Clonostachin, a Novel Peptaibol That Inhibits Platelet Aggregation

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A novel peptaibol, designated clonostachin, was isolated from cultures of *Clonostachys* sp. F5898 by HP-20 and silica gel column chromatographies and reverse-phase HPLC. The structure of clonostachin was determined by Edman and chemical degradations, positive ion FAB-MS, EI-MS, and NMR analyses. Clonostachin was a linear tetradecapeptide with an *N*-terminal acetyl group and a *C*-terminal sugar alcohol. Clonostachin inhibited ADP-induced aggregation of human platelets by 80% at 150 μM.

During the course of screening for blood coagulation inhibitors of microbial origin, we have found a novel peptaibol, clonostachin, as an active compound. In this paper, we describe the isolation, structure elucidation and biological activity of clonostachin.

Materials and Methods

Materials

Human venous blood was drawn from healthy volunteers in 13 mM sodium citrate. L-Isovaline (Iva) was purchased from Acros, α-amino isobutyric acid (Aib) and ADP were obtained from Sigma. Malt extract agar, oatmeal agar and corn meal agar were purchased from Difco, U.S.A. Czapek-Dox agar was obtained from Eiken chemical, Japan. YpSs agar was prepared as follows: soluble starch (15.0 g) and agar (20.0 g) was dissolved in 400 ml of distilled water by heating in a boiling bath, followed by adding 600 ml of distilled water containing malt extract (4.0 g), K_2HPO_4 (1.0 g) and $MgSO_4 \cdot 7H_2O$ (0.5 g).

Microorganism

The producing strain *Clonostachys* sp. F5898 was isolated from a soil sample collected in Koganei-shi, Tokyo and subcultured on potato glucose agar slants at 25°C.

Amino Acid Analysis

Clonostachin (0.5 mg) was hydrolyzed in 6 N HCl at 110°C for 24 hours. The amino acids released were converted into the *N*-trifluoroacetyl (*N*-TFA) *n*-butyl ester derivatives as described by PANDEY *et al.*[1]. The derivatives were analyzed by GC/MS using a Chirasil-L-Val capillary column (0.25 mm × 25 m, GL-Sciences, Japan).

Trifluoroacetylation

Clonostachin (0.2 mg) was hydrolyzed in 0.5 M NaOH at 25°C for 18 hours. The mixture was neutralized with 1 M HCl and evaporated to dryness. The residue was suspended with 0.1 ml of ethyl acetate and incubated with 0.2 ml of trifluoroacetic anhydride at room temperature for 20 minutes[2].

Determination of the Configuration of Iva

Clonostachin (0.1 mg) was hydrolyzed in 6 N HCl at 110°C for 24 hours. After removing HCl by evaporation, the resulting amino acids were converted into phenylthiohydantoin (PTH) derivatives as follows. The amino acids were dissolved in 20 μl of ice-cold coupling buffer (ethanol - H_2O - triethylamine - phenylisothiocyanate, 7 : 1 : 1 : 1, by volume). After incubation at 50°C for 10 minutes under nitrogen gas, the mixture was evaporated to dryness at 50°C under high vacuum. The residue was dissolved in 30 μl of methanol - HCl (1 : 1, by volume) and incubated at 50°C for 5 minutes under nitrogen gas. The mixture was evaporated to dryness and the resulting PTH-amino acids were fractionated by HPLC on an Inertsil PREP-ODS column (6 × 250 mm, GL Sciences, Japan) using CH_3CN - 10 mM aqueous sodium acetate, pH 4.5 (39.5 : 60.5). The fraction containing PTH-Iva was extracted with ethyl acetate and the organic extract was applied to HPLC on a CHIRALCEL OJ-R column (4.6 × 150 mm, Daicel Chemical, Japan). The column was developed with CH_3CN - 10 mM aqueous sodium acetate, pH 4.5 (39.5 : 60.5) at 40°C at a rate of 0.5 ml/minute. The identification of the absolute configuration of Iva was carried out by the co-chromatography with the PTH-derivatives of authentic L- and D,L-Iva.

Platelet-rich Plasma and Platelet-poor Plasma

Venous blood drawn from healthy volunteers who had not taken any drugs for 2 weeks prior to the study was mixed with 1/9 vol of 0.11 M trisodium citrate.

Platelet-rich plasma and platelet-poor plasma were prepared by centrifuging blood at $200 \times g$ for 10 minutes and at $2000 \times g$ for 10 minutes, respectively[3].

Thromboelastography

Citrated blood (300 μl) was mixed with 40 μl of PBS (150 mM NaCl and 20 mM sodium phosphate, pH 7.4) containing a test sample. Blood coagulation was initiated by adding 60 μl of 1.29% $CaCl_2$ and the thromboelastograms were recorded at 37°C using a TE-700 Clot Tracer (Erma Inc. Japan).

Platelet Aggregation

Clonostachin dissolved in platelet-poor plasma (750 μl) was placed in a disposable polystyrene cuvette. Following incubation at 37°C for 5 minutes, 750 μl of platelet-rich plasma were added and the suspension was stirred at 1000 rpm for 5 minutes at 37°C. The aggregation of platelets was induced by adding 10 μl of ADP and the change in turbidity was monitored at 600 nm at 37°C on a Model 320 spectrometer (Hitachi, Japan)[3,4].

General Procedures

UV spectra were taken on a Hitachi 320 spectrometer. IR spectra were recorded on a Hitachi IR-810 spectrometer. Mass spectra were taken on a JMS-SX102A (JEOL, Japan). NMR spectra were measured on a JEOL LAMBDA 500 spectrometer.

Results

Taxonomy

Strain F5898 showed slow growth on potato glucose, Czapek-Dox, YpSs, malt extract, oatmeal and corn meal agars at 25°C, but did not grow at 37 and 45°C. Colonies on potato glucose agar attained a diameter of ~20 mm after 7 days at 25°C. Colonies were plain, initially white, then became powdery and pale cream after prolonged incubations, and partly pale orange to pale brown where conidia and aerial hyphae developed. The reverse of the colony was pale yellow. The submerged hyphae branched irregularly, producing chlamydospores. The conidiophores ($\sim 100 \sim 300 \times 2 \sim 4$ μm) were hyaline, septate, smooth-walled, branched twice to several times penicillately at upper portion and ending in phialides. The phialides ($10 \sim 15 \times 2 \sim 4$ μm) were hyaline, ampulliform, slightly swollen at the base and bent away from main axis. The conidia ($2 \sim 2.5 \times 4 \sim 6$ μm) were hyaline, elliptical or long oval and adhered to each other obliquely, producing long chains (~100 μm).

These characteristics of the strain F5898 were closely related to those of *Clo. cylindrospora*[5]. However, the strain F5898 showed slower growth on malt agar, produced fewer chlamydospores and had smaller conidia than that of the type (7.0 ~ 8.0 μm). Thus, this strain was identified as *Clonostachys* sp. F5898.

Fermentation and Isolation

The strain F5898 on agar slant was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of medium consisting of 3.5% glucose, 1.0% corn starch, 2.0% soybean meal, 0.5% peptone, 0.5% meat extract, 0.3% yeast extract, 0.2% NaCl, 0.05% KH_2PO_4, 0.05% $MgSO_4$, 0.01% CB442 (antifoam, Nippon Oil and Fat Co., Japan) and cultured at 25°C for 3 days on a rotary shaker (180 rpm). One ml of this seed culture broth was transferred to each of twenty-five 500-ml Erlenmeyer flasks containing 100 ml of the same medium and cultured at 25°C for 7 days on a rotary shaker (180 rpm). The cultured broth (2.5 liters) was filtered to separate the mycelium from the broth. The mycelium was extracted three times with 500 ml of acetone. The acetone extract was concentrated to give an aqueous solution, which was then combined with the culture filtrate. The pooled solution (2.4 liters, pH 8.0) was adsorbed to a Diaion HP-20 column (35×210 mm, Mitsubishi Chemical Industries, Japan). After washing with water (1.5 liters), the column was developed with MeOH (1.5 liters). The active fractions were concentrated to dryness, giving 8.4 g of an oily residue, which was then applied to a silica gel column (45×300 mm, Wakogel C-200). After washing the column with dichloromethane - methanol (6 : 4, 8.4 liters), the active compound was eluted with dichloromethane - methanol (5 : 5, 8.4 liters). The active fractions were purified by two successive chromatographies on silica gel columns (15×510 mm and 15×340 mm) with a solvent system of ethyl acetate-methanol. The resulting active compound (0.53 g) was further purified by HPLC on an Inertsil PREP-ODS column (30×250 mm) developed with acetonitrile - water (52.5 : 47.5) at a rate of 25 ml/minute. Active fractions were concentrated to give an aqueous solution, which was then lyophilized giving 26 mg of purified clonostachin.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of clonostachin are shown in Table 1. The molecular formula of the agent was established to be $C_{78}H_{134}N_{14}O_{25}$ from the results of positive HRFAB-MS and NMR spectra and amino acid analysis. The IR absorption bands at 3400 (NH), 1650 (amide I, CO) and 1540 cm^{-1} (amide II, NH), the amide proton signals at 6.7 ~ 8.5 ppm in the ^1H NMR spectrum and signals in the CO and C_α region in the ^{13}C NMR spectrum suggested a peptide structure for

clonostachin. Its negative reactivity with ninhydrin and the presence of a singlet methyl proton at δ_H 2.0 ppm in the ^{1}H NMR spectrum indicated the *N*-terminus to be acetylated. Amino acid analysis of the acid hydrolysate (after converting amino acids into *N*-TFA butyl ester derivatives) established the composition of Aib, D,L-Iva, L-Hyp, L-Leu, L-Ile in a molar ratio of 3 : 4 : 4 : 2 : 1. The ratio of D-Iva to L-Iva was 1 : 1 as determined by HPLC on a CHIRALCEL OJ-R column.

The ^{1}H-^{1}H COSY and HMBC spectra suggested the presence of a sugar alcohol, which was linked through an ester bond to the carboxyl terminus of the molecule (Fig. 1). The presence of a sugar alcohol was further confirmed by the following experiments. The alkaline hydrolysate of clonostachin was trifluoroacetylated and analyzed by GC/MS on a Chirasil-L-Val column. A peak of a hexa-*O*-trifluoroacetyl sugar alcohol was detected at a retention time of 6.8 minutes. The mass fragmentation pattern of this sugar alcohol derivative was identical to those of hexa-*O*-trifluoroacetyl derivative of mannitol (Fig. 2).

In the positive FAB mass spectrum of the alkaline hydrolysate of clonostachin, some sequence-specific fragment ions at *m/z* 128, 241, 354, 453, 566 (B-series) were observed (Fig. 3). From this result, *N*-terminal acyl amino acid residue was assigned to be *N*-acetyl Aib. This residue was released by the treatment with conc. HCl at 50°C for 10 minutes, exposing a new α-amino group. This finding enabled determination of the peptide sequence by Edman degradation. Following the first Edman cycle which released *N*-acetyl Aib, the PTH-amino acid derivatives were detected as shown in Fig. 4. A peptide fragment obtained after a 6-cycled degra-

Table 1. Physico-chemical properties of clonostachin.

Nature	White powder
Molecular formula	$C_{78}H_{134}N_{14}O_{25}$
HRFAB-MS (*m/z*)	
Found:	1689.9626 $(M+Na)^+$
Calcd:	1689.9542 for $C_{78}H_{134}N_{14}O_{25}Na$
IR ν_{max} (KBr) cm^{-1}	3400, 1650, 1540, 1079
Amino acid composition	Aib (3), L-Hyp (4), L-Leu (2), L-Ile (1), L-Iva (2), D-Iva (2)

Fig. 1. NMR analyses of the *C*-terminal structure of clonostachin.

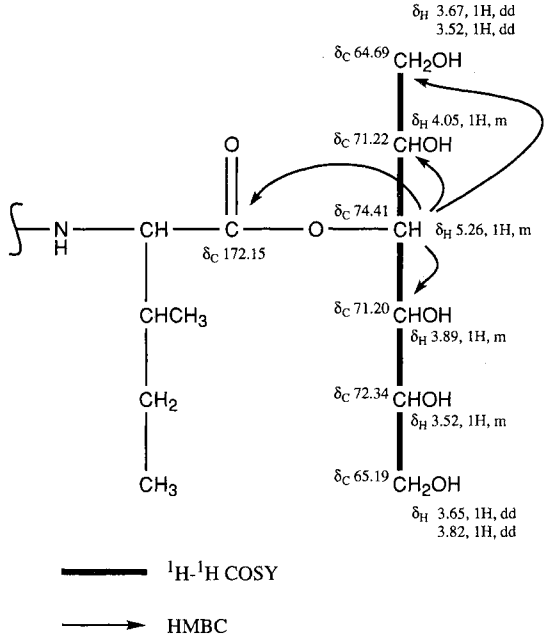


Fig. 2. EI-mass spectrum of the *O*-TFA derivative of the sugar alcohol released by alkaline hydrolysis of clonostachin.

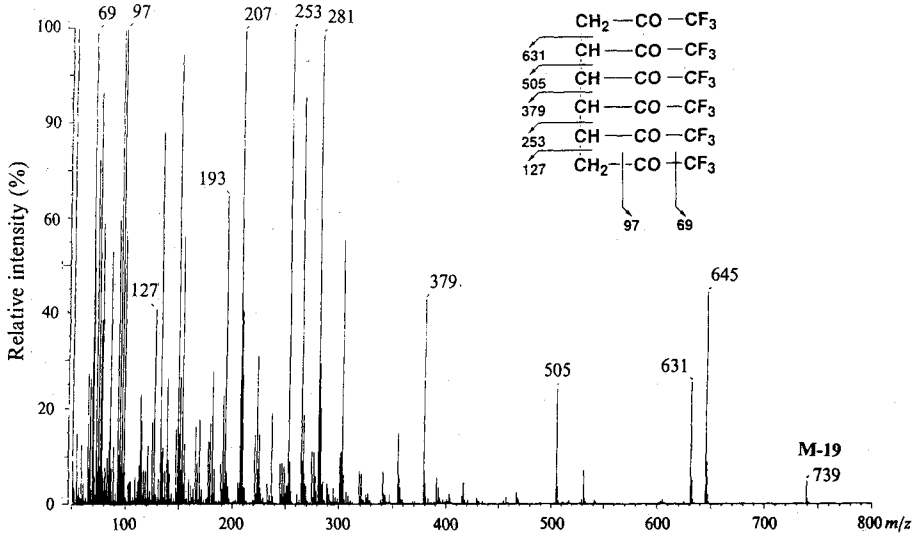


Fig. 3. FAB-MS spectrum of the alkaline hydrolysate of clonostachin.

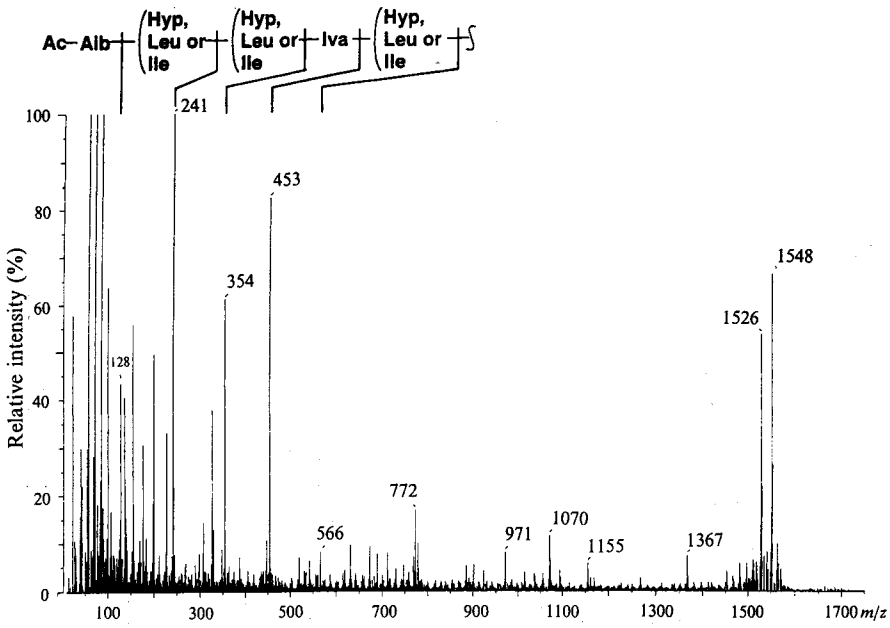
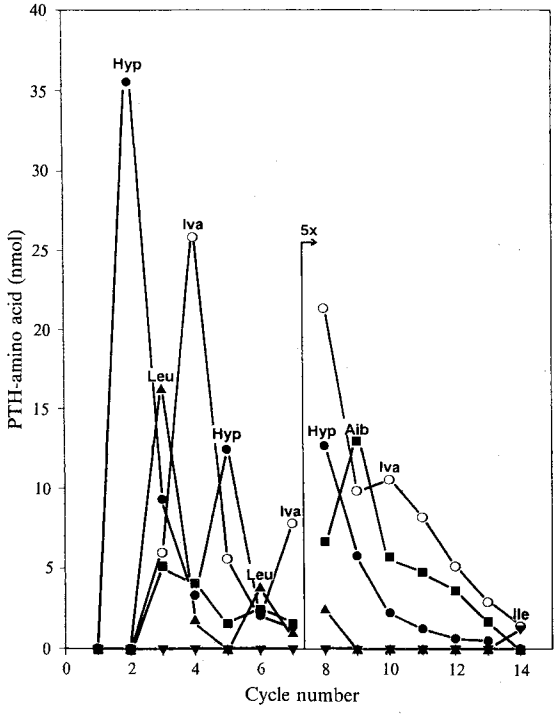


Fig. 4. Edman degradation of clonostachin.

● Hyp, ■ Aib, ○ Iva, ▲ Leu, ▼ Ile.



dation showed a protonated molecular ion peak at m/z 825.5142 ($C_{39}H_{69}O_{11}N_8$) in the HRFAB-MS spectrum. In addition, the Y″-series sequence-specific fragment ions were detected (summarized in Fig. 5). To assign the positions of two L-Iva and two D-Iva residues, the configuration of Iva released at the 4th, 7th and 10th Edman cycles was determined by HPLC on a CHIRALCEL OJ-R column. The results indicated that Iva was in the D configuration at the 4th cycle and the L configuration at the 7th and 10th cycles. Accordingly, the remaining Iva (at position 13) was assigned to be in the D configuration. Thus, the structure of clonostachin was proposed as depicted in Fig. 6.

Biological Properties

Clonostachin inhibited blood coagulation at a concentration of 50～100 μM as determined by thromboelastography (Fig. 7). In the elastograms, this compound's effect was predominantly on the amplitude, which represents the elasticity of the blood clot. Clonostachin had no effect on the reaction time, which represents the time required for the initiation of clotting. The clotting of platelet-rich plasma, but not of platelet-poor plasma, was similarly inhibited by clonostachin. These results suggested that clonostachin affected platelet aggregation. The effect of clonostachin on the platelet aggregation was investigated turbidimetrically (Fig. 8). At a concentration of 150 μM, clonostachin inhibited 50% the reversible aggregation of platelets induced by a low concentration of ADP. Further, the irreversible platelets aggregation, which had been induced by 10 μM ADP, was inhibited 80% by the agent at this concentration.

Fig. 5. FAB-MS spectrum of the octapeptide derived from clonostachin by a six-cycled Edman degradation.

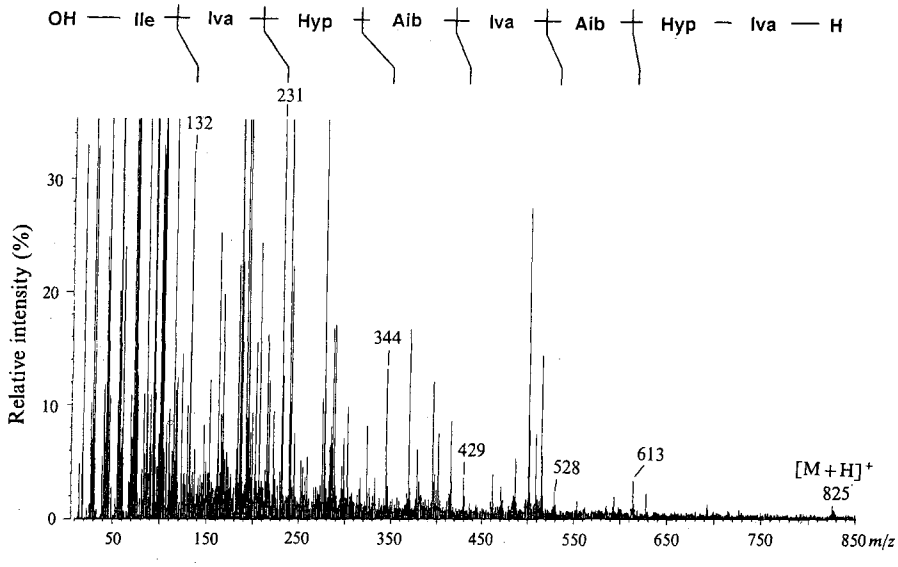


Fig. 6. Proposed structure of clonostachin.

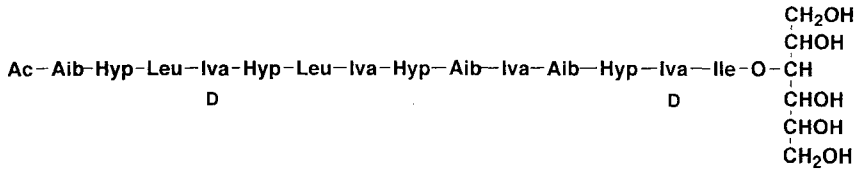
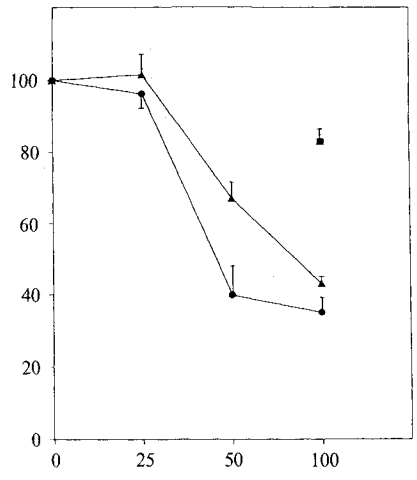
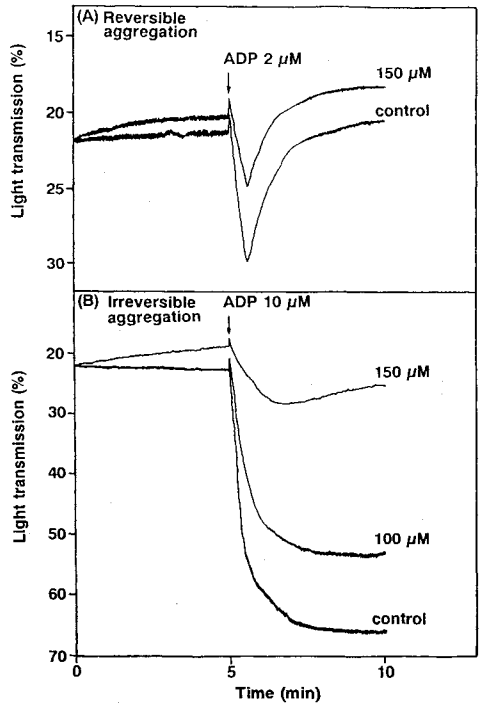


Fig. 7. Effect of clonostachin on the coagulation of whole blood (●), platelet-rich plasma (▲) and platelet-poor plasma (■).



Clonostachin in PBS was added to whole blood, platelet-rich plasma and platelet-poor plasma, then blood coagulation was initiated by adding $CaCl_2$. The coagulation was recorded using a thromboelastograph and the maximum amplitude on thromboelastogram was measured. Each value represents the mean ± S.D. from triplicate determinations.

Fig. 8. Effect of clonostachin on platelet aggregation induced by ADP.



The reversible aggregation was induced by 2 μM ADP (A) and the irreversible aggregation by 10 μM ADP (B). The spectrometer was adjusted so that platelet-rich plasma has 0% light transmission and platelet-poor plasma has 100% transmission.

Discussion

Linear peptides containing several α,α-dialkylated amino acid residues such as Aib and Iva, an *N*-terminal acyl group and a *C*-terminal amino alcohol are collectively referred to as peptaibols. Although the *C*-terminus of the molecule is an ester-linked sugar alcohol instead of amide-linked amino alcohol, clonostachin has other features of a peptaibol in that it contains seven residues of α,α-dialkyl amino acids (Aib and Iva) and an *N*-terminal acetyl group. Thus, clonostachin represents the first member of a new class of peptaibol.

Clonostachin inhibited both reversible and irreversible platelet aggregations. Recently, peptide antagonists for platelet GP IIb/IIIa receptor have been reported[6,7]. GP IIb/IIIa binds fibrinogen and this binding is involved in the mechanism of platelet aggregation[8]. Since clonostachin does not inhibit the binding of ^{125}I-fibrinogen to platelets (data not shown), a mechanism other than GP IIb/IIIa antagonism should be involved.

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