PYRROLOSTATIN, A NOVEL LIPID PEROXIDATION INHIBITOR FROM *Streptomyces chrestomyceticus*

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL PROPERTIES

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Pyrrolostatin, a new lipid peroxidation inhibitor, was isolated from the culture of *Streptomyces chrestomyceticus* EC40. The structure was determined to be 4-geranylpyrrole-2-carboxylic acid on the basis of its spectroscopic and physico-chemical properties. Pyrrolostatin inhibited lipid peroxidation in rat brain homogenate.

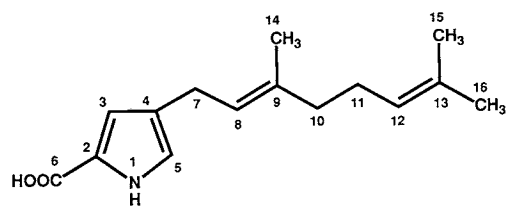
Generation of free radicals has been recently suggested to play a major role in the progression of a wide range of pathological disturbances including myocardial and cerebral ischemia[1,2], atherosclerosis[3], renal failure[4], inflammation[5] and rheumatoid arthritis[6]. Subsequent peroxidative disintegration of cells and organellar membranes has been especially implicated in various pathological processes[7]. In the screening program of free radical scavenging substances from microorganisms which are expected to be useful as therapeutic agents for these diseases, we isolated carazostatin[8] and neocarazostatins[9], as reported previously. Further screening has resulted in the isolation of a novel substance, pyrrolostatin from the mycelium of *Streptomyces chrestomyceticus* EC40 (Fig. 1). Pyrrolostatin has shown an inhibitory activity against lipid peroxidation in rat brain homogenate. In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structure elucidation and biological properties of pyrrolostatin.

Materials and Methods

Taxonomic Studies

The producing organism, strain EC40, was isolated from a soil sample collected in Brazil. The media and procedures used for cultural and physiological characterization of strain EC40 were described by SHIRLING and GOTTLIEB[10]. The culture was observed after incubation at 27°C for 3 weeks. The color names used in this study were based on the Color Standard of Nippon Shikisai Co., Ltd. Chemical composition of the cells was determined using the methods of LECHEVALIER and LECHEVALIER[11]. Detailed observation of mycelial and spore morphologies was performed with a light microscope and a scanning electron microscope (Model S-800, Hitachi Co., Ltd., Japan). The strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba-shi, Japan, under the name of *Streptomyces chrestomyceticus* EC40 and the accession No. FERM BP-3032.

Fig. 1. Structure of pyrrolostatin.



Fermentation

Culture medium consisted of glycerol 3.0%, fish meal 2.0% and $CaCO_3$ 0.2%, and pH of the medium was adjusted to 7.0 before autoclaving. In the case of fermentation in tank fermentors, 0.02% of Nissan Disfoam CA-123 (Nihon Yushi Co., Ltd., Japan) was added to the medium as an antifoam agent.

Analytical Procedures

The content of pyrrolostatin was monitored during purification by silica gel TLC (Kieselgel 60 F_{254}, E. Merck; developed with $CHCl_3$-MeOH (5:1)). Pyrrolostatin on the TLC plate was visualized as a brown spot under a UV light (254 nm).

IR and UV spectra were recorded with a Jasco A-3 spectrophotometer and a Hitachi U-3200 spectrophotometer, respectively. ^1H and ^{13}C NMR spectra were measured in CD_3OD with a JEOL JNM-GX500 spectrometer. Mass spectrum was recorded with a VG Analytical ZAB-HF mass spectrometer.

In vitro Inhibitory Activity against Lipid Peroxidation

Rat brain homogenate was prepared according to the method of KUBO *et al.*[12] with some modification. In brief, a male Wistar rat weighing about 300 g was anaesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally), and the brain was perfused transcardially with 10 mM phosphate buffered saline (pH 7.4) for complete removal of blood from the brain. After killing by decapitation, the whole brain except cerebellum was immediately homogenized with a teflon homogenizer for 30 seconds in 15 ml of ice-cold 100 mM phosphate buffer (pH 7.4). The reaction mixture of the assay consisted of 0.5% (w/v) of the homogenate, 100 μM of sodium ascorbate as an initiator for generation of oxygen radicals and a sample dissolved in MeOH. $FeSO_4$, which strongly promotes lipid peroxidation together with ascorbate, was added to the mixture, if necessary. The mixture was incubated at 37°C for 1 hour under reciprocal agitation. Malondialdehyde (MDA) was stoichiometrically formed in the reaction mixture depending on the concentration of lipid peroxides. MDA thus formed was further allowed to react with thiobarbituric acid[13] for spectrophotometric quantification at 532 nm. Percent inhibition was calculated as follows: $(1-(T-B)/(C-B)) \times 100$ (%), in which T, C and B are A_{532} readings of the drug treatment, the control (peroxidation without a drug) and the 0 time control (no peroxidation), respectively.

In Vivo Antihypoxic Activity

Male *ddY* mice weighing about 30 g were treated intraperitoneally with a sample suspended in 10% cremophor EL (Sigma Chemical Co., U.S.A.). Thirty minutes after treatment, 2.7 mg/kg of KCN in saline was injected intravenously. Immediately after injection, mice started seizuring and died within 30 seconds under a transient hypoxic state. Protective activity against hypoxia was expressed in terms of percent survival.

Results

Taxonomy of the Producing Strain

Strain EC40 produced aerial mycelia with curved or loosely spiral spore chains which were comprised 20 or more spores per chain with a smooth surface. Neither sporangia, zoospores nor sclerotia were observed. Spores were cylindrical to ovoid, $0.4 \sim 0.6 \times 0.3 \sim 0.5$ μm in size, and their surface was smooth (Fig. 2). The color of the aerial mycelium and spore mass was white to dark yellowish brown. The reverse side of colonial growth was yellowish gray to dark yellow. No soluble pigments were observed. LL-Diaminopimelic acid was detected in whole-cell hydrolysates of the culture. Based on these properties, strain EC40 was considered to belong to the genus *Streptomyces*.

Among the known species of *Streptomyces*, *Streptomyces chrestomyceticus* and *Streptomyces albus* subsp. *albus* were recognized to be similar to strain EC40 by the survey of previous literatures[14~19] and ACTINOBASE, the database of actinomycetes (Japan Collection of Microorganisms, The Institute of Physical and Chemical Research). Side-by-side comparison of strain EC40 with *S. chrestomyceticus* and

Fig. 2. Scanning electron micrographs of spore chains of strain EC40.

Bars below pictures indicate 6 μm (A) and 1.5 μm (B), respectively.

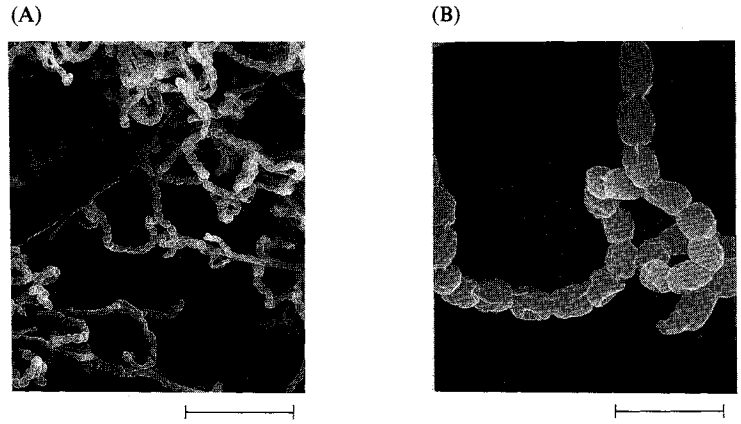


Table 1. Cultural characteristics of strain EC40 and other related strains.

Medium		Strain EC40	*S. chrestomyceticus* JCM4735	*S. albus* subsp. *albus* JCM4177
Sucrose-nitrate agar	G:	Moderate	Good	Poor
	AM:	Poor, white	Good, white	Poor
	RC:	Yellowish gray	Light olive gray	Light olive gray
	SP:	None	None	None
Glucose-asparagine agar	G:	Poor	Good	Moderate
	AM:	None	Poor	Poor
	RC:	Yellowish white	Light olive gray	Yellowish white
	SP:	None	None	None
Glycerol-asparagine agar (ISP No. 5)	G:	Moderate	Moderate	Good
	AM:	Poor, dull yellow orange	Poor, white	Good, white
	RC:	Dull yellow orange	Yellowish white	Yellowish white
	SP:	None	None	None
Inorganic salts-starch agar (ISP No. 4)	G:	Good	Good	Good
	AM:	Good, white	Good, white	Good, white
	RC:	Yellowish white	Pale yellow	Yellowish white
	SP:	None	None	None
Tyrosine agar (ISP No. 7)	G:	Good	Good	Good
	AM:	Moderate, yellowish white	Good, white	Good, white
	RC:	Dull yellow	Yellowish white	Yellowish white
	SP:	None	None	None
Nutrient agar	G:	Poor	Good	Good
	AM:	None	Poor	Good, white
	RC:	Pale yellow	Pale yellowish brown	Pale yellowish brown
	SP:	None	None	None
Yeast extract-malt extract agar (ISP No. 2)	G:	Moderate	Good	Good
	AM:	Poor, dark yellowish brown	Moderate, light olive gray	Good, white
	RC:	Dark yellow	Pale yellowish brown	Pale yellowish brown
	SP:	None	None	None
Oatmeal agar (ISP No. 3)	G:	Moderate	Good	Good
	AM:	Moderate, white	Good, white	Moderate, white
	RC:	Yellowish white	Yellowish white	Pale yellow
	SP:	None	None	None

G, Growth; AM, aerial mycelium; RC, reverse side color; SP, soluble pigment.

Table 2. Physiological characteristics of strain EC40 and other related strains.

	Strain EC40	*S. chrestomyceticus* JCM4735	*S. albus* subsp. *albus* JCM4177
Growth temperature range (°C)	15~37	15~40	15~40
Melanoid production on:			
Tyrosine agar	−	−	−
Peptone-yeast extract-Fe agar	−	−	−
Tryptone-yeast extract medium	−	−	−
Hydrolysis of starch	−	−	−
Liquefaction of gelatin	−	−	−
Coagulation of milk	−	−	−
Peptonization of milk	+	+	+
Reduction of nitrate	+	+	−
Utilization of:[a]			
L-Arabinose	−	+	+
D-Xylose	±	+	+
D-Glucose	+	+	+
D-Fructose	−	+	+
Sucrose	±	±	±
Inositol	−	+	+
L-Rhamnose	±	−	±
Raffinose	+	+	−
D-Mannitol	+	+	+
D-Galactose	+	+	+
D-Sorbitol	−	+	+
D-Mannose	+	+	+
Maltose	±	+	+

+, Positive; ±, weakly positive; −, negative.
[a] Basal medium: PRIDHAM-GOTTLIEB's inorganic salts agar (ISP No. 9).

S. albus subsp. *albus* was then carried out. The results of cultural and physiological comparison of strain EC40 and these two species are summarized in Tables 1 and 2, respectively.

Cultural characteristics resemble one another among these three strains. The aerial mycelia of *S. albus* subsp. *albus* show good growth on glycerol-asparagine agar and nutrient agar, while poor or no growth is observed for those of strain EC40 and *S. chrestomyceticus*. Furthermore, *S. albus* subsp. *albus* has different physiological characteristics from strain EC40 and *S. chrestomyceticus* in the reduction of nitrate and the utilization of raffinose. In conclusion, the cultural and physiological properties of strain EC40 are more closely related to *S. chrestomyceticus* than to *S. albus* subsp. *albus*. Accordingly, strain EC40 was designated as *S. chrestomyceticus* EC40.

Fermentation

One loopful of the agar slant culture of *Streptomyces chrestomyceticus* EC40 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the medium. The flask was shaken on a rotary shaker for 4 days at 27°C. One hundred ml of the culture was inoculated into a 50-liter tank fermentor containing 30 liters of the medium. The fermentation was continued for 4 days at 27°C with an air-flow rate of 0.6 v/v/m. Thirty liters of the seed culture was then inoculated into a 1,600-liter tank fermentor containing 800 liters of the medium. Fermentation was carried out at 27°C for 6 days with an air-flow rate of 0.6 v/v/m and an agitation rate of 80 rpm.

Isolation

The isolation procedure of pyrrolostatin is summarized in Fig. 3. Eight hundred liters of the fermentation

Fig. 3. Isolation procedure of pyrrolostatin.

Whole broth (800 liters)
↓ filtered with Celite (23 kg)
Mycelial cake
↓ extracted with acetone (270 liters)
concentrated
Oily material (16 liters)
↓ extraction with EtOAc (60 liters)
concentrated *in vacuo*
Oily material
↓ silica gel column chromatography
eluted with 10 liters of hexane-acetone (5:1)
concentrated *in vacuo*
Crude material (38 g)
↓ Sephadex LH-20 column chromatography
eluted with MeOH
concentrated *in vacuo*
Pyrrolostatin (1.7 g)

Table 3. Physico-chemical properties of pyrrolostatin.

Appearance	Pale yellowish powder
MP (°C)	121~124 (dec.)
Molecular formula	$C_{15}H_{21}NO_2$
HREI-MS (M^+, m/z)	
Calcd:	247.1572
Found:	247.1609
UV λ_{max} nm (ε)	237 (8,500), 271 (16,100)
IR (KBr) ν cm^{-1}	3360, 2970, 2920, 1690, 1440, 1350, 1120, 970

Table 4. ^{13}C and ^{1}H chemical shifts of pyrrolostatin in CD_3OD.

Position	^{1}H NMR δ_H (multiplicity, J=Hz)	^{13}C NMR δ_C (multiplicity)
1-NH	—	—
2	—	123.5 (s)
3	6.66 (m)	116.3 (d)
4	—	126.3 (s)
5	6.68 (m)	122.4 (d)
6	—	164.5 (s)
7	3.16 (d, 7.3)	26.3 (t)
8	5.31 (t, 7.3)	124.9 (d)
9	—	136.2 (s)
10	2.03 (m)	40.7 (t)
11	2.11 (m)	27.7 (t)
12	5.10 (t, 7.0)	125.4 (d)
13	—	132.2 (s)
14	1.68 (s)	16.0 (q)
15	1.60 (s)	17.7 (q)
16	1.67 (s)	25.8 (q)

broth was filtered with Celite (23 kg) to yield mycelia. The mycelia were extracted with acetone (270 liters), and the extract was concentrated to 16 liters of the aqueous suspension, which was extracted with EtOAc (60 liters). The organic layer was dehydrated with anhydrous Na_2SO_4 and then concentrated *in vacuo*. The crude oil was suspended in 500 ml of hexane and chromatographed on a silica gel column (Wako gel C-200, 10 × 50 cm) by eluting with 10 liters of hexane-acetone (5:1). The active fractions were pooled and concentrated to give a crude material (38 g), which was suspended in MeOH and further chromatographed on a Sephadex LH-20 column (5 × 100 cm) with MeOH. The active fractions were pooled and concentrated *in vacuo* to afford pyrrolostatin (1.7 g) as a pale yellowish powder.

Physico-chemical Properties

Physico-chemical properties of pyrrolostatin are summarized in Table 3. It was soluble in lower alcohols and benzene, sparingly soluble in $CHCl_3$, acetone, EtOAc and *n*-hexane, and insoluble in H_2O. The Rf value of pyrrolostatin on the silica gel TLC plate developed with $CHCl_3$-MeOH (5:1) was 0.42. The molecular formula was confirmed to be $C_{15}H_{21}NO_2$ by HREI-MS. IR absorption spectrum indicated the existence of conjugated carboxylic acid (1690 cm^{-1}).

Structure Elucidation

The ^{1}H and ^{13}C NMR spectral data are shown in Table 4. These NMR data coupled with ^{1}H-^{1}H and ^{13}C-^{1}H COSY showed the existence of a geranyl group in pyrrolostatin. Since treatment of pyrrolostatin in $CHCl_3$ with ethereal diazomethane gave the monomethyl ester ($C_{16}H_{23}NO_2$, FD-MS, m/z 261 $(M)^+$,

δ_H 3.74 (OCH_3)), the presence of one carboxylic acid residue was proved. Based on the data of ^{13}C chemical shifts (116.3 (d), 122.4 (d), 123.5 (s) and 126.3 (s)) and UV absorption, the remaining element (C_4H_3N) in pyrrolostatin was determined to a di-substituted pyrrole ring.

The connectivities of these partial structures were determined by the heteronuclear multiple-bond correlation (HMBC) experiment. The HMBC spectrum showed long range couplings of 7-H (CH_2) in the geranyl group to three pyrrole carbons (δ_C 116.3 (d), 122.4 (d) and 126.3 (s)) (Fig. 4). Therefore, the geranyl group was proved to attach at C-4 (δ_C 126.3) in the pyrrole ring. As C-3 (δ_C 116.3) and C-5 (δ_C 122.4) were both protonated, the remaining carboxylic acid was deduced to attach at C-2 (δ_C 123.5) by elimination. From these results, we determined the total structure of pyrrolostatin to be 4-geranylpyrrole-2-carboxylic acid as shown in Fig. 1.

Fig. 4. Long range couplings observed in the HMBC spectrum of pyrrolostatin (arrows).

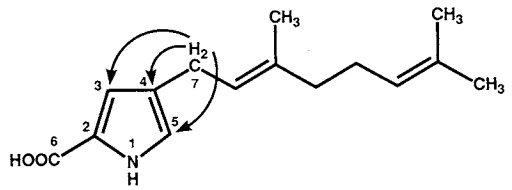


Fig. 5. The NOE and long range couplings observed in *N,O*-dimethylpyrrolostatin.

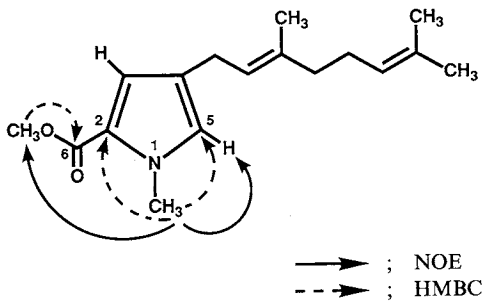
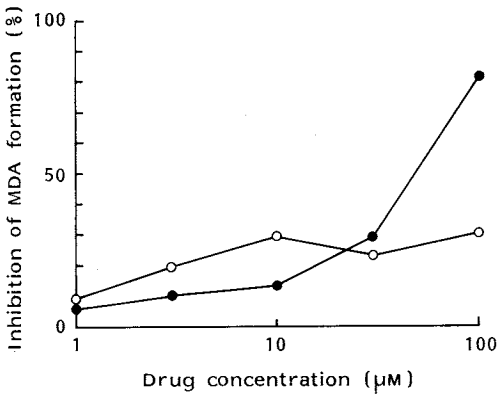


Fig. 6. Inhibitory effect of pyrrolostatin on lipid peroxidation.

● Pyrrolostatin, ○ α-tocopherol.



For confirmation of the above structure, the *N,O*-dimethyl derivative of pyrrolostatin ($C_{17}H_{25}NO_2$, FD-MS, *m/z* 275 $(M)^+$, δ_H 3.74 (OCH_3) and δ_H 3.81 (NCH_3)) was prepared by methylation with MeI in the presence of NaH in DMF. Nuclear Overhauser effects (NOE) and long range couplings observed in this derivative completely supported the structure of pyrrolostatin (Fig. 5).

Biological Activities

Inhibitory effect of pyrrolostatin on lipid peroxidation in rat brain homogenate is shown in Fig. 6. As IC_{50} of pyrrolostatin was 49.0 μM, pyrrolostatin is more inhibitory than α-tocopherol

Table 5. Effect of Fe^{2+} addition on inhibitory activity of 100 μM pyrrolostatin on lipid peroxidation.

Fe^{2+} (μM)	Inhibitory activity (%)
0	81.5
1.0	50.1
5.0	34.8

Table 6. Protective effect of pyrrolostatin on KCN-induced hypoxia in mice.

Drug	Dose[a] (mg/kg)	N[b]	Survival	Survival rate (%)
Pyrrolostatin	200	7	7	100
	100	7	2	29
Indel oxazine·HCl	50	16	15	94
Vehicle	—	13	1	8

[a] Drug was administered intraperitoneally 30 minutes before injection of KCN (2.7 mg/kg, intravenously).
[b] The number of treated mice.

(IC_{50}; $>100\,\mu M$), a well known antioxidant. The activity of pyrrolostatin decreased dose-dependently when Fe^{2+} was added to the reaction mixture as a promoter of lipid peroxidation (Table 5).

Pyrrolostatin showed strong protective activity against KCN-induced acute hypoxia in mice when administered at 200 mg/kg intraperitoneally (Table 6). This activity is as strong as that of indeloxazine hydrochloride, one of brain protective agents on the market.

Pyrrolostatin showed low toxicity; no death was observed at 200 mg/kg after intraperitoneal injection to mice ($n=7$).

Discussion

In the course of screening for free radical scavenging substances from microorganisms, we isolated the novel compound, pyrrolostatin. Pyrrolostatin had an inhibitory activity against lipid peroxidation in rat brain homogenate. Interestingly, the inhibitory activity of pyrrolostatin decreased in the presence of Fe^{2+} added to the reaction mixture, which could be explained by the formation of hydroxyl radicals (HO·) by FENTON's reaction[20] leading to promote lipid peroxidation. The inhibitory activity of desferrioxamine, a well known iron chelator, against lipid peroxidation also decreased in the presence of Fe^{2+} (data not shown). Since the lipid peroxidation-inhibitory mechanism of desferrioxamine is suggested to depend on suppression of HO· radical formation by chelation of intrinsic Fe^{2}[21], it may be natural for the inhibitory activity of desferrioxamine to decrease if a large amount of Fe is added to the reaction mixture. Pyrrolostatin as well as desferrioxamine may also prevent formation of HO· radicals by chelation of the intrinsic metal. This hypothesis is further supported by the structural characteristics of pyrrolostatin. Chemically speaking, the pyrrole-2-carboxylic acid moiety in pyrrolostatin is favorable to chelate metal ions.

In addition to the *in vitro* activity, pyrrolostatin showed a protective activity against acute hypoxia in mice. These results suggest that pyrrolostatin might be expected to be useful for the alleviation of tissue damages which are due to generation of free radicals and subsequent peroxidative disintegration of cell membranes.

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