TWO NEW INHIBITORS OF PHOSPHOLIPASE A_2 PRODUCED BY *PENICILLIUM CHERMESINUM*

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL PROPERTIES

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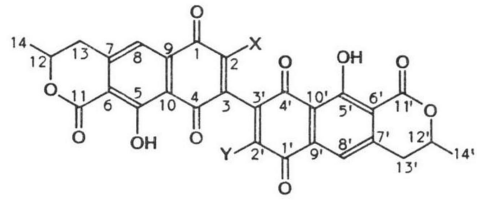
Plastatin and the known fungal metabolite, luteosporin, have been isolated from fermentations of *Penicillium chermesinum* as inhibitors of porcine pancreatic phospholipase A_2 (PLA_2). Structure **1** for plastatin was deduced from its spectroscopic properties. Plastatin and luteosporin inhibited pancreatic PLA_2 competitively with *Ki* values of 0.89 μM and 12.8 μM, respectively. PLA_2 preparations from *Naja naja* and *Crotalus adamanteus* were not significantly inhibited by plastatin and luteosporin.

Phospholipase A_2 [EC 3.1.1.4] (PLA_2) catalyzes the specific hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl-*sn*-phosphoglycerides. This enzyme is one of several that may initiate the arachidonic acid cascade which leads to the production of prostaglandins and leukotrienes, compounds of prime importance in the inflammation process. During the course of screening for naturally-occurring PLA_2 inhibitors, we isolated plastatin (**1**) and luteosporin (**2**, Fig. 1) as metabolites of *Penicillium chermesinum*. Taxonomy of the producing organism, fermentation, isolation, structure determination and biological properties are discussed in this paper.

Fig. 1. Structures of plastatin and luteosporin.



1 (Plastatin) X=NH_2, Y=OH
2 (Luteosporin) X=Y=OH
3 (Xanthomegnin) X=Y=OCH_3

Taxonomy

Penicillium chermesinum (SC 12,932) was isolated from a soil sample collected in Nova Scotia, Canada. It is a filamentous fungus belonging to the Fungi Imperfecti. Microscopic examination of a slide culture grown on Czapek agar revealed brush-like conidiophores amidst trailing cottony aerial hyphae. Spores occurred in chains from phialides arranged in verticils borne terminally on unbranched conidiophores. The spores were hyaline, single celled, oval and finely echinulate, averaging 3.0 μm in diameter. The spore bearing phialides had a mean length of 7.3 μm and mean diameter of 2.1 μm. The conidiophores varied considerably in length: short ones ranged from 20~28 μm; intermediates, 43~73 μm; and long, 107~112 μm. The above characteristics are consistent with a generic designation of a *Penicillium* belonging to the section *Monoverticillata*, series *decumbens* according to the classification of RAPER and THOM[1] and RAMIREZ[2].

The colony morphology was determined on the diagnostic media employed by RAMIREZ[2]. Ob-

servations were made at 18 days. On Czapek agar, the colony surface was rosaceous while the reverse was light brown with patches of burgundy red. On Czapek yeast extract agar, the colony centers were white tinted lightly with pink and radial ridges were evident on the surface. The leading edge of the colony was buff to dusty pink, with accumulation of droplets of clear exudate. The reverse surface was tan with streaks of reddish-brown. On malt extract agar, sporulation was abundant and was green with yellow sectors while the reverse was gold to honey colored.

SC 12,932 was identified as a strain of *Penicillium chermesinum* based on the above description.

Fermentation

Seed cultures of *P. chermesinum* ATCC 20700 were prepared by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks containing 100 ml of the following medium: 0.3 % yeast extract, 0.3 % malt extract, 0.5 % Tryptone and 1.0 % glucose in distilled water. The flasks were incubated at 25°C on a rotary shaker (300 rpm, 5-cm stroke) for approximately 48 hours.

A 10 % (v/v) transfer was made from the seed culture flasks to 500-ml Erlenmeyer flasks containing 100 ml portions of the same medium as was used for the germination stage. The flasks were incubated at 25°C for approximately 144 hours, with the same operating conditions as described for the germinator flasks. At the end of the incubation period, the contents of the flasks were pooled and the pool filtered to remove the mycelial cake.

Assay of Phospholipase A_2

Phospholipase A_2 (PLA_2) from porcine pancreas as a suspension in 3.2 M $(NH_4)_2SO_4$, PLA_2 from *Naja naja* and from *Crotalus adamanteus* venom (lyophilized powders) and deoxycholic acid were obtained from Sigma Chemical Company, St. Louis, MO. The pancreatic PLA_2 preparation was specific for the hydrolysis of fatty acids from the 2-position of triglycerides. This was demonstrated by incubating the enzyme with L-α-[β-*palmitoyl*-1-^{14}C]phosphatidyl choline, dipalmitoyl (Amersham, Arlington Heights, IL), and identifying the reaction products by TLC.

Several assay methods for phospholipases have been reported[3,4]. For screening purposes a plate assay was developed using a saline egg yolk preparation in agar as the substrate. The saline egg yolk solution consisted of 10 egg yolks mixed with 300 ml sterile 0.85 % NaCl. After centrifugation (2,000 × *g* for 5 minutes) the supernatant was stored at −90°C in aliquots. To 95 ml of 0.5% agar were added 4 ml of the saline egg yolk solution, 1 ml of 1.0 M phosphate buffer, pH 7.7, and 240 units of porcine pancreatic phospholipase A_2. Fermentation broths from a variety of microorganisms were screened as PLA_2 inhibitors after adjustment of the broths to pH 7. Paper discs saturated with clarified broths were placed on the PLA_2-egg yolk agar and incubated for 1 ~ 2 hours at 37°C. An inhibitor of phospholipase A_2 prevented clearing of the opaque egg yolk around the disc.

For quantitative measurements of inhibition, a pH-stat assay was used. Pancreatic PLA_2 was dialyzed against distilled water to remove the ammonium sulfate, lyophilized and then rehydrated for use in 0.25 mM Tris-HCl, pH 8.0. The reaction mixture was prepared from a stock solution containing 20 ml aqueous egg yolk suspension (one egg yolk mixed thoroughly with 30 ml distilled water and centrifuged at 2,000 × *g* for 5 minutes), 30 ml of 0.65 M NaCl, 0.50 ml of 200 mM $CaCl_2$ and 3.0 ml of 150 mM deoxycholic acid. A 5.0 ml aliquot, equilibrated at 25°C, was then adjusted to pH 8 with 0.1 M NaOH. Inhibitor was added to the reaction mixture in a volume of 100 μl. Reaction was initiated by addition of 20 units of PLA_2 (20 μl). The rate of addition of 5 mM NaOH needed to maintain a pH of 8 was recorded automatically and related to enzymatic activity, using a Radiometer TTT 60

Fig. 2. Isolation of plastatin and luteosporin.

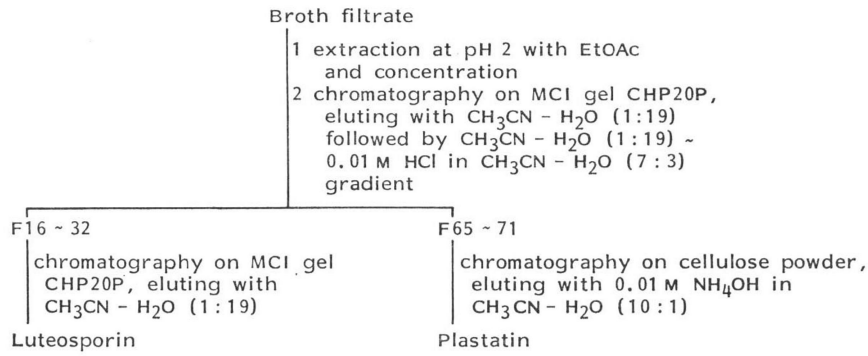


Table 1. Physico-chemical properties of plastatin and luteosporin.

	Plastatin	Luteosporin
Appearance	Orange red crystals	Black crystals
MP	>270°C (dec)	>300°C
TLC (silica gel, lower phase, $CHCl_3$ - MeOH - H_2O, 6: 3: 1) Rf	0.35	0.22
IC_{50} (μg/ml)	1.6	25
IR (KBr) cm^{-1}	3400, 1720, 1675, 1610	1720, 1670, 1570, 1540
UV nm ($E^{1\%}_{1cm}$) (H_2O, pH 2~10)	215 (670), 236 (475), 280 (500), 398 (130)	215 (595), 233 (404), 281 (534), 388 (131)

titrator and Autoburette ABU 12.

Kinetic studies were performed using five substrate concentrations, prepared by diluting a freshly-prepared stock substrate with 0.36 M NaCl, 1.9 mM $CaCl_2$ and 8.4 mM deoxycholic acid. Two inhibitor concentrations and a control containing no inhibitor were assayed in duplicate at each substrate concentration.

Time dependence for inhibition was studied by preparing 500 μl volumes of the enzyme (100 μl) and an inhibitor (or enzyme and buffer as control). Aliquots of 120 μl were removed from the incubation mixtures at 15 minutes intervals and enzyme activity was monitored as described above. A zero time value was determined by adding 20 μl of the enzyme to substrate containing 100 μl of inhibitor (or buffer as a control).

Isolation

Plastatin and luteosporin[5] were isolated from fermentation broths as outlined in Fig. 2. The inhibitors were extracted from the acidified broth filtrate with ethyl acetate. The separation of plastatin from luteosporin was accomplished by reverse-phase chromatography on MCI gel CHP20P (Mitsubishi Chemical Industries, Ltd., Japan) eluting with a linear gradient prepared from acetonitrile - water (1 : 19) and 0.01 M HCl in acetonitrile - water (7: 3). The fractions were combined on the basis of TLC (silica gel, lower phase of $CHCl_3$ - MeOH - H_2O, 6 : 3 : 1, visible light and Rydon visualization). Final purification of plastatin was effected by chromatography on cellulose powder eluting with 0.01 M NH_4OH in CH_3CN - H_2O (10 : 1). Luteosporin was purified further by reverse-phase chromatography on MCI gel CHP20P eluting with CH_3CN - H_2O (1 : 19).

Table 2. ^{1}H NMR data for plastatin and luteosporin in DMSO-d_6.

Plastatin		Luteosporin	
Chemical shifts (δ)[a]	Assignment	Chemical shifts (δ)[a]	Assignment
1.40	14, 14′	1.45	14
3.00, 3.15	13, 13′	3.00, 3.20	13
4.65	12, 12′	4.70	12
5.40	2′ (OH)	5.80	2 (OH)
7.48	8[b]	7.45	8
7.55	8′[b]	13.50	5 (OH)
7.65	2 (NH_2)		
13.60	5 (OH)[c]		
14.45	5′ (OH)[c]		

[a] ppm Downfield from TMS using DMSO (2.49 ppm) as an internal standard.
[b, c] Interchangeable.

Table 3. ^{13}C NMR data for plastatin and luteosporin in DMSO-d_6.

Plastatin		Luteosporin	
Chemical shifts (δ)[a]	Assignment	Chemical shifts (δ)[a]	Assignment
20.0, 20.0	C-14, C-14′	20.0	C-14
35.1, 35.1	C-13, C-13′	35.0	C-13
73.6, 73.6	C-12, C-12′	73.9	C-12
103.5, 113.8	C-3, C-3′	113.9	C-3
114.2, 114.7	C-10, C-10′	114.6	C-10
116.0, 116.1	C-8, C-8′	116.5	C-8
117.7, 117.8	C-6, C-6′	117.8	C-6
132.6, 133.4	C-9, C-9′	132.2	C-9
147.7, 148.5	C-7, C-7′	148.6	C-7
158.1, 160.0	C-2, C-2′	158.2	C-2
160.5, 160.6	C-11, C-11′	160.8	C-11
161.6, 161.9	C-5, C-5′	161.6	C-5
180.0, 180.4	C-1, C-1′	180.0	C-1
185.2, 188.2	C-4, C-4′	187.3	C-4

[a] ppm Downfield from TMS, using DMSO-d_6 (39.5 ppm) as an internal standard.

Physico-chemical Properties of Plastatin and Luteosporin

The less active component of the fermentation, luteosporin (**2**, IC_{50}=25 μg/ml), is a water-soluble (at pH 7) and weakly acidic PLA_2 inhibitor, mp >300°C. The physico-chemical properties of luteosporin are given in Table 1. The molecular weight and empirical formula were determined by fast atom bombardment MS (FAB-MS)[6]. Both positive and negative ion spectra led to the conclusion that the molecular weight was 546. The exact mass observed for $C_{28}H_{19}O_{12}$ $(M+H)^+$ was 547.082 (theory 547.087).

The ^{1}H and ^{13}C NMR data are given in Tables 2 and 3, respectively. The carbon connectivity in luteosporin was determined by a C-H long range selective proton decoupling (LSPD) experiment. In LSPD (in D_2O), the doublets at δ_C 186.3 (C-1) and 116.7 (C-10) collapsed to two singlets and the multiplet at δ_C 117.6 (C-6) reduced to a simpler multiplet upon irradiation of the signal at δ_H 7.60 (C-8 proton). Upon irradiation of the methylene protons (δ_H 3.00), multiplets at δ_C 117.6 (C-6) and 147.4 (C-7) collapsed to a doublet (J_{CH} 5.1 Hz) and a singlet, respectively. Irradiation of the methine (δ_H 4.70) gave a doublet (J_{CH} 5.1 Hz) and a diffuse triplet from the multiplets at δ_C 117.6 (C-6) and 147.4 (C-7),

Fig. 3. DIXON plot of inhibition of pancreatic PLA_2 by plastatin.

Substrate concentrations were: S_0, ○ (the standard egg yolk mixture used in routine assays); 0.50 S_0, □; 0.40 S_0, △; 0.33 S_0, ●.

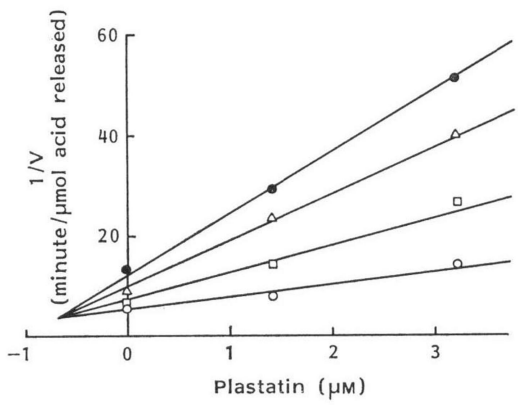


Table 4. Inhibition of PLA_2 activities by plastatin and luteosporin.

Reactions were initiated by the addition of PLA_2 to a mixture of inhibitor and substrate as described in the text. Reaction rates were monitored for 2.8 minutes.

Source of PLA_2	Inhibition (%)	
	Plastatin (36 μM)	Luteosporin (72 μM)
Porcine pancreas	96	67
Naja naja	3	3
Crotalus adamanteus	1	0

respectively. These data are consistent with structure **2** for luteosporin and the results obtained with xanthomegnin[7] (**3**, Fig. 1). The identity of luteosporin was confirmed by conversion to xanthomegnin[8], identical to an authentic sample provided by A. ZEECK.

The most active component of the fermentation, plastatin (**1**, $IC_{50}=1.6$ μg/ml) is a water-soluble (at pH 7.0) and weakly acidic inhibitor, mp >270°C (dec). The physico-chemical properties of plastatin are given in Table 1. Plastatin gave a positive reaction towards sodium dithionite indicating the presence of a quinone system. The IR and UV spectral data (Table 1) also indicated the presence of a quinone system in the molecule. The 1720 cm^{-1} band in the IR spectrum is attributed to a six-membered lactone. Plastatin has the molecular formula $C_{28}H_{19}NO_{11}$ as confirmed by microanalysis and the high resolution FAB-MS. The mass spectrum of plastatin showed peaks at *m/z* 546 $(M+H)^+$ and 568 $(M+Na)^+$ together with abundant 547 $(M+2H)^+$ and 548 $(M+3H)^+$ peaks corresponding to the semiquinone and hydroquinone[9], respectively.

The ^1H NMR data for plastatin are given in Table 2. The signals at δ 5.40, 7.65, 13.60, 14.45 (exchangeable with D_2O), were assigned to the C-2′ hydroxylic proton, the C-2 amino protons, the C-5 hydroxylic proton and the C-5′ hydroxylic proton, respectively.

The ^{13}C NMR data are given in Table 3. The assignments were made by comparison with the data obtained for luteosporin. The 10 ppm difference in chemical shift of C-3 is attributed to the replacement of the C-2 hydroxyl by an amino group.

The structure of plastatin was established by synthesis from xanthomegnin. Xanthomegnin gave plastatin as one of several products on its reaction with ammonium hydroxide followed by relactonization with 1 N HCl.

Biological Properties

Plastatin and luteosporin were competitive inhibitors of pancreatic PLA_2 with *Ki* values of 0.89 μM and 12.8 μM, respectively. A typical DIXON plot[10] for evaluation of plastatin inhibition is presented in Fig. 3. Over a one-hour period, no time dependence was observed for inhibition. This activity is in contrast to PLA_2 inhibition by manoalide (another small molecular weight inhibitor isolated from a marine sponge) which requires preincubation for maximal activity[11].

As shown in Table 4, inhibitory potencies of plastatin and luteosporin were compared using PLA_2

from various sources. No significant inhibition was observed with two snake venom PLA_2 preparations when assayed at inhibitor concentrations in great excess of the *Ki* values for the porcine enzyme.

Experimental

NMR spectra were determined on Joel GX 400 and FX 270 spectrometers. IR spectra were recorded on a Perkin-Elmer model 621 spectrometer. UV-visible spectra were recorded on a Perkin-Elmer model 202 spectrophotometer. Mass spectra were determined on a VG Analytical model ZAB 1F spectrometer.

Isolation of Plastatin and Luteosporin

At harvest, the cells from a 20-liter fermentation of *P. chermesinum* were separated by filtration. The broth filtrate was adjusted to pH 2.0 with HCl and extracted with EtOAc (2×10 liters). The aqueous layer was discarded and the combined EtOAc extracts concentrated to 1.6 liters. The EtOAc concentrate was stored at 4°C for 24 hours. The aqueous layer visible after storage was removed and extracted with EtOAc (2×75 ml). The combined EtOAc extracts were concentrated to an aqueous slurry *in vacuo*, adjusted to pH 7 with NaOH, and concentrated to dryness *in vacuo*. The residue was then suspended in CH_3CN - H_2O (1: 19, 3×25 ml) and chromatographed on MCI gel CHP20P (75~150 μ, 5×36 cm). The column was eluted with CH_3CN - H_2O (1: 19, 1 liter) followed by a linear gradient of CH_3CN - H_2O (1: 19) to 0.01 M HCl in CH_3CN - H_2O (7: 3) (3 liters). The fractions were combined on the basis of TLC assay (Table 1) and bioactivity (PLA_2 inhibition). Fractions that contained luteosporin were combined and concentrated *in vacuo*. The residue (1.29 g) was further purified on a column of MCI gel CHP20P (75~150 μ, 2.5×100 cm) eluting with CH_3CN - H_2O (1: 19, 320 ml). The active fractions were combined and concentrated *in vacuo* to give luteosporin as fine black crystals (500 mg).

The bioactive fractions containing plastatin from the MCI gel CHP20P column, were combined and concentrated *in vacuo*. The residue (385 mg) was dissolved in 0.01 M NH_4OH in CH_3CN - H_2O (2: 1, 3 ml), and chromatographed on cellulose powder (2.5×25 cm) eluting with 0.01 M NH_4OH in CH_3CN - H_2O (10: 1). The fractions containing plastatin were combined and concentrated *in vacuo* to dryness. The residue was dissolved in H_2O (20 ml) and precipitated with 0.02 M HCl (20 ml, pH *ca* 2.0). The suspension was extracted with EtOAc (3×50 ml), keeping most of the precipitate with the EtOAc layer. The combined EtOAc extracts were concentrated to dryness *in vacuo* to give 172 mg of plastatin as fine red crystals.

Conversion of Xanthomegnin to Plastatin

Xanthomegnin (100 mg) was stirred with conc NH_4OH (10 ml) at room temp for 15 hours and then the reaction mixture was concentrated *in vacuo*. The residue was suspended in H_2O (10 ml), pH adjusted to 2 with 1 M HCl and extracted with BuOH (4×10 ml). The BuOH extracts were combined and concentrated *in vacuo*. The residue was suspended in 1 M HCl (10 ml) and stirred at room temp for 48 hours. The reaction mixture was adjusted to pH 7 with 1 M NaOH and then concentrated to dryness *in vacuo*. The crude material was purified on a MCI gel CHP20P (75~150 μ, 1.1×30 cm) column eluting with a linear gradient of CH_3CN - H_2O (1: 19) to 0.01 M HCl in CH_3CN - H_2O (7: 3) (300 ml), to give plastatin (3.7 mg) and several unidentified products. The plastatin thus produced was identical to the plastatin isolated from *P. chermesinum*.

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