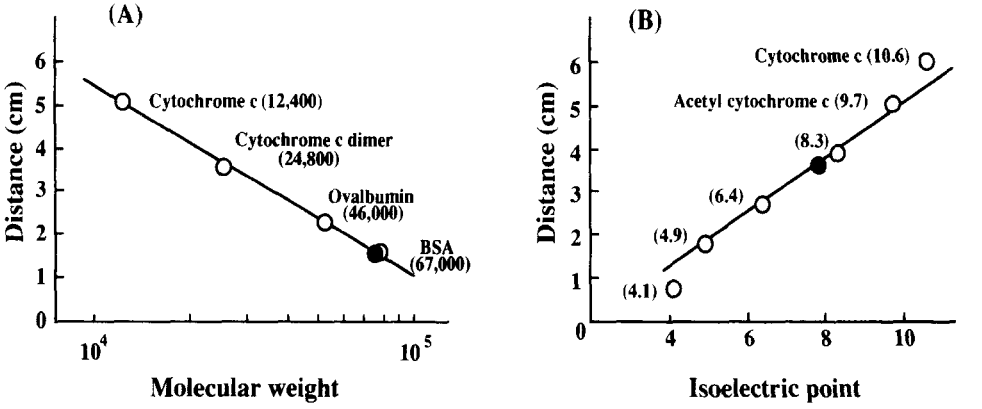

FIGURE 2 Determination of molecular weight and isoelectric point of S-PLI by SDS-PAGE (A) and electrofocusing (B). ○, marker proteins; ●, S-PLI.

Amino Acid Composition and Sugar Contents

The amino acid composition of S-PLI is shown in Table 2. The number of amino acid residues was calculated by assuming four cysteine residues per S-PLI molecule. Neutral amino acids such as alanine or leucine were the main constituent among the components of S-PLI.

The total sugar contents of S-PLI were estimated to be approximately 4.7% (w/w) by the phenol-sulfuric acid method. Thin layer chromatography of the hydrolysate of S-PLI by trifluoroacetic acid showed one staining spot with the Rf value and specific color of glucose. Consequently, S-PLI was regarded as a glycoprotein which is composed of 609 amino acids and 19 glucose residues. From the results of these analyses, the molecular weight was calculated to be 71,228 and this value was similar to that estimated by SDS-polyacrylamide gel electrophoresis.

TABLE 2
Amino acid and sugar contents of S-PLI.

Amino acid	Composition	
	g per 100 g of S-PLI	No. of residues per molecule
Lysine	2.98	18
Histidine	2.13	13
Arginine	7.44	46
Aspartic acid	3.27	20
Glutamic acid	6.10	38
Serine	7.39	45
Glycine	6.18	38
Threonine	7.40	46
Alanine	11.48	71
Proline	6.84	42
Valine	7.45	46
Methionine	1.88	12
Cysteine	0.65	4
Isoleucine	4.72	29
Leucine	11.60	71
Tyrosine	4.98	31
Phenylalanine	2.80	17
Tryptophan[a]	3.57	22
Total amino acid	98.86	609
Glucose[b]	4.70	19

[a] Determined spectrophotometrically. [b] Determined by phenol-sulfuric acid and TLC.

Effects of pH and Temperature on the Stability

Stability of S-PLI was tested in buffer solutions of various pHs at 37°C for 60 min. Residual inhibitory activity against phospholipase C was measured using lecithin as substrate at pH 8.0. As shown in Figure 3(a), S-PLI retained almost 100% of initial inhibitory activity within a wide range of pH from 3.0–10.0.

The inhibitor was exposed to various temperatures for 60 min at pH 6.0, followed by assay of inhibitory activity. As shown in Figure 3(b), S-PLI was stable up to 40°C, but gradual inactivation occurred above 40°C.

Effects of pH and Temperature on Inhibitory Activity

The inhibitory activity of S-PLI against PLC was measured over the pH range 5.0–9.0 at 37°C. The inhibitor and lecithin in the incubation mixture (600 μl) for the assay were used

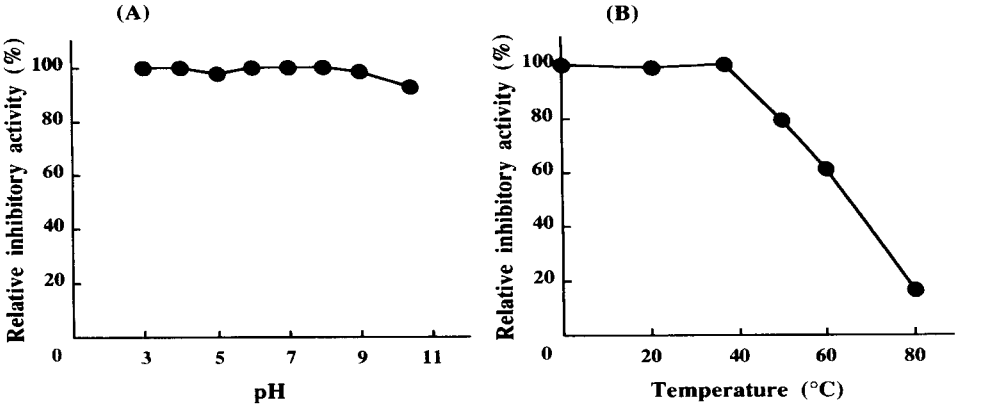

FIGURE 3 Effects of pH (A) and temperature (B) on the stability of S-PLI. The inhibitor was used at concentration of 0.58 μg in the incubation mixture (600 μl) and the residual inhibitory activities after treatment by pH and temperature were measured by the assay described in MATERIALS AND METHODS using lecithin (3.3 mM) as substrate. Relative inhibitory activities were expressed as 100% at pH 6 and 0°C, respectively.

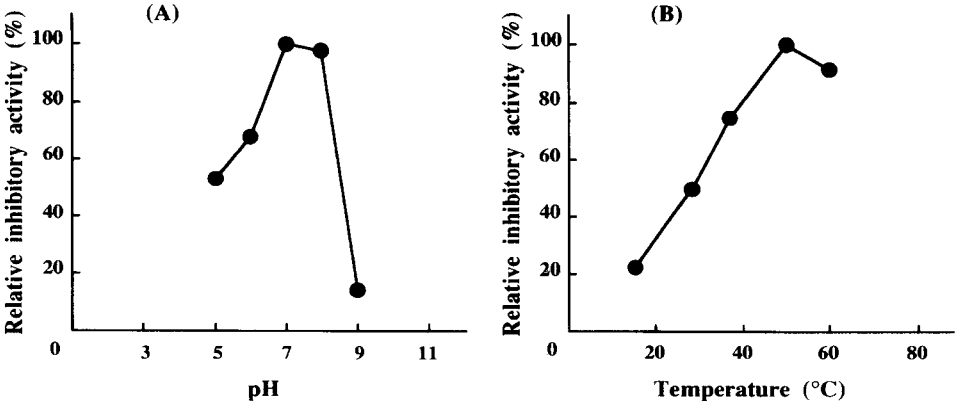

FIGURE 4 Effects of pH (A) and temperature (B) on the inhibitory activity of S-PLI. The inhibitor was used at concentration of 0.58 μg in the incubation mixture (600 μl) and the inhibitory activity was measured by the assay described in MATERIALS AND METHODS using lecithin (3.3 mM) as substrate.

at concentrations of 0.58 μg and 3.3 mM, respectively. As shown in Figure 4(a), the activity of S-PLI was observed to be pH-dependent with a maximum at 7.0 and subsequently decreased with increasing pH.

The inhibitory activity of S-PLI at various temperatures from 15°C to 60°C at pH 8.0 is shown in Figure 4(b). The concentrations of the inhibitor and lecithin in the incubation mixture were the same as those described in the above experiment. The inhibitory activity

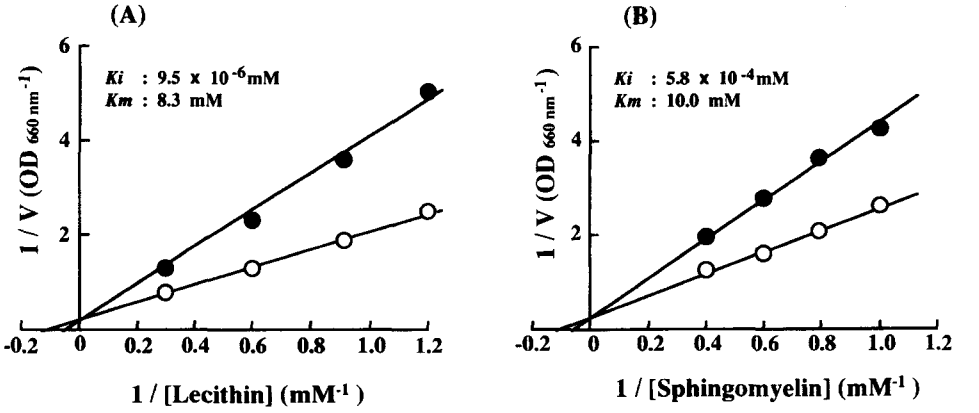

FIGURE 5 Lineweaver-Burk plots of substrate concentration against the rate of hydrolysis by PLC with (•) and without S-PLI (○). The inhibitor concentrations used were 1.5×10^{-5} mM in the incubation mixtures of lecithin (0.8 mM ~ 3.3 mM) and 7.2×10^{-4} mM in the incubation mixtures of sphingomyelin (1.0 mM ~ 2.5 mM respectively).

was also dependent on temperature as that of pH. S-PLI showed weak inhibitory activity at low temperature, and the activity increased with rising temperature, reaching a maximum at around 50°C.

K_i Values of S-PLI against PLC

The type of inhibition was determined by a double-reciprocal Lineweaver-Burk plot[37] of substrates concentration against the rate of hydrolysis by PLC from *Clostridium perfringens* in the presence and absence of S-PLI. As shown in Figure 5, S-PLI inhibited PLC in a competitive manner with respect to lecithin and sphingomyelin, but the inhibitor showed different K_i values with respect to both substrates. The K_m value of the PLC and K_i value of S-PLI with respect to lecithin was 8.3 mM and 9.5×10^{-6} mM (Figure 5(a)), respectively. With respect to sphingomyelin (Figure 5(b)), the K_m value of the enzyme was 10.0 mM and the K_i value of the inhibitor was 61 fold higher than that with respect to lecithin.

Inhibitory Spectrum

The effects of S-PLI on various phospholipases were investigated. As shown in Table 3, S-PLI strongly inhibited phospholipase C's from *C. perfringens* and *B. cereus* and phosphatidylinositol specific phospholipase C (PI-PLC), and weakly inhibited sphingomyelinase and phosphodiesterase I. In contrast, S-Hemolysin, phospholipase A_2, phospholipase B, phospholipase D, acid phosphatase and alkaline phosphatase were not inhibited by the inhibitor. The results suggest that S-PLI is extremely specific for PLC.

PI-PLC hydrolyzes inositol phospholipid to produce diacylglycerol and inositol trisphosphate. The former activates protein kinase C, and the latter increases intracellular calcium concentration, resulting in the induction of platelet thrombus, inflammation and various

TABLE 3
Inhibitory spectrum of S-PLI.

Enzyme	Origin	Substrate (final concn., mM)	IC_{50} (μg/inc. mix.)
Phospholipase C	*Clostridium perfringens*	Lecithin (3.3)	0.58
Phospholipase C	*Bacillus cereus*	Lecithin (3.3)	0.65
PI-Phospholipase C	*Bacillus cereus*	Phosphatidylinositol (2.0)	2.34
Sphingomyelinase	*Streptomyces* sp.	Sphingomyelin (3.3)	22.57
S-Hemolysin (Phospholipase C)	*Streptomyces* sp. A-6288	Lecithin (3.3)	>43.00
Phospholipase A_2	Porcine pancreas	Palmitoyl-NBD-PC (5 μM)	>43.00
Phospholipase B	*Vibrio* sp.	Lecithin (3.3)	>43.00
Phospholipase D	*Streptomyces chromofuscus*	Phosphatidylethanolamine (5.0)	>43.00
Acid phosphatase	Wheat germ	*p*-Nitrophenylphosphate (5.4)	>43.00
Alkaline phosphatase	Bovine intestine	*p*-Nitrophenylphosphate (5.4)	>43.00
Phosphodiesterase I	*Crotalus atrox* venom	Thymidine 5′-monophosphate *p*-nitrophenyl ester (0.2)	8.35

diseases. Therefore, S-PLI is expected to be an antagonist of an intracellular calcium regulatory enzyme and can also be a promising reagent for exploring the mechanism of the signal transduction. In conclusion, as far as we know, S-PLI is the first reported example of a glycoproteinaceous inhibitor of microbial origin with exclusively specific inhibitory activity against phospholipase C.

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