PROPIOXATINS A AND B, NEW ENKEPHALINASE B INHIBITORS

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

YOSHINORI INAOKA, HIDETSUNE TAMAOKI, SHUJI TAKAHASHI†, RYUZO ENOKITA† and TAKAO OKAZAKI†

Bio-Science Research Laboratories and †Fermentation Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

(Received for publication May 1, 1986)

A soil isolate of actinomycete, strain SANK 60684, was found to produce new enkephalinase B inhibitors, propioxatins A and B. The presence of both LL- and *meso*-2,6-diaminopimelic acid, glycine and galactose in the cell wall assigned this strain to genus *Kitasatosporia*. From the morphological, cultural and physiological characteristics, this strain was determined to be *Kitasatosporia setae*. The *Ki* values of propioxatins A and B were 1.3×10^{-8} M and 1.1×10^{-7} M, respectively, for enkephalinase B. All other proteases examined except aminopeptidases, which were slightly inhibited, were not inhibited by these two compounds.

In the course of our extensive screening program for new enkephalinase B inhibitors, we obtained new substances, propioxatins A and B, from the culture broth of a soil isolate.

Enkephalins (Met-enkephalin and Leu-enkephalin) are generally short-lived molecules, being rapidly degraded into inactive derivatives. Therefore, their analgesic function cannot be expected to persist long after administration[1]. This degradation system in the brain includes aminopeptidases existing in the soluble fraction and the membrane[2~5], enkephalinase A[6,7] and enkephalinase B[8]. Aminopeptidases hydrolyze the Tyr-Gly bond and release Tyr, enkephalinase A hydrolyzes the Gly-Phe bond and releases Tyr-Gly-Gly, and enkephalinase B hydrolyzes the Gly-Gly bond and releases Tyr-Gly. An enkephalinase inhibitor would be expected to act as an analgesic because of the protection of enkephalin degradation *in vivo*. Some synthetic enkephalinase A inhibitors such as thiorphan[9] have been studied but specific inhibitors for enkephalinase B have not been found. Thus, we searched for an enkephalinase B inhibitor in culture filtrates of microorganisms and found two new inhibitors, which consisted of L-prolyl-L-valine peptide with *N*-acyl containing hydroxamic acid at the *N*-terminal end. We named these two inhibitors propioxatins A and B. In this paper, we report on the taxonomy of the producing organism, fermentation, isolation and biological properties of propioxatins A and B.

Materials and Methods

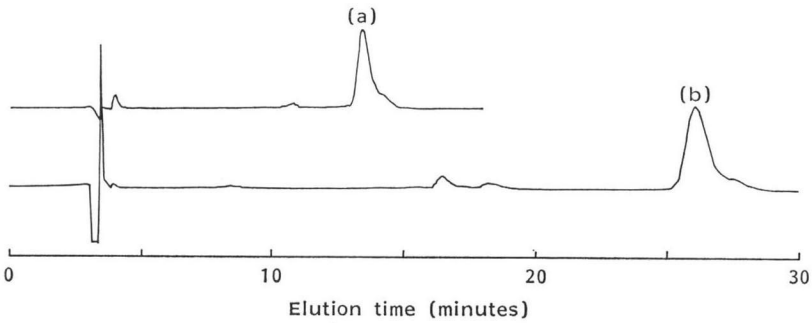
Taxonomic Studies

The strain, SANK 60684, was isolated from soil sample collected in Kanagawa prefecture, Japan.

Morphological and physiological properties of strain SANK 60684 were determined by media and methods described by SHIRLING and GOTTLIEB[10] and those recommended by WAKSMAN[11] along with several supplementary tests.

Observations of the cultures were made after incubation at 28°C for 2 weeks unless otherwise stated. Color names were assigned according to "Guide to Color Standard" (Nippon Shikisai Kenkyusho, Tokyo, Japan).

Fig. 1. HPLC profiles of propioxatins A (a) and B (b).
Propioxatins A and B were eluted with acetonitrile - H_2O (17: 83) containing 0.1% TFA on TSK gel ODS-120A (0.46×25 cm) and the absorbance at 230 nm was monitored.



Physiological examinations were carried out in terms of carbon utilization on the ISP No. 9 medium containing 1.0% carbon source, tolerance to 1 to 14% NaCl on the ISP No. 2 medium, permissive temperature range for growth on the ISP No. 2 medium by the use of a Temperature Gradient Incubator (Toyo Kagaku Sangyo Co., Japan).

Chemical analyses of strain SANK 60684 were performed in terms of cell wall type[12], whole cell sugar pattern[13], acyl-type in cell wall[14] and mycolic acid[15].

Fermentation

The seed medium and production medium (pH 7.2) contained glucose 3%, soy bean meal 3%, pressed yeast 1%, $CaCO_3$ 0.4% and $MgSO_4 \cdot 7H_2O$ 0.2% in tap water. A loopful amount of mycelium of an agar culture was incubated to 500 ml of the medium in a 2 liter-flask and shake-cultured for 4 days at 28°C. Fermentation was carried out in two 30-liter jar fermentors containing 15 liters of the medium. After inoculation of 250 ml of the seed culture, the fermentation was continued at 28°C for 4 days with agitation (150 rpm) and aeration (15 liters/minute). The culture broths were filtrated with Hyflo Super-Cel.

Isolation and Purification of Propioxatins A and B

A culture filtrate (28 liters) was passed through a column of Diaion HP-20 (5.6 liters bed volume) and washed with distilled water. The propioxatins A and B were eluted with 24 liters of 50% aq EtOH and the eluate was concd under reduced pressure to 2 liters. After the pH was adjusted to 2.0, propioxatins A and B were extracted with equal volume of butanol. The extract was adjusted to pH 7.0 and propioxatins A and B were re-extracted with 2 liters of distilled water. The solution was diluted with 10 mM of AcOH to 10 liters and applied to a DEAE-Sephadex A-25 column (4.5×35 cm) previously equilibrated with 10 mM AcOH. After the column was washed with the same solution, propioxatins A and B were eluted with a linear gradient from 10 mM (2.5 liters) to 1 M AcOH (2.5 liters). The active fractions were pooled and lyophilized. The crude powder (700 mg) was dissolved in 50 ml of 10 mM of AcOH and applied to a DEAE-Toyopearl 650S column (2.2×28 cm) previously equilibrated with 10 mM AcOH. After the column was washed with the same solution, propioxatins A and B were eluted with a linear gradient from 10 mM (500 ml) to 1 M AcOH (500 ml). The active fractions were pooled and lyophilized. The crude powder (300 mg) was dissolved with 0.5 ml of 15% aq acetonitrile containing 0.1% TFA, applied to a TSK gel ODS-120A column (0.78×30 cm) and eluted with the same solution. The obtained propioxatins A and B fractions were concd under reduced pressure separately and dissolved in small volumes of H_2O. After lyophilization, 14 mg of propioxatin A and 4 mg of propioxatin B were obtained as a colorless pure powder. The HPLC profiles of propioxatins A and B are shown in Fig. 1.

Enzyme Assay

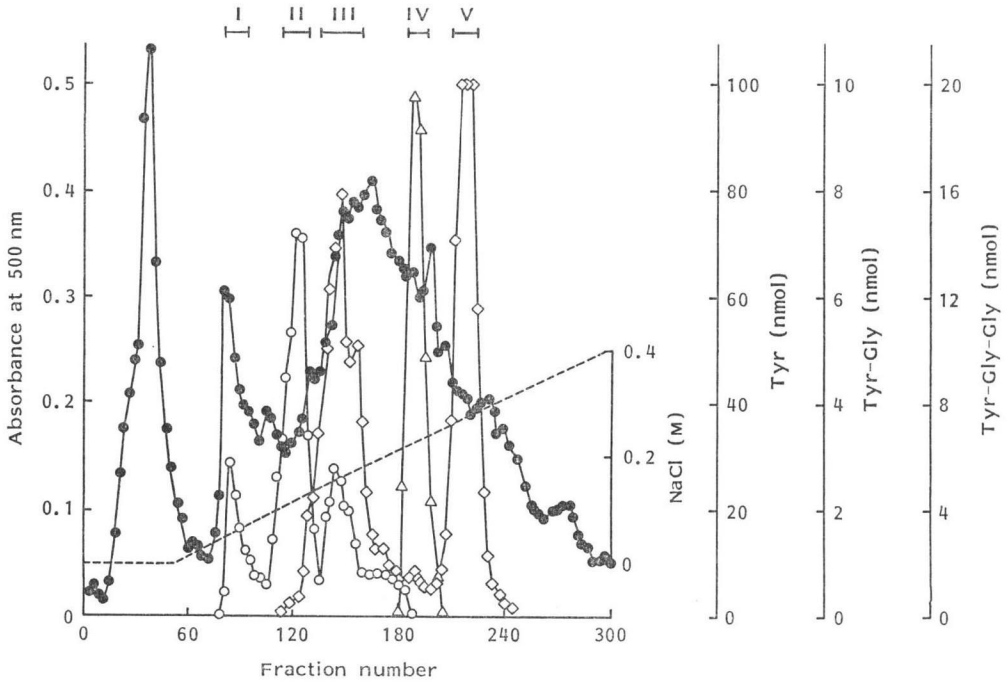
Assays of enkephalinase B and other enkephalin degrading enzymes were carried out by a HPLC

Fig. 2. DEAE-Sephacel chromatography of solubilized rat brain membrane.

The dialyzed crude extract was applied to a column (3.5×35 cm) previously equilibrated with 5 mM sodium phosphate buffer, pH 7.0, containing 1% Triton X-100.

After the column was washed with the same buffer, enzymes were eluted with linear sodium chloride gradient of 0 (2.5 liters) to 0.4 M (2.5 liters) (------) in the same buffer and fractions of 20 ml were collected.

Aliquots of each fraction were assayed for enkephalin degrading enzyme activity (50 μl) and determined for protein concentration (●) (100 μl). Nano mols of Tyr (◇), Tyr-Gly (△) and Tyr-Gly-Gly (○) were indicated.



system with Met-enkephalin as a substrate in order to obtain enkephalin degrading enzyme inhibitors. A 100 μl amount of a reaction mixture containing 1 mM Met-enkephalin, enzyme preparation and 0.1 M sodium phosphate buffer, pH 7.0, was incubated at 37°C for 15 minutes and the reaction was stopped by adding 10 μl of 2 N HCl. A 20 μl portion of the mixture was applied to a Develosil ODS-5 column (4.6×250 mm) to identify the reaction products, Tyr, Tyr-Gly and Tyr-Gly-Gly. Elution was carried out at 25°C with a 10 mM potassium phosphate - MeOH mixture (100 : 5) at a flow rate of 1.0 ml/minute. The reaction products were monitored with an emission maximum at 304 nm of Tyr on excitation at 275 nm using a Shimadzu RF-530 fluorescence spectromonitor. Elution times of Tyr, Tyr-Gly and Tyr-Gly-Gly were 4.5, 6.6 and 6.2 minutes, respectively. The amount of the products eluted were calculated with known concentrations of standard samples of Tyr, Tyr-Gly and Tyr-Gly-Gly.

Preparation and Separation of Membrane-bound Enkephalin Degrading Enzymes

Preparation and separation of crude enzymes from rat brain membrane were carried out according to a method of GORENSTEIN and SNYDER[8] with slight modifications. Brains from young male Wister rats (100 g) were homogenized in 10 volumes of 50 mM Tris-HCl buffer, pH 7.7, with a Polytron. The homogenate was centrifuged at 50,000×g for 15 minutes and washed three times with the same buffer. The membrane pellet was solubilized by resuspension with 5 volumes of 50 mM Tris-HCl buffer, pH 7.7, containing 1% Triton X-100 and then incubated at 37°C for 60 minutes. Solubilized enzymes were obtained after centrifugation of the mixture at 100,000×g for 60 minutes.

Plate 1. Light micrograph of spores of strain SANK 60684.

On sucrose - nitrate agar, 28°C, 8 days (×150).

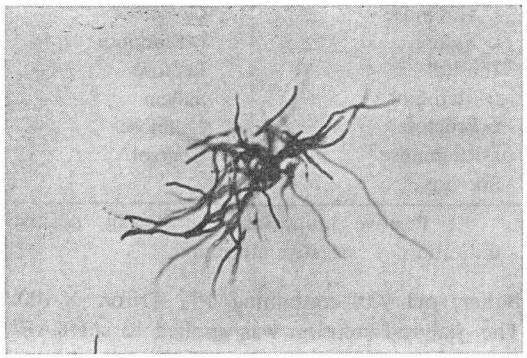


Plate 2. Scanning electron micrograph of spores of strain SANK 60684.

On potato extract - carrot extract agar, 28°C, 8 days.

A mark equals 1 μm.

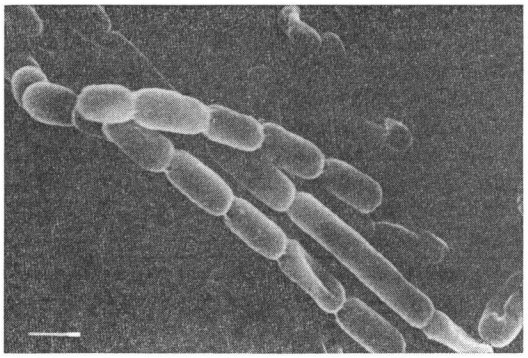


Table 1. Cultural characteristics of strain SANK 60684.

Yeast extract - malt extract agar (ISP 2)	G:	Abundant, flat to raised, pale brown (2-8-9)
	AM:	Good, velvety, brownish white (1-8-6)
	R:	Pale yellowish brown (6-7-9)
	SP:	None
Oatmeal agar (ISP 3)	G:	Abundant, flat, light brownish gray (2-7-8)
	AM:	Abundant, velvety, brownish white (1-8-6)
	R:	Pale yellowish brown (4-7-8)
	SP:	Pale yellowish brown (3-7-8)
Inorganic salts - starch agar (ISP 4)	G:	Good, flat, pale yellowish brown (4-8-9)
	AM:	Moderate, velvety, brownish white (1-8-6)
	R:	Grayish yellow brown (3-6-8)
	SP:	None
Glycerol - asparagine agar (ISP 5)	G:	Abundant, flat, brownish white (1-9-6)
	AM:	Abundant, velvety, brownish white (1-8-6)
	R:	Pale yellowish brown (3-7-8)
	SP:	None
Sucrose - nitrate agar	G:	Moderate, flat, pale yellowish orange (2-9-9)
	AM:	Poor, white
	R:	Pale yellowish orange (2-9-9)
	SP:	None
Potato extract - carrot extract agar	G:	Good, flat, pale yellowish orange (2-9-9)
	AM:	Good, velvety, brownish white (1-8-6)
	R:	Brownish white (1-8-6)
	SP:	None

G; Growth, AM; aerial mycelium, R; reverse, SP; soluble pigment.

This Triton-solubilized preparation was thoroughly dialyzed against 5 mM sodium phosphate buffer, pH 7.0, containing 1% Triton X-100 and then applied to a DEAE-Sephacel column (3.5×35 cm) previously equilibrated with the same buffer. The enzymes were eluted with the same buffer containing a linear gradient of sodium chloride to 0.4 M (2.5 liters+2.5 liters). Fig. 2 shows the elution pattern of enkephalin degrading enzymes.

Fractions I and II were used as enkephalinase A-I and -II, and fractions III and V were used as aminopeptidase-I and -II, respectively. Enkephalinase B from fraction IV was further purified as follows: the collected fraction of enkephalinase B was dialyzed thoroughly against 5 mM sodium phosphate

Table 2. Physiological properties of strain SANK 60684.

Nitrate reduction	Positive
Starch hydrolysis	Positive
Gelatin liquefaction	Negative
Milk peptonization	Positive
Milk coagulation	Positive
Melanin formation*	Negative
Casein decomposition	Positive
Tyrosine decomposition	Negative
Xanthine decomposition	Negative
Growth temperature range**	6~38°C
Optimum temperature range**	19~28°C
Sodium chloride tolerance	2<3%

* Tryptone - yeast extract broth (ISP 1), peptone - yeast extract - iron agar (ISP 6), tyrosine agar (ISP 7).

** Yeast extract - malt extract agar (ISP 2).

Table 3. Carbohydrate utilization of strain SANK 60684.

D-Glucose	+	Raffinose	−
L-Arabinose	±	Galactose	+
D-Xylose	±	D-Mannose	±
Inositol	−	Lactose	−
D-Mannitol	−	Salicin	−
D-Fructose	−	Cellulose	−
L-Rhamnose	−	Control	−
Sucrose	−		

+; Positive utilization, ±; weakly positive utilization, −; negative utilization.

buffer, pH 7.0, containing 1% Triton X-100. The dialyzed solution was applied to a DEAE-Toyopearl 650S column (1.6×27 cm) previously equilibrated with the same buffer. The column was washed with the same buffer and the enzyme was eluted with the buffer containing a linear concentration gradient of sodium chloride to 0.4 M (500 ml+500 ml). The enzyme fractions were collected and used for screening and assay of propioxatins A and B. A cytosolic aminopeptidase from rat brain was prepared from a supernatant obtained by centrifugation at 50,000×*g* of a homogenate and partially purified by ammonium sulfate precipitation (30~60% saturation) and Mono Q ion exchange chromatography.

Assay for Inhibitory Activity Used for the Screening and Isolation of Propioxatins A and B

The reaction mixture for inhibitory activity contained 80 μl of enzyme solution in 0.1 M sodium phosphate buffer, pH 6.5 (optimum pH of enkephalinase B), 10 μl of inhibitor solution and 10 μl of 1 mM Met-enkephalin in the same buffer (total volume 100 μl). The reaction was stopped by adding 10 μl of 2 N HCl after incubation at 37°C for 15 minutes. A 20 μl aliquot of reaction product, Tyr-Gly, was analyzed as mentioned above. The reaction was also carried out without inhibitor as a control. The concentration of the inhibitor required for 50% inhibition (IC_{50}) was calculated using 10 mM Met-enkephalin instead of 1 mM.

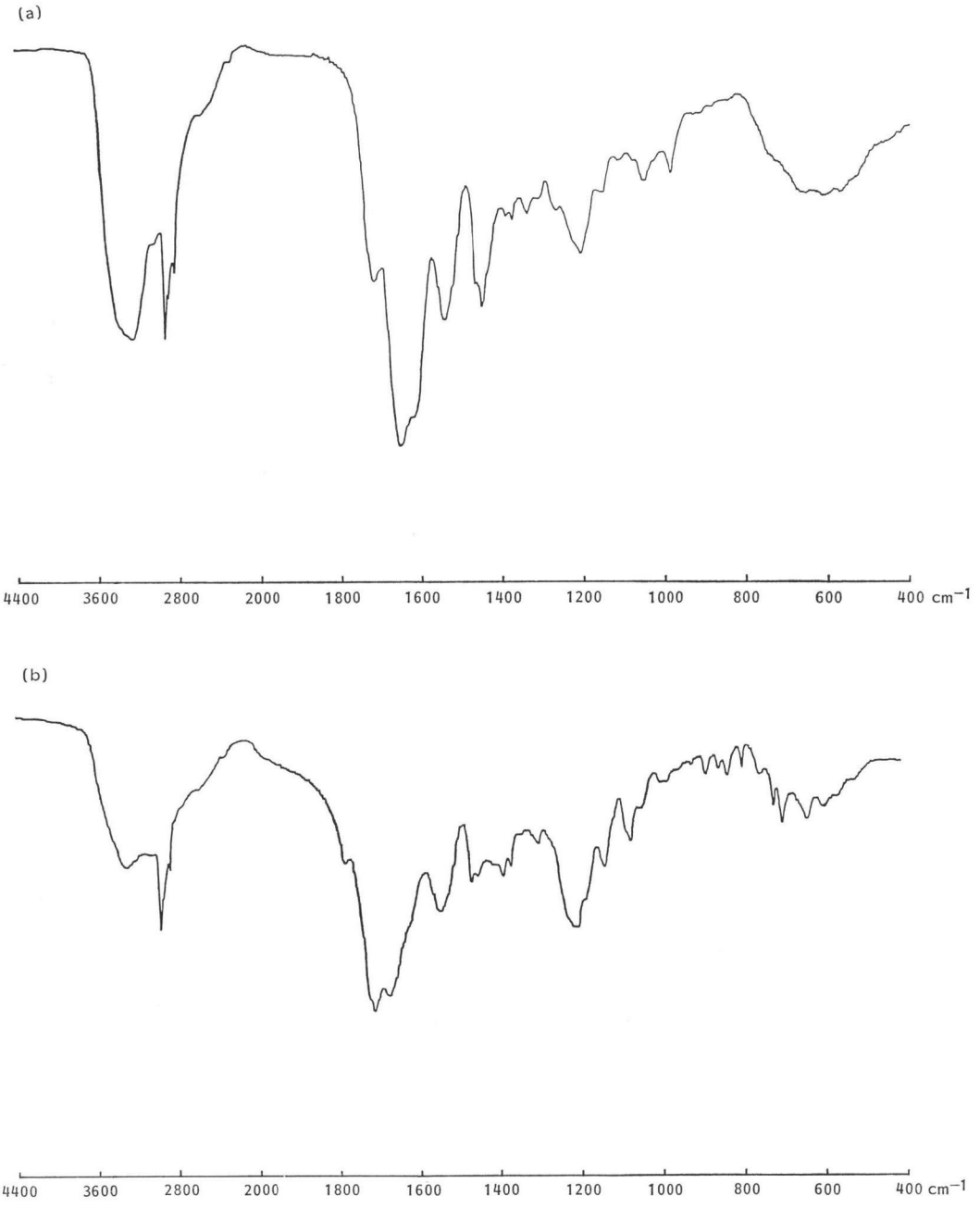
Results and Discussion

Taxonomic Studies of Strain SANK 60684

Mature spore chains produced in the aerial mycelium were long, with 10 to 50 or more spores per chain. The morphology of the spore chains was determined as *Rectiflexibiles*, as shown in Plate 1. The spores were 0.4~0.8 by 0.8~3.0 μm in size with a smooth surface, as shown in Plate 2.

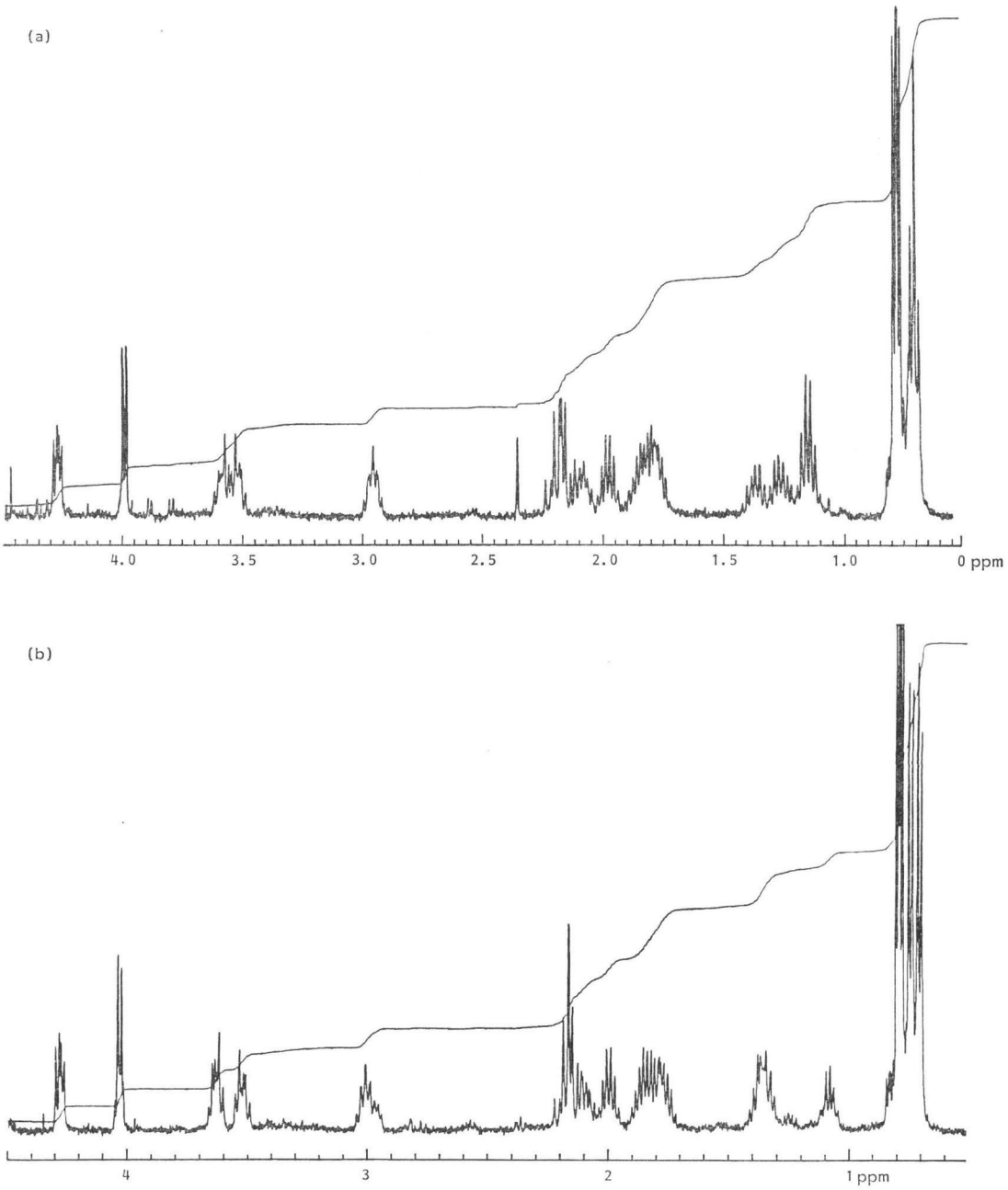
The cultural characteristics of strain SANK 60684 on various media are shown in Table 1. Physiological properties and carbohydrate utilization are summarized in Tables 2 and 3, respectively. The cell wall of strain SANK 60684 contained both *meso*- and LL-2,6-diaminopimelic acid (molecular ratio, 2 : 1) as the diamino acid and glycine. Galactose was detected in whole-organism hydrolysates. Mycolic acid was not detected. A glycolate test indicated that strain SANK 60684 had *N*-acetylglucosamine in its wall. The results of the taxonomic studies mentioned above showed that strain SANK 60684 belongs to genus *Kitasatosporia*. Among known species of genus *Kitasatosporia*[16~18], the characteristics of strain SANK 60684 were found to be closely related to those of *Kitasatosporia setalba*[19] (*K. setae*[19,20]), except in the utilization of galactose and decomposition of tyrosine. Strain SANK 60684 utilized galactose but did not decompose tyrosine while *K. setae*

Fig. 3. IR spectra of propioxatins A (a) and B (b).



was the opposite. These differences were not sufficient to consider strain SANK 60684 as a new species. It was, therefore, concluded that strain SANK 60684 belonged to the species *K. setae* and it was designated as *K. setae* SANK 60684. Progeny of the strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaragi prefecture, Japan with an accession number of FERM P-7581.

Fig. 4. ^{1}H NMR spectra of propioxatins A (a) and B (b).



Properties of Propioxatins A and B

Propioxatins A and B were soluble in water, methanol, ethanol but insoluble in ethyl acetate, chloroform, benzene and ether. They gave negative ninhydrin and Sakaguchi reaction. Physicochemical properties of the propioxatins were as follows: Propioxatin A: MP 106~110°C; $[\alpha]_D^{25}$ −70.9° (*c* 1.0, H_2O).

Fig. 5. ^{13}C NMR spectrum of propioxatin A in D_2O.

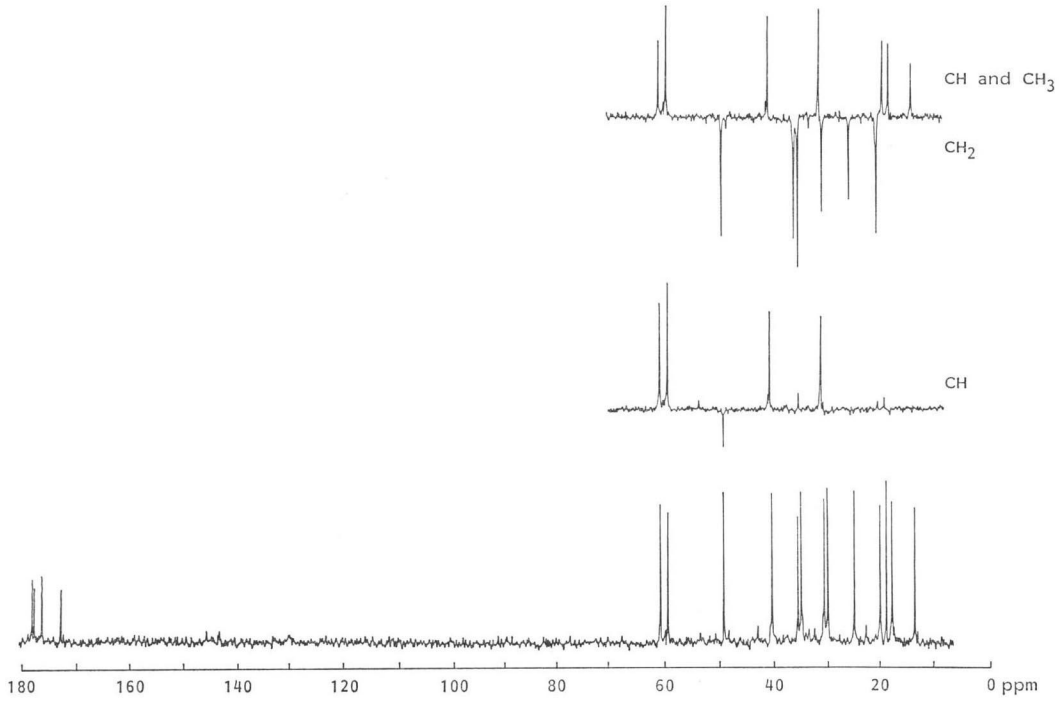


Fig. 6. Inhibition by propioxatins A and B of hydrolysis of Met-enkephalin by enkephalinase B.
(a) Propioxatin A, *Ki* 1.3×10^{-8} M: Met-enkephalin 0.1 mM (○), 1 mM (●).
(b) Propioxatin B, *Ki* 1.1×10^{-7} M: Met-enkephalin 0.1 mM (□), 1 mM (■).

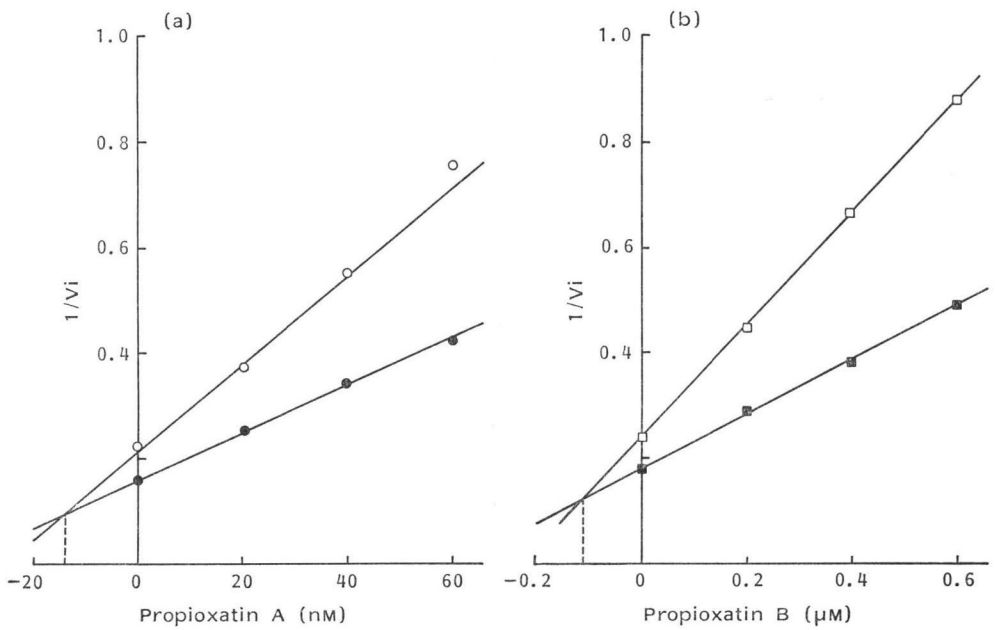


Table 4. Inhibitory activities of propioxatins A and B on enkephalin degrading enzymes of rat brain membrane.

Enzymes	IC_{50} (μM)	
	Propioxatin A	Propioxatin B
Enkephalinase A-I	>100	>100
Enkephalinase A-II	>100	>100
Aminopeptidase-I	0.94	3.8
Aminopeptidase-II	20	2.8
Enkephalinase B	0.036	0.34
Soluble-aminopeptidase	>100	1.6

Table 5. Inhibitory activities of propioxatins against various proteases and peptidases.

Enzymes	IC_{50} (μM)	
	Propioxatin A	Propioxatin B
Leu-aminopeptidase	2.6	1.7
Aminopeptidase M	0.39	2.7
Carboxypeptidase A	>100	>100
Carboxypeptidase B	>100	>100
Chymotrypsin	>100	>100
Trypsin	>100	>100
Elastase	>100	>100
Pepsin	>100	>100
Thermolysin	>100	>100
Papain	>100	>100

Anal Calcd for $C_{17}H_{29}N_3O_6$: C 54.99, H 7.82, N 11.32, O 25.88.
Found: C 54.67, H 7.51, N 11.67, O 26.15.

The molecular formula was determined to be $C_{17}H_{29}N_3O_6$ by elemental analysis and FAB-MS *m/z* 372 (M+1). Propioxatin B: MP 84~90°C; $[\alpha]_D^{25}$ −51.3° (*c* 1.0, H_2O).

Anal Calcd for $C_{18}H_{31}N_3O_6$: C 56.10, H 8.05, N 10.91, O 24.94.
Found: C 50.59, H 7.19, N 9.59, O 32.63.

The molecular formula was determined to be $C_{18}H_{31}N_3O_6$ by elemental analysis and FAB-MS *m/z* 386 (M+1). The IR spectra of propioxatins A and B are shown in Fig. 3. The ^1H NMR spectra of propioxatins A and B in deuterium oxide (external TMS as the reference) are shown in Fig. 4 and also the ^{13}C NMR spectrum of propioxatin A is shown in Fig. 5.

The structure determination is described in a subsequent paper.

Inhibitory Activities of Propioxatins A and B

Dixon plots of propioxatins A and B for enkephalinase B are shown in Fig. 6. Propioxatins A and B were both competitive inhibitors; the *Ki* values being 1.3×10^{-8} M and 1.1×10^{-7} M, respectively. Inhibitory activities of propioxatins A and B for other enkephalin degrading enzymes from rat brain are shown as IC_{50} values in Table 4. Aminopeptidases were also inhibited by both propioxatins A and B but these activities were over 10-fold lower than for enkephalinase B. Inhibitory activities of propioxatins A and B for other proteases and peptidases are also shown in Table 5 as IC_{50} values. Leu-aminopeptidase and aminopeptidase M were inhibited but others were not. The propioxatins were specific inhibitor for enkephalinase B, with propioxatin A being more specific than propioxatin B.

References

1) Chang, J. K.; B. T. W. Fong, A. Pert & C. B. Pert: Opiate receptor affinities and behavioral effects of enkephalin: structure-activity relationship of ten synthetic peptide analogues. Life Sci. 18: 1473~1482, 1976
2) Hambrook, J. M.; B. A. Morgan, M. J. Rance & C. F. C. Smith: Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. Nature 262: 782~783, 1976
3) Mark, N.; A. Grybaum & A. Neidle: On the degradation of enkephalins and endorphins by rat and mouse brain extracts. Biochem. Biophys. Res. Commun. 74: 1552~1559, 1977
4) Meek, J. L.; H. Y. T. Yang & E. Costa: Enkephalin catabolism in vitro and in vivo. Neuropharmacology 16: 151~154, 1977
5) Knight, M. & W. A. Klee: The relationship between enkephalin degradation and opiate receptor occupancy. J. Biol. Chem. 253: 3843~3847, 1978

6) MALFROY, B.; J. P. SWERTS, A. GUYON, B. P. ROQUES & J. C. SCHWARTZ: High-affinity enkephalin-degrading peptidase in brains is increased after morphine. Nature 276: 523~526, 1978
7) SULLIVAN, S.; H. AKIL & J. O. BARCHAS: *In vitro* degradation of enkephalin: Evidence for cleavage at the Gly-Phe bond. Commun. Psychopharmacol. 2: 525~531, 1978
8) GORENSTEIN, C. & S. H. SNYDER: Two distinct enkephalinases: Solubilization, partial purification and separation from angiotensin converting enzyme. Life Sci. 25: 2065~2070, 1979
9) ROQUES, B. P.; M. C. FOURNIÉ-ZALUSKI, E. SOROCA, J. M. LECOMTE, B. MALFROY, C. LLORENS & J.-C. SCHWARTZ: The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. Nature 288: 286~288, 1980
10) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
11) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, identification and descriptions of genera and species. Williams and Wilkins Co., Baltimore, 1961
12) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell-wall preparation from strains various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
13) LECHEVALIER, M. P. & H. A. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
14) UCHIDA, K. & K. AIDA: Acyltype of bacterial cell wall; its simple identification by colorimetric method. J. Gen. Appl. Microbiol. 23: 249~260, 1977
15) HECHT, S. T. & W. A. CAUSEY: Rapid method for the detection and identification of mycolic acids in aerobic actinomycetes and related bacteria. J. Clin. Microbiol. 4: 284~287, 1976
16) TAKAHASHI, Y.; Y. IWAI & S. OMURA: Two new species of the genus *Kitasatosporia*, *Kitasatosporia phosalacinea* sp. nov. and *Kitasatosporia griseola* sp. nov. J. Gen. Appl. Microbiol. 30: 377~387, 1984
17) SHIMAZU, A.; S. HIGARA, T. ENDO, K. FURIHATA & N. ŌTAKE: *Kitasatosporia melanogena*, a new species of *Actinomycetales*. Abstracts of Ann. Meeting of Soc. for Actinomycetes, Japan. p. 9, Osaka, June 15, 1984
18) ŌMURA, S.; M. MURATA, H. HANAKI, K. HINOTOZAWA, R. ŌIWA & H. TANAKA: Phosalacine, a new herbicidal antibiotic containing phosphinothricin. Fermentation, isolation, biological activity and mechanism of action. J. Antibiotics 37: 829~835, 1984
19) ŌMURA, S.; Y. TAKAHASHI, Y. IWAI & H. TANAKA: *Kitasatosporia*, a new genus of the order *Actinomycetales*. J. Antibiotics 35: 1013~1019, 1982
20) ŌMURA, S.; Y. TAKAHASHI, Y. IWAI & H. TANAKA: Revised nomenclature of *Kitasatosporia setalba*. Int. J. Syst. Bacteriol. 35: 221, 1985