PI-200 AND PI-201, NEW PLATELET AGGREGATION INHIBITORS PRODUCED BY *Streptomyces* sp. A7498

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES, STRUCTURE DETERMINATION AND BIOLOGICAL PROPERTIES

YUKO KISHIMURA, AKIRA KAWASHIMA, TERUMI KAGAMIZONO, MICHIO YAMAGISHI, KEITA MATSUMOTO, YUTAKA KAWASHIMA and KAZUNORI HANADA

Research Center, Taisho Pharmaceutical Co., Ltd.,
Ohmiya-shi, Saitama 330, Japan

(Received for publication October 15, 1991)

Two new platelet aggregation inhibitors, PI-200 and PI-201 were isolated from the fermentation broth of *Streptomyces* sp. A7498. PI-200 and PI-201 inhibited ADP-induced aggregation of rabbit platelets with an IC_{50} of 3.8×10^{-4} M and 7.1×10^{-4} M, respectively.

In the course of our screening for platelet aggregation inhibitors from microbial origin, we isolated two new compounds, designated PI-200 and PI-201, from the fermentation broth of *Streptomyces* sp. A7498.

In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and structure determination of the new compounds.

Materials and Methods

Taxonomic Studies

The strain A7498 was isolated from a soil sample collected at Urawa City, Saitama Prefecture, Japan.

Growth characteristics and carbohydrate utilization were determined by the methods of the International Streptomyces Project (ISP)[1]. The spores and mycelia of the strain were observed with a scanning electron microscope (model S-2500, Hitachi Co., Ltd.). The diaminopimelic acid of the cell wall was analyzed by HPLC in the hydrolysates of the whole cell grown in a medium (glucose 10 g, yeast extract 10 g per liter, pH 7.0) for 96 hours at 28°C.

Fermentation

The growth of *Streptomyces* sp. A7498 on a mature slant culture was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of sterile seed medium composed of oatmeal 2%, glucose 2%, meat extract 0.3%, NaCl 0.3%, $Fe_2(SO_4)_3$ 0.04%, $MnCl_2$ 0.04% (pH 7.0) and cultured at 28°C for 72 hours on a rotary shaker with a 7-cm throw at 220 rpm. Then 100 ml of this seed culture was transferred to a 5-liter jar fermenter containing 3 liters of the same medium and cultured at 28°C for 72 hours under aeration of 3 liters/minute and agitation of 500 rpm.

Structural Studies

The IR spectrum was recorded on a Perkin-Elmer 1760 IR Fourier Transform Spectrometer. The ^1H and ^{13}C NMR spectra were measured on a Jeol GX-400 spectrometer with TMS as the internal standard. Optical rotations were measured on a Jasco DIP-360 polarimeter. UV spectra were recorded on a Hitachi 220A spectrophotometer. MP's were determined on a Yanagimoto micro melting point apparatus and were uncorrected. MS spectra were obtained on a JMS-SX 102 spectrometer.

For X-ray crystallography, a single crystal PI-201 with dimensions of $0.50 \times 0.40 \times 0.20$ mm was used,

and the intensity values were collected on an MAC Science MXC 18 diffractometer, with in a range of $20 < 130°$, a total of 1,428 unique reflections were obtained above the threshold $[|F_o| > 3\sigma(|F_o|)]$ and corrected for Lorentz and polarization factors. The structure of PI-201 was solved by a SUN 4 computer using the SHELXS 86 program system.

Platelet Aggregation Test

Platelet aggregation was measured turbidimetrically[2] with a platelet aggregation tracer (model PAM-6C, MEBANIX, Inc.). Rabbit blood was collected through a polyethylene catheter from the carotid artery of a male NZW rabbit into a plastic tube that contained one volume of 3.0% sodium citrate to nine volumes of blood. After obtaining platelet rich plasma by centrifugation, the platelet number was adjusted to $30 \sim 70 \times 10^4$ cells/μl. Platelets were preincubated for 2 minutes at 37°C with the test compound, and ADP was added to a final concentration of 5 μM.

Results and Discussion

Taxonomic Studies

The vegetative mycelium developed well without fragmentation. The aerial mycelium branched monopodially and formed spiral chains (Fig. 1). The spores had a warty surface and were oval in shape with a size of 0.9×1.1 μm. The appearance of strain A7498 on nine solid media is presented in Table 1. The vegetative mycelium grew on both synthetic and complex media. The aerial mycelium was gray to grayish brown. Melanoid and other soluble pigments were not produced. Analysis of whole cell hydrolysates of strain A7498 showed the

Fig. 1. Scanning electron micrograph of strain A7498.

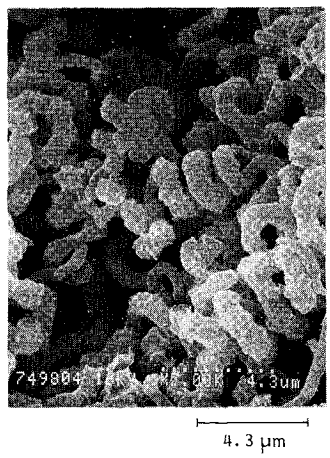


Table 1. Cultural properties of strain A7498.

Medium	Cultural properties	Medium	Cultural properties
Sucrose-nitrate agar	G: Moderate	Nutrient agar	G: Moderate
	A: None		A: None
	R: White		R: Brown
	S: None		S: None
Glucose-asparagine agar	G: Moderate	Yeast extract-malt	G: Moderate
	A: None	extract agar	A: Grayish brown
	R: Cream		R: Brown
	S: None		S: None
Glycerol-asparagine	G: Moderate	Oatmeal agar	G: Good
agar	A: Grayish white		A: Grayish brown
	R: Cream		R: Yellowish brown
	S: None		S: None
Inorganic salts-starch	G: Moderate	Peptone-yeast	G: Moderate
agar	A: Grayish white	extract iron agar	A: None
	R: Cream		R: Brown
	S: None		S: None
Tyrosine agar	G: Moderate		
	A: None		
	R: Pale brown		
	S: None		

G; Growth, A; aerial mass color, R; reverse side color, S; soluble pigment.

presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

In Table 2, the physiological properties of strain A7498 are presented. The strain grew at temperatures ranging from 18 to 40°C with the optimum range from 24 to 37°C. The strain utilized various carbon sources (Table 3). Based on the taxonomic properties described above, strain A7498 was classified in the genus *Streptomyces* and deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the name of *Streptomyces* sp. A7498 under the accession No. FERM P-11537.

Table 2. Physiological properties of strain A7498.

Liquefaction of gelatin	+
Coagulation of milk	+
Peptonization of milk	+
Hydrolysis of starch	+
Formation of melanoid pigment	−
Optimum growth temperature	24~37°C

+; Positive, −; negative.

Table 3. Utilization of carbon sources by strain A7498.

L-Arabinose	+
D-Glucose	+
D-Xylose	+
D-Fructose	+
Inositol	+
D-Mannitol	+
Sucrose	+
L-Rhamnose	+
Raffinose	+

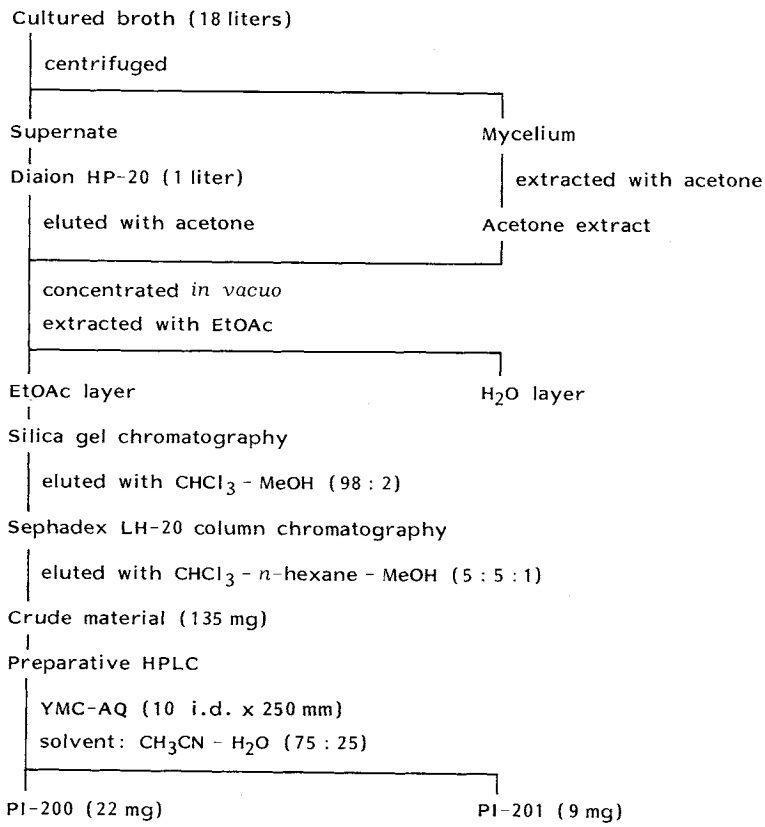
+; Utilized.

Isolation

The isolation scheme is shown in Fig. 2.

The cultured broth (18 liters) was centrifuged and the mycelia were extracted with acetone (10

Fig. 2. Isolation procedure for PI-200 and PI-201.



liters). The supernate was passed through a column of Diaion HP-20 (1 liter). After washing with 2 liters of water, the adsorbed material was eluted with acetone (2 liters).

The acetone layer was combined and concentrated *in vacuo*. This concentrate was extracted with ethyl acetate (2 liters × 2). The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The resulting material (18.7 g) was applied to a silica gel column (Wakogel C-200, 0.8 liter) and washed successively with $CHCl_3$ and $CHCl_3$ - MeOH (99 : 1), followed by elution with a mixture of $CHCl_3$ - MeOH (98 : 2) to give PI-200 and PI-201. The fractions which contained these new compounds were combined and dried up yielding 700 mg of brown oily residue. This residue was dissolved in a mixture of chloroform, *n*-hexane and methanol (5 : 5 : 1) and subjected to a Sephadex LH-20 column (350 ml) which was developed with the same solvent to give a pale yellow syrup (135 mg).

Further purification was carried out by preparative reverse-phase HPLC (YMC-AQ 10 i.d. × 250 mm) with a solvent system of acetonitrile and water (3 : 1) to yield PI-200 (22 mg) and PI-201 (9 mg) as colorless oil. Crystallization from $CHCl_3$ gave colorless plates of PI-201.

Physico-chemical Properties

The physico-chemical properties of PI-200 and PI-201 are summarized in Table 4.

PI-200 and PI-201 are soluble in methanol, chloroform, ethyl acetate, but almost insoluble in water. Their MW's and molecular formulae were determined by FAB-MS and elemental analysis to be *m/z* 262 ($C_{17}H_{26}O_2$) and 280 ($C_{17}H_{28}O_3$), respectively. The IR spectra of PI-200 showed absorptions of δ-lactone at 1729 cm^{-1}. PI-201 had bands characteristic of hydroxyl groups at 3455 cm^{-1} and carbonyl functions at 1689 cm^{-1}.

Structure Determination

The ^1H and ^{13}C NMR data of PI-200 and PI-201 are shown in Table 5.

INEPT experiment with PI-201 indicated the presence of a carbonyl carbon, two olefinic carbons, three methyl carbons, six methylene carbons, three methine carbons, an oxymethine carbon and a quaternary carbon. Two partial structures A and B (Fig. 3) were revealed by ^1H-^1H COSY and CH-COSY spectra, where two sets of allylic couplings for the methine proton at δ_H 5.07 (9-H) had the methyl proton at δ_H 1.77 (17-H) and the methine proton at δ_H 2.63 (11-H). The HMBC spectrum of PI-201 supported the partial structures by cross peaks as follows: 15-H→14-C, 13-C; 17-H→9-C, 10-C, 11-C; 9-H→17-C, 11-C; 3-H→8-C, 4-C. Partial structures A and B were connected by a cross peak for the singlet methyl signal at δ_H 1.35 (16-H) and 1-C, 3-C and 11-C. Further, the methine proton at δ_H 2.03 (3-H) was correlated

Table 4. Physico-chemical properties of PI-200 and PI-201.

	PI-200	PI-201
Appearance	Colorless oil	Colorless plates
MP		138～140°C
Molecular formula	$C_{17}H_{26}O_2$	$C_{17}H_{28}O_3$
FAB-MS (positive) *m/z*	263 $(M+H)^+$, 301 $(M+K)^+$	281 $(M+H)^+$
Elementary analysis		
Calcd:	C 77.89, H 10.00	C 72.78, H 10.08
Found:	C 77.23, H 10.06	C 72.17, H 10.00
$[\alpha]_D^{25}$ (*c* 0.5, $CHCl_3$)	+10.3°	−98.4°
UV λ_{max}^{MeOH} nm (ε)	212 (2,025)	210 (2,044)
IR ν_{max}^{KBr} cm^{-1}	2931, 1729	3455, 2923, 1689

Table 5. ^{13}C and ^{1}H NMR chemical shift assignments and coupling data of PI-200 and PI-201 in $CDCl_3$.

	PI-200		PI-201	
	^{13}C[a,b]	^{1}H[c,d]	^{13}C	^{1}H
C-1	177.8 (s)		184.2 (s)	
C-2	46.1 (s)		50.4 (s)	
C-3	39.9 (d)	2.28 (1H, m)	40.6 (d)	2.03 (1H, m)
C-4	26.6 (t)	1.25~1.38 (1H), 1.47~1.63 (1H)	23.6 (t)	1.06~1.24 (1H), 1.46~1.62 (1H)
C-5	24.5 (t)	1.25~1.38 (1H), 1.46~1.64 (1H)	26.3 (t)*	1.04~1.19 (1H), 1.56~1.71 (1H)
C-6	23.1 (t)	1.26~1.46 (2H)	22.6 (t)*	1.18~1.26 (1H), 1.30~1.46 (1H)
C-7	29.5 (t)	1.40~1.60 (2H)	31.5 (t)	1.40~1.52 (1H), 1.53~1.65 (1H)
C-8	35.4 (d)	2.22 (1H, m)	34.7 (d)	2.43 (1H, m)
C-9	128.8 (d)	5.43 (1H, m)	124.7 (d)	5.07 (1H, br s)
C-10	133.2 (s)		137.3 (s)	
C-11	41.5 (d)	2.50 (1H, br d, 13.0)	39.9 (d)	2.63 (1H, m)
C-12	26.8 (t)	1.88 (1H, ddd, 3.7, 4.9, 13.8)	38.1 (t)	1.34~1.50 (1H), 1.54~1.71 (1H)
C-13	82.0 (d)	4.20 (1H, m)	75.0 (d)	3.58 (1H, m)
C-14	29.2 (t)	1.65~1.79 (2H)	30.4 (t)	1.36~1.60 (2H)
C-15	9.2 (q)	0.95 (3H, t, 7.5)	10.2 (q)	1.00 (3H, t, 7.5)
C-16	17.6 (q)	1.01 (3H, s)	20.6 (q)	1.35 (3H, s)
C-17	19.8 (q)	1.65 (2H, s)	22.8 (q)	1.77 (3H, s)

[a] Measured at 100 MHz; chemical shifts in ppm from TMS.
[b] Multiplicity was determined by INEPT data.
[c] Measured at 400 MHz; chemical shifts in ppm from TMS.
[d] Multiplicity, coupling constants (J=Hz) and peak area.
* Exchangeable.

Fig. 3. Partial structures of PI-201.

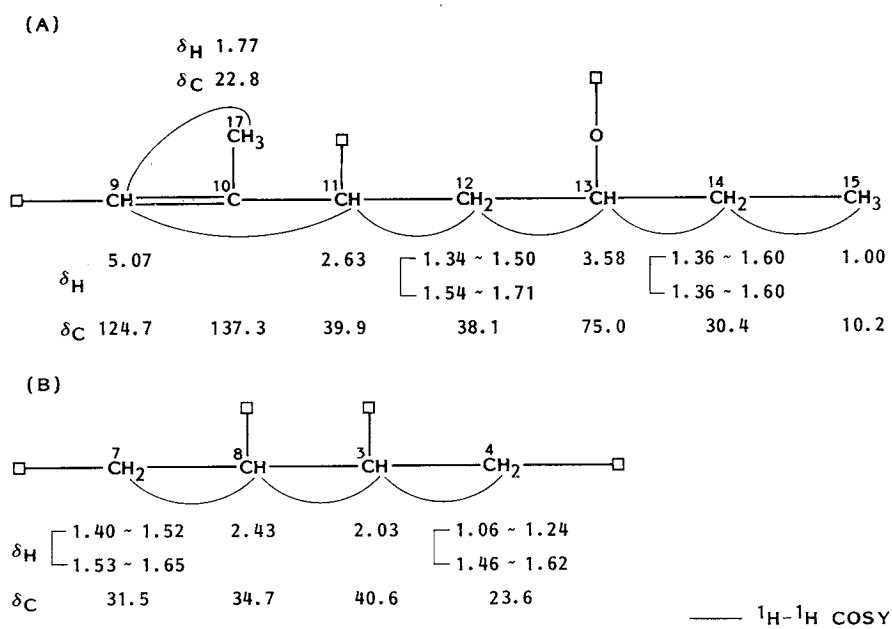
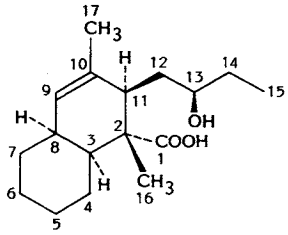
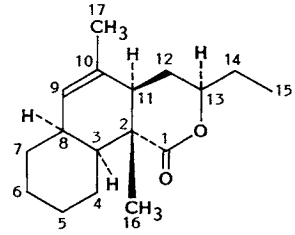


Fig. 4. Structures of PI-200 and PI-201.



PI-201

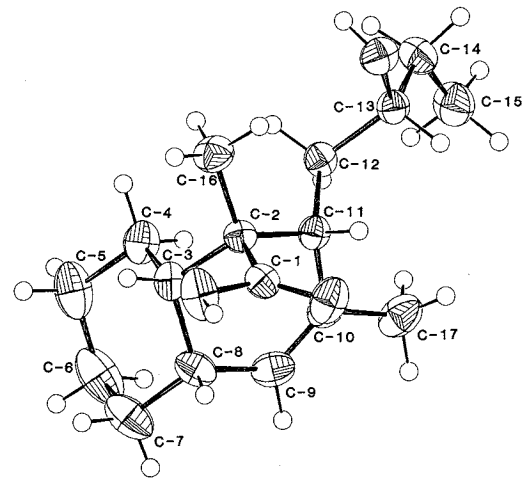


PI-200

with olefinic carbon at δ_C 124.7 (9-C). Based on these results the structure of PI-201 was inferred to be as shown in Fig. 4 except for the stereochemistry.

X-ray analysis was carried out to determine the structure of PI-201. A single crystal of PI-201 was obtained by recrystallizing from chloroform. The crystal data ($C_{17}H_{28}O_3$, 280.00) indicated the following dimensions: Monoclinic $P2_1$; $a=13.924(2)$, $b=8.411(2)$, $c=6.8375(5)$Å, $\beta=102.24(3)°$, $Z=2$, $V=782.6$Å^3, $D_C=1.19$ g/cm^3. All the non-hydrogen atoms were refined with anisotropic thermal parameters. Twenty-eight H atoms appeared on a difference Fourier map and were refined isotropically, the final R being 0.028 and Rw being 0.037. A computer-generated perspective the final X-ray model of PI-201 is shown in Fig. 5. The absolute stereochemistry is under study.

Fig. 5. Perspective view of PI-201.



The ^{1}H and ^{13}C NMR data of PI-200 were similar to those of PI-201. Comparison of the ^{1}H NMR data for PI-200 with those of PI-201 revealed a significant difference at C-13. The 13-H signal is shifted downfield by 0.62 ppm to δ_H 4.20, suggesting the existence of a lactone ring in PI-200. Thus the structure of PI-200 was determined to be as shown in Fig. 4.

Biological Properties

The effects of PI-200 and PI-201 on rabbit platelet aggregation induced by ADP was estimated turbidimetrically. The IC_{50} values of PI-200 and PI-201 were 3.8×10^{-4} M and 7.1×10^{-4} M, respectively. These values indicated that PI-200 and PI-201 were less active than adenosine ($IC_{50}=0.5\times10^{-4}$ M) which has been known to possess antiplatelet activity.

The antimicrobial activities of PI-200 and PI-201 were evaluated by the paper-disk diffusion assay. PI-200 revealed a slight antimicrobial activity against *Staphylococcus aureus* 209P JC at 1 mg/ml (growth inhibitory diameter, 9.4 mm), but was not effective against *Escherichia coli* NIHJ JC-2, *Pseudomonas aeruginosa* P-32, *Candida albicans* TIMM0239, *Candida neoformans* TIMM0354, *Trichophyton mentagrophytes* No. 81028 and *Aspergillus fumigatus* TIMM0063 at the same concentration. PI-201 did not

inhibit the growth of the tested organisms mentioned above at 1 mg/ml.

Acknowledgments

The authors wish to thank Ms. Y. TSUCHIDA for NMR spectra.

References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
2) BORN, G. V. R. & M. J. CROSS: The aggregation of blood platelets. J. Physiol. 168: 178, 1968