TETRAFIBRICIN, A NOVEL FIBRINOGEN RECEPTOR ANTAGONIST

I. TAXONOMY, FERMENTATION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

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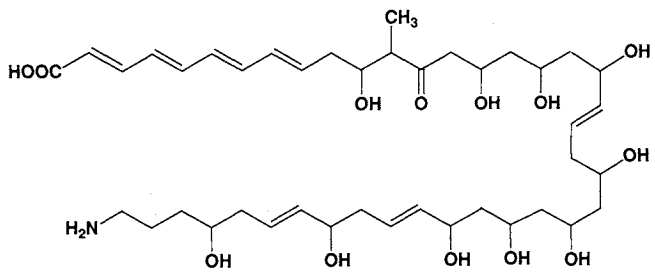
Tetrafibricin is a novel fibrinogen receptor antagonist produced by *Streptomyces neyagawaensis* NR0577. It was isolated from the culture broth by Diaion HP-21 adsorption, MeOH extraction, MCI GEL CHP-20P column chromatography, preparative HPLC and Toyopearl HW-40 SF column chromatography. The physico-chemical properties of tetrafibricin indicated that the structure of tetrafibricin is different from the known peptide fibrinogen receptor antagonists and closely related to the polyene macrolide antibiotics. Tetrafibricin strongly inhibited the binding of fibrinogen to its receptors with an IC_{50} of 46 nM. It also inhibited ADP-, collagen-, and thrombin-induced aggregation of human platelets with IC_{50}s of 5.6, 11.0 and 7.6 μM, respectively.

Platelet aggregation plays a key role during normal haemostasis and thrombosis. Platelets first adhere and spread onto the thrombogenic components of the vascular subendothelium at the sites of vascular lesions. When stimulated by an agonist such as ADP, collagen or thrombin, the fibrinogen receptors (GPIIb/IIIa), which exist as Ca^{2+}-dependent heterodimer complexes, acquire the ability to bind fibrinogen through some conformational changes within the molecules. Fibrinogen binding to the receptors on the surface of platelets is prerequisite for platelet aggregation. Thus, fibrinogen receptor antagonism is a good target for a platelet aggregation inhibitor. In recent years, many types of fibrinogen receptor antagonists have been reported, and most are peptide mimetics of RGDS (Arg-Gly-Asp-Ser), which is the minimal sequence in fibrinogen that is considered necessary to recognize fibrinogen receptors during aggregation[1~3]. Therefore, the search for non-peptide platelet aggregation inhibitors of microbial origin has been considered desirable.

In the course of our screening program for fibrinogen receptor binding antagonists, we isolated a non-peptidic antagonist, tetrafibricin (Fig. 1), from the culture broth of *Streptomyces neyagawaensis* NR0577. It inhibited the binding of fibrinogen to its receptors with an IC_{50} of 46 nM and inhibited

Fig. 1. The structure of tetrafibricin.



aggregation of human platelets induced by ADP, collagen, and thrombin. In this paper, we report on the taxonomy, production, isolation, physico-chemical properties, and biological activities of tetrafibricin. We describe the structural elucidation of tetrafibricin in the accompanying paper[4].

Taxonomy of Strain NR0577

The producing organism, strain NR0577, was isolated from a soil sample collected in Kiinaga-shima, Mie Prefecture, Japan. Taxonomic studies were carried out according to the procedures of the International Streptomyces Project[5]. The aerial mycelium formed spiral chains of spores having a rugose or warty surface (Fig. 2). Sclerotic granules, sporangia, and flagellated spores were not observed. The cultural characteristics of strain NR0577 grown

Fig. 2. Scanning electron micrograph of strain NR0577 (oatmeal yeast extract agar).

Bar represents 1.0 μm.

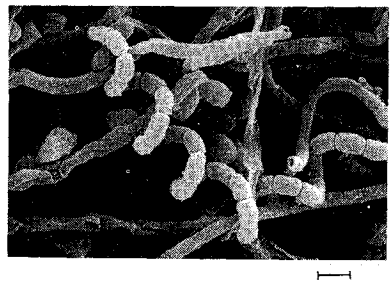


Table 1. Cultural characteristics of strain NR0577 and *Streptomyces neyagawaensis* IFO 13477.

		NR0577	*S. neyagawaensis* IFO 13477
Yeast extract-malt extract agar (ISP 2)	G:	Good, pale yellowish brown	Good, pale yellowish brown
	AM:	Abundant, white~light gray	Abundant, white~light gray
	R:	Pale yellowish brown	Pale yellowish brown
	SP:	None	None
Oatmeal agar (ISP 3)	G:	Moderate, colorless	Moderate, colorless
	AM:	Moderate, white~light gray, black (hygroscopic)	Moderate, white~light gray
	R:	Pale yellowish brown	Pale yellowish brown
	SP:	Pale yellowish brown	Dull yellow orange
Inorganic salts-starch agar (ISP 4)	G:	Moderate, pale yellowish brown	Moderate, pale yellowish brown
	AM:	Moderate, white~light gray	Moderate, white~light gray
	R:	Pale yellow orange	Pale yellowish brown
	SP:	None	Pale yellowish brown
Glycerol-asparagine agar (ISP 5)	G:	Good, pale yellow orange	Good, pale yellowish brown
	AM:	Thin, white~light gray	Thin, white
	R:	Pale yellow orange	Pale yellow orange
	SP:	Pale yellow orange	Pale yellow orange
Tyrosine agar (ISP 7)	G:	Moderate, grayish yellow brown	Moderate, dark yellowish brown
	AM:	Thin, white~light gray	Scant, light gray
	R:	Dark yellowish brown	Dark yellowish brown
	SP:	Pale yellowish brown	Grayish yellow brown
Glucose-asparagine agar	G:	Moderate, colorless	Good, colorless
	AM:	None	Thin, white
	R:	Colorless	Colorless
	SP:	None	None
Sucrose-nitrate agar	G:	Good, colorless	Good, colorless
	AM:	None	Thin, white
	R:	Colorless	Colorless
	SP:	None	None
Nutrient agar	G:	Good, light brownish gray	Moderate, dark yellowish brown
	AM:	None	Scant, light gray
	R:	Pale yellowish brown	Dark yellowish brown
	SP:	Pale yellowish brown	Pale yellowish brown

The color names used in this table were based on the Color Standard (Nihon Shikisai Co., Ltd.).
Abbreviations: G, growth; AM, aerial mycelium; R, reverse side color; SP, soluble pigment.

Table 2. Physiological characteristics of strain NR0577 and *Streptomyces neyagawaensis* IFO 13477.

	NR0577	*S. neyagawaensis* IFO 13477		NR0577	*S. neyagawaensis* IFO 13477
Gelatin liquefaction	+	+	Utilization of:		
Starch hydrolysis	+	+	Adonitol	−	−
Milk coagulation	−	−	L-Arabinose	+	+
Milk peptonization	+	−	D-Fructose	+	+
Nitrate reduction	+	−	D-Glucose	+	+
H_2S production	+	+	Inositol	+	+
Melanin production:			D-Mannitol	+	+
ISP medium 1	+	+	Melezitose	+	+
ISP medium 6	+	+	Raffinose	+	+
ISP medium 7	+	+	L-Rhamnose	+	+
NaCl tolerance	7%	7%	Sucrose	+	+
Temperature range for growth	15~42°C	15~42°C	D-Xylose	+	+
			L-Asparagine	+	+
Optimum temperature for growth	27~37°C	30~37°C	L-Phenylalanine	+	+

+, Positive; −, negative.

on various agar media at 27°C for 15 days are shown in Table 1. The aerial mass was colored in shades of gray. Hygroscopic aereas were observed on the aerial mycelium of aging cultures on oatmeal agar and inorganic salts - starch agar. The color of vegetative growth was colorless to grayish yellow brown. Melanoid pigments and other soluble pigments were produced. The physiological characteristics are shown in Table 2. The whole cell hydrolysates of strain NR0577 contained LL-diaminopimelic acid. Based on the morphological characteristics and cell wall type, strain NR0577 was considered to belong to the genus *Streptomyces*.

Among the known species of this genus, strain NR0577 showed the closest resemblance to *Streptomyces neyagawaensis* Yamamoto, Nakazawa, Horii, and Miyake[6]. The microbiological characteristics of strain NR0577 were directly compared with those of *S. neyagawaensis* IFO 13477. *S. neyagawaensis* IFO 13477 formed black hygroscopic aereas on the aerial mycelium of aging cultures. As shown in Tables 1 and 2, the taxonomic characteristics of strain NR0577 were very similar to those of *S. neyagawaensis*, except for milk peptonization and nitrate reduction. These differences were too small to permit regarding strain NR0577 as a different species. Therefore, strain NR0577 was identified as a strain of *S. neyagawaensis* and designated *S. neyagawaensis* NR0577.

Fermentation

The frozen mycelial suspension of *Streptomyces neyagawaensis* NR0577 (1.5 ml) was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a medium consisting of glucose 2.0%, Toast soya 2.0%, NaCl 0.25%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $MnCl_2 \cdot 4H_2O$ 0.0005%, $CaCO_3$ 0.32%, and Nissan Disfoam CA-115 0.05%. The inoculated flask was incubated on a rotary shaker at 27°C for 3 days at 190 rpm, and 2 ml of vegetative inoculum was transferred into a 500-ml baffled Erlenmeyer flask containing 100 ml of the same medium as above followed by incubation on a rotary shaker at 27°C at 190 rpm. The production of tetrafibricin reached maximum after 5-day incubation. A large scale fermentation was carried out in a 200-liter fermentor. Three liters of the seed culture prepared by the above method were transferred into the fermentor containing 150 liters of the production medium described above. The fermenter was run at 27°C for 5 days with aeration of 150 liters/minute and agitation of 350 rpm.

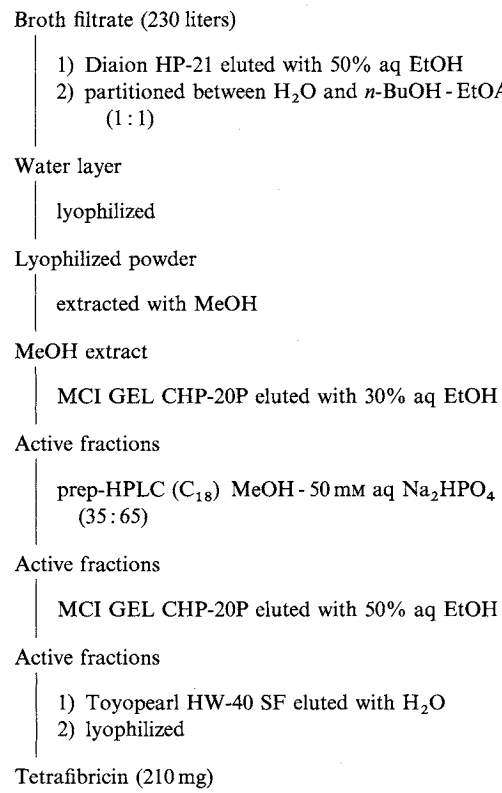
Assay Method

Biotinylated human fibrinogen and assay samples were added to micro titer wells coated with purified GPIIb/IIIa. After washing the free fibrinogen, peroxidase conjugated streptavidin (avidin-POD) was added to each well. After washing the excess avidin-POD, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added to each well. The binding was assessed by measuring the absorbance at 415 nm using a microplate photometer. Detailed assay methods were described by MORI *et al.*[7].

Isolation

Tetrafibricin was isolated from the culture broth. The isolation scheme is shown in Fig. 3. To the filtrate (230 liters) was added 50 liters (wet volume) of Diaion HP-21. The mixture was stirred at room temperature for 30 minutes and stored at 4°C for 15 hours. The HP-21 resin was collected by filtration and washed with water (150 liters) and 10% aqueous EtOH (150 liters). The resin was then treated with 50% aqueous EtOH (250 liters). The 50% aqueous EtOH solution was concentrated under reduced pressure to a volume of about 20 liters, which was then partitioned between a water and *n*-BuOH - EtOAc mixture (1 : 1). The water layer was concentrated under reduced pressure to a volume of about 5 liters and then lyophilized to give 180 g of brown powder. The lyophilized powder was suspended in 8 liters of MeOH and stirred at room temperature for 1 hour. After removal of insoluble part, the MeOH solution was concentrated under reduced pressure to give 31.3 g of yellow syrup. The syrup was dissolved in 500 ml of 15% aqueous EtOH and applied onto a column (1.5 liters) of MCI GEL CHP-20P. The column was developed with 15% aqueous EtOH, 17.5% aqueous EtOH, and 30% aqueous EtOH. The fractions were monitored by the HPLC analysis* and solid phase GPIIb/IIIa binding assay. The active fractions containing tetrafibricin were combined, concentrated under reduced pressure, and lyophilized. This lyophilized powder was purified by preparative-HPLC under the following chromatographic conditions (column: Capcellpak C_{18} AG (30 mm i.d. × 250 mm); solvent: MeOH - 50 mM aqueous Na_2HPO_4 (35 : 65); flow rate: 25 ml/minute; detection: UV 320 nm; retention time: 24 minutes). The eluate containing tetrafibricin was adjusted to pH 7 with 1 N HCl and diluted with water to 10% conc. MeOH solution. The solution was applied onto a column of MCI GEL CHP-20P (1 liter). The column was washed with 15% aqueous EtOH and eluted with 50% aqueous EtOH. The eluate was concentrated under reduced pressure and lyophilized. The

Fig. 3. Isolation procedure of tetrafibricin.



* HPLC conditions; column: Capcellpak C_{18} SG (4.6 mm i.d. × 25 cm; solvent: MeOH - 50 mM aq Na_2HPO_4 (35 : 65); flow rate: 1.0 ml/minute; detection: UV 320 nm.

Fig. 4. IR spectrum of tetrafibricin (KBr).

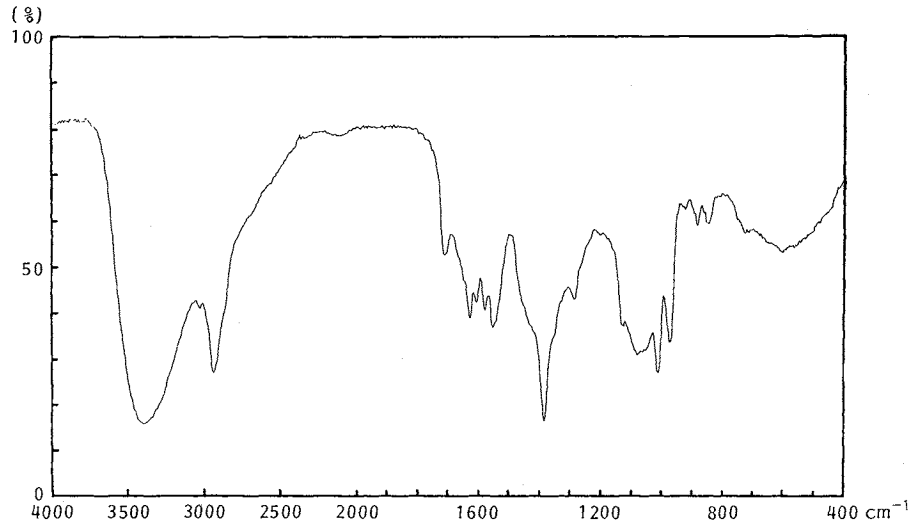


Table 3. Physico-chemical properties of tetrafibricin.

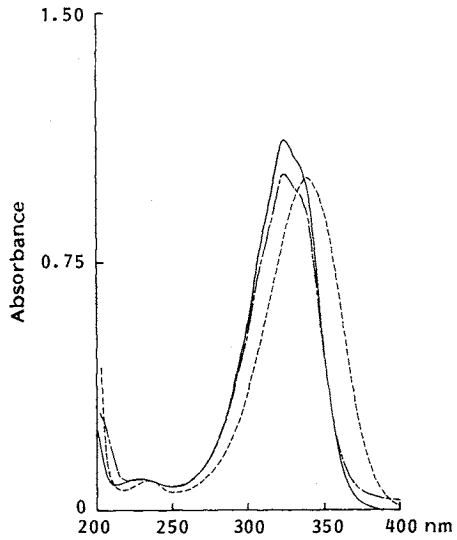
Appearance	Pale yellow powder
Molecular formula	$C_{41}H_{67}NO_{13}$
HRFAB-MS (*m/z*)	
Calcd:	782.4691
Found:	782.4676 $(M+H)^+$
UV $\lambda_{max}^{H_2O}$ nm (ε)	229 (4,200), 323 (48,400)
$\lambda_{max}^{0.05\,N\,HCl}$	235 (3,700), 339 (43,500)
$\lambda_{max}^{0.05\,N\,NaOH}$	229 (4,100), 322 (43,900)
IR ν_{max} KBr cm^{-1}	3400, 2940, 1710, 1630, 1550, 1390, 1010, 975
$[\alpha]_D^{24}$	$-38.9°$ (*c* 1.0, H_2O)
HPTLC[a] (Rf)	0.6
HPLC[b] (t_R)	14 minutes

[a] HPTLC plate RP-18: E. Merck Art. No. 13124, solvent: MeOH - 50 mM aq Na_2HPO_4 (2:1); detection: ninhydrin spray and UV 254 nm.

[b] Column: Shiseido Capcellpak C_{18} SG (4.6 mm i.d. × 25 cm); eluant: MeOH - 50 mM aq Na_2HPO_4 (35:65); flow rate: 1 ml/minute; detection: UV 320 nm.

Fig. 5. UV spectra of tetrafibricin (in H_2O).

—— water, ---- 0.05 N HCl, —·— 0.05 N NaOH.



lyophilized powder was dissolved in 6 ml of water. The solution was applied onto a column of Toyopearl HW-40 SF (3 liters) and the column was developed with water. The fractions containing tetrafibricin were combined, concentrated under reduced pressure and lyophilized to give 210 mg of tetrafibricin as a pale yellow powder.

Physico-chemical Properties

The physico-chemical properties of tetrafibricin are summarized in Table 3. Tetrafibricin was soluble in water, MeOH, EtOH, *N*,*N*-dimethylformamide, pyridine, and DMSO, but insoluble in *n*-hexane, $CHCl_3$, and EtOAc. The presence of primary amino and carbonyl groups was suggested by the positive color reactions to ninhydrin and 2,4-dinitrophenylhydrazine, respectively. The IR spectrum of tetrafibricin

(Fig. 4) suggested the presence of hydroxy and/or amino (3400, 1100 ~ 1000 cm^{-1}) and carbonyl (1710 cm^{-1}) functionalities. The UV spectrum (Fig. 5) in H_2O showed absorption maxima at 229 and 323 nm, which were attributable to a tetraenoic acid chromophore[8]. The molecular formula ($C_{41}H_{67}NO_{13}$) was determined from positive ion HRFAB-MS and ^{13}C NMR spectral data. The ^1H and ^{13}C NMR spectra of tetrafibricin in D_2O are shown in Figs. 6 and 7. These physico-chemical properties indicated that the structure of tetrafibricin is different from the known peptide fibrinogen receptor antagonists, such as decorsin[9], echistatin[10], kistrin[11], trigramin[11] and bitan[11], and is rather closely related to the polyene macrolide antibiotics.

Biological Activity

Tetrafibricin strongly inhibited the binding of fibrinogen to the GPIIb/IIIa receptor with an IC_{50} of

Fig. 6. ^1H NMR spectrum of tetrafibricin (400 MHz, in D_2O).

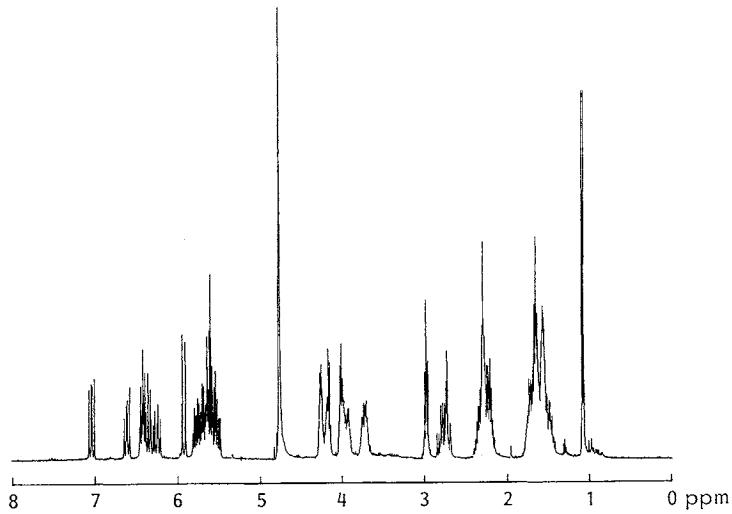
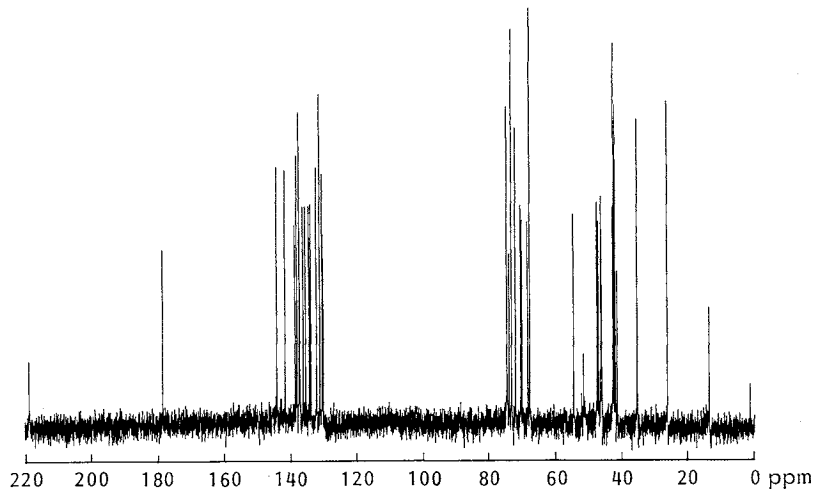


Fig. 7. ^{13}C NMR spectrum of tetrafibricin (100 MHz, in D_2O).



46 nM in a competitive manner and was about 3-fold more potent than RGDS (IC_{50} = 120 nM). Tetrafibricin also inhibited human platelet aggregation induced by ADP, collagen, and thrombin with IC_{50}s of 5.6, 11.0, and 7.6 μM (RGDS: 116, 118, and 135 μM), respectively, indicating that tetrafibricin was an antagonist of platelet fibrinogen receptors. Tetrafibricin had no antibacterial and antifungal activity. The IC_{50} value of cytotoxicity against HeLa cell was greater than 25 μM. Detailed biological activity will be reported elsewhere[12,13].

Discussion

Several fibrinogen receptor antagonists of natural origin have recently been reported. Decorsin[9] was isolated from the North American leech, *Macrobdella decora*. Disintegrins such as echistatin[10], kistrin[11], trigramin[11], and bitan[11] were isolated from the venom of various snakes. All these antagonists of natural origin are peptides containing the RGD (Arg-Gly-Asp) sequence. The inhibitory activity of these antagonists is presumably due to the binding of the RGD sequence of the receptors. With respect to the synthetic inhibitors of platelet aggregation *via* antagonism of fibrinogen receptors, many peptide mimetics containing an RGD sequence[1~3] have been synthesized. Thus, all known fibrinogen receptor antagonists are either peptides or peptide mimetics. Tetrafibricin has a novel structure and is an non-peptidic antagonist isolated from the natural products.

A novel and potent fibrinogen receptor antagonist, tetrafibricin, was isolated from the culture broth of *Streptomyces neyagawaensis* NR0577. The physico-chemical data indicated that tetrafibricin is closely related to the polyene macrolide antibiotics and is completely different from the known natural fibrinogen receptor antagonists. Tetrafibricin inhibited the binding of fibrinogen to its receptors in GPIIb/IIIa solid phase assay with an IC_{50} value of 46 nM and also inhibited human platelet aggregation induced by ADP, collagen, and thrombin with IC_{50}s in the μM range. In summary, tetrafibricin is a potent fibrinogen receptor antagonist with unique structure and will be useful in the study of fibrinogen-binding and platelet aggregation.

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