PANCLICINS, NOVEL PANCREATIC LIPASE INHIBITORS

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

MASAE MUTOH, NAOKI NAKADA*, SHOKO MATSUKUMA, SHOICHI OHSHIMA, KIYOSHI YOSHINARI, JUNKO WATANABE and MIKIO ARISAWA

Nippon Roche Research Center,
200 Kajiwara, Kamakura, Kanagawa 247, Japan

(Received for publication July 13, 1994)

Panclicins A, B, C, D, and E are novel pancreatic lipase inhibitors isolated from *Streptomyces* sp. NR 0619. Structurally, panclicins A, B, C, D, and E are analogues of tetrahydrolipstatin (THL), which contains a β-lactone and a *N*-formyl leucine ester, and the IC_{50}s of panclicins A, B, C, D, and E for porcine pancreatic lipase are 2.9, 2.6, 0.62, 0.66, and 0.89 μM, respectively. The potency of the inhibitory activity of each compound is attributed to the amino acid moiety of each structure. The panclicins are either glycine-type compounds such as panclicins C, D, E, which are two to threefold more potent than THL, or they are alanine-type compounds such as panclicins A and B, which are less potent than the glycine compounds. The inhibitory profiles of the panclicins for other lipases such as post-heparin plasma lipases and bacterial lipases are similar to those for pancreatic lipase. Panclicins A, B, C, D, and E, in a manner similar to THL, irreversibly inhibit pancreatic lipase. However, the compounds don't irreversibly inhibit the enzyme as strongly as THL does.

Obesity is a risk factor for hypercholesterolemia, hypertension and diabetes etc., and to a certain extent is related to a high nutritional fat intake. Pancreatic lipase plays a key role in dietary triglyceride absorption[1,2]. It catalyzes the hydrolysis of fatty acids from triacylglycerol with colipase at the surface of their droplets, which are emulsified by bile salts under physiological conditions[3]. A number of inhibitors of pancreatic lipase including lipstatin, which was isolated from *Streptomyces toxytricini* in 1987[4,5], have been described so far. Lipstatin is a very potent and irreversible inhibitor of pancreatic lipase having an unusual β-lactone structure incorporated into its hydrocarbon backbone. Chemical modification of lipstatin resulted in a hydrogenated derivative, tetrahydrolipstatin (THL)[4~10]. THL has advantage over lipstatin in stability, and is currently in the clinical stage. We continued our screening with the aim of (i) to find more selective and potent inhibitors of pancreatic lipase, and (ii) to discover a compound with THL hydrocarbon backbone. As its result, panclicins A, B, C, D, and E were isolated from *Streptomyces* sp. NR 0619. They were found to be analogues of THL. In this paper, we report on the taxonomy, production, isolation and biological activities of panclicins A, B, C, D, and E. The structure determination of panclicins A, B, C, D, and E are described in the accompanying paper[11].

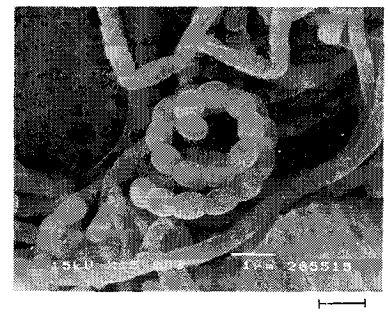
Taxonomy of strain NR 0619

Strain NR 0619 was isolated from a soil sample collected in Yamaga-machi, Oita Prefecture, Japan. Taxonomic studies were carried out according to the procedures of the International Streptomyces Project[12]. The aerial mycelium formed spiral chains of spores with 20 to 40 spores per chain. The spores were subspherical ($0.5 \sim 0.7 \times 0.7 \sim 1.0$ μm), and their surface was smooth (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed. Fragmentation of the vegetative mycelium was not observed on any of the agar media tested. The cultural characteristics of strain NR 0619 grown on various agar media

at 27°C for 14 days are shown in Table 1. Aerial mass color was light gray. Hygroscopic areas were observed on the aerial mycelium of aging cultures on oatmeal agar. The color of vegetative growth was pale yellow to grayish yellow brown. Melanoid pigments were produced. Soluble pigments were not distinctive. The physiological characteristics and carbohydrate utilization are shown in Tables 2 and 3, respectively. The whole-cell hydrolysates of strain NR 0619 contained L,L-diaminopimelic acid. Based on the morphological characteristics and cell wall type, we assigned strain NR 0619 to the genus *Streptomyces* and designated it *Streptomyces* sp. NR 0619.

Fig. 1. Scanning electron micrography of strain NR 0619 (water agar).

Bar represents 1.0 μm.



Fermentation

The frozen mycelial suspension of *Streptomyces*

Table 1. Cultural characteristics of strain NR 0619.

Medium		Cultural characteristics	Medium		Cultural characteristics
Yeast extract-malt extract agar (ISP 2)	G:	Moderate, yellowish brown	Tyrosine agar (ISP 7)	G:	Good, light brownish gray
	AM:	Moderate, white		AM:	Abundant, light gray
	R:	Yellowish brown		R:	Yellowish brown
	SP:	None		SP:	Shade of brownish gray
Oatmeal agar (ISP 3)	G:	Good, dull yellow orange	Glucose-asparagine agar	G:	Moderate, yellowish gray
	AM:	Abundant, light gray		AM:	None
	R:	Dull yellow orange		R:	Yellowish gray
	SP:	Pale brown		SP:	None
Inorganic salts-starch agar (ISP 4)	G:	Moderate, grayish yellow brown	Sucrose-nitrate agar	G:	Good, pale yellow
	AM:	Moderate, light gray		AM:	Abundant, light gray
	R:	Pale yellowish brown		R:	Yellowish gray
	SP:	None		SP:	None
Glycerol-asparagine agar (ISP 5)	G:	Moderate, yellow orange	Nutrient agar	G:	Moderate, pale yellowish brown
	AM:	Moderate, light gray		AM:	Thin, white
	R:	Yellow orange		R:	Pale yellowish brown
	SP:	None		SP:	Pale yellowish brown

Abbreviations: G, growth; AM, aerial mycelium; R, reverse side color; S, soluble pigment.

Table 2. Physiological characteristics of strain NR 0619.

Gelatin liquefaction	−
Starch hydrolysis	+
Milk coagulation	−
Milk peptonization	−
Nitrate reduction	+
Melanin production	
ISP medium No. 1	+
ISP medium No. 6	+
ISP medium No. 7	+
Temperature range for growth	10~37°C
Optimum temperature for growth	27~30°C

+, Positive; −, negative.

Table 3. Carbohydrate utilization of strain NR 0619.

Adonitol	−	D-Mannose	+
L-Arabinose	±	Melezitose	−
Cellobiose	+	Melibiose	+
D-Fructose	+	Raffinose	+
D-Galactose	+	L-Rhamnose	−
D-Glucose	+	Salicin	+
Glycerol	+	Soluble starch	+
Inositol	+	Sucrose	+
Lactose	+	Trehalose	+
Maltose	+	D-Xylose	+
D-Mannitol	+		

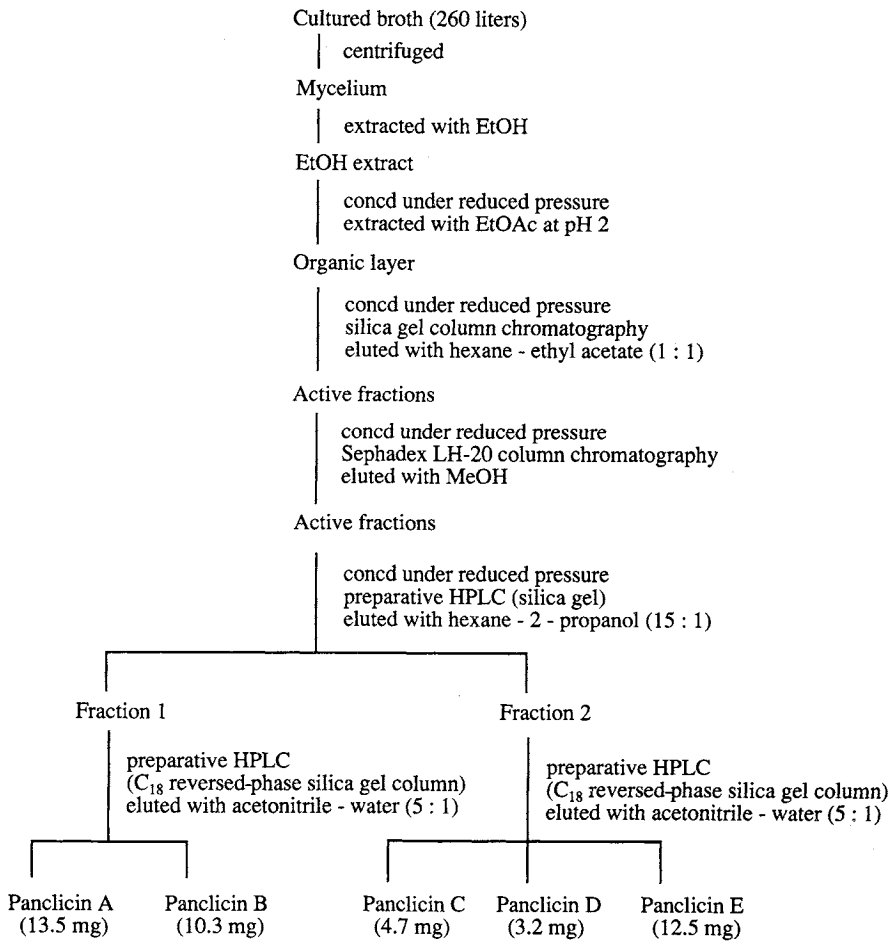
+, Positive; ±, doubtful; −, negative.

sp. NR 0619 (1.5 ml) was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a medium consisting of 2.0% glucose, 2.0% potato starch, 0.5% yeast extract, 2.0% Toast soya, 0.25% NaCl, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnCl_2 \cdot 4H_2O$, 0.32% $CaCO_3$ and 0.05% Nissan disfoam CA-115 (adjusted to pH 7.0 before sterilization). The inoculated flask was incubated on a rotary shaker at 27°C for 4 days at 220 rpm, and 12 ml of the vegetative inoculum was transferred into 3-liter baffled Erlenmeyer flasks containing 600 ml of the same medium as described above, followed by incubation on a rotary shaker at 27°C for 3 days at 100 rpm. Then, 3 liters of the resultant vegetative inoculum were transferred into a 200-liter fermentor containing 150 liters of the fermentation medium consisting of 1.5% dextrin (Difco), 1.0% lactose, 0.65% dried distiller's soluble, 0.35% peptonized milk, 0.25% baker's yeast and 0.17% Tohshiba silicon TSA737 (adjusted to pH 7.0 before sterilization). Jar fermentation was carried out at 27°C for 3 days with an agitation rate of 250 rpm, air flow rate of 150 liters/minute and internal pressure of 0.5 kg/cm^2.

Isolation of Panclicins

The isolation was carried out by monitoring the inhibition of porcine pancreatic lipase. The isolation procedure of panclicins is outlined in Fig. 2. The mycelium, collected by centrifugation from the cultured

Fig. 2. Isolation procedure of panclicins A, B, C, D, and E.



broth (260 liters), was extracted with ethanol (60 liters). The extract was evaporated under reduced pressure and the residue was suspended in water (30 liters). The suspension was adjusted to pH 2 with 6N HCl and extracted with ethyl acetate (30 liters). The organic layer was concentrated under reduced pressure, and the residue was applied to a column (10×20 cm) of silica gel (silica gel 60, Merck) pre-packed with hexane. The column was washed with hexane (4.5 liters) and hexane-ethyl acetate (2:1, 4.5 liters), and the active principle was eluted with hexane-ethyl acetate (1:1, 4.5 liters). The active eluate was concentrated under reduced pressure and chromatographed on a Sephadex LH-20 column (6×35 cm) developed with MeOH. The active fractions were collected and concentrated under reduced pressure to give a syrup (1.2 g). The syrup was separated by preparative HPLC in a silica gel column (YMC pack A003, 20×250 mm) with hexane-2-propanol (15:1) into active fraction 1 (retention time, 12.5~15.5 minutes, 90 mg) and active fraction 2 (retention time, 17~21 minutes, 100 mg). Fraction 1 was separated by preparative HPLC in a C_{18} reversed-phase silica gel column (Capcell pak C_{18}, 15×250 mm; Shiseido) with acetonitrile-water (5:1) at a flow rate of 10.6 ml/minute to give 13.5 mg of panclicin A (retention time, 15 minutes) and 10.3 mg of panclicin B (retention time, 16.2 minutes). Fraction 2 was also separated by preparative HPLC under the same conditions as those of fraction 1 to give 4.7 mg of panclicin C (retention time, 12 minutes), 3.2 mg of panclicin D (retention time, 16 minutes), and 12.5 mg of panclicin E (retention time, 23 minutes).

Biological Activities

Experimental

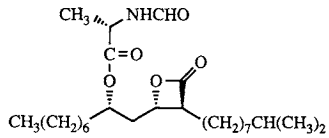
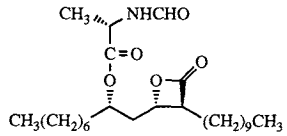
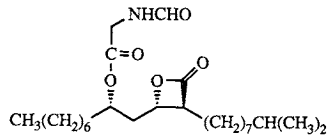
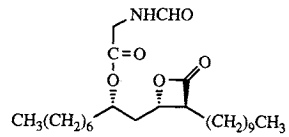
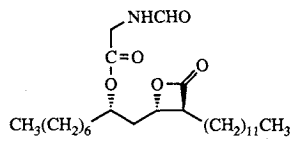
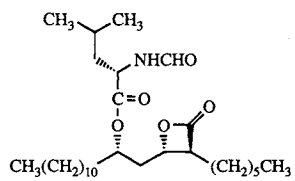
The lipolytic activity of pancreatic lipase was measured by the method of WEIBEL *et al.*[4] (standard assay). The hydrolysis of triolein to fatty acids was followed at pH 8 for 12 minutes at room temperature using a recording pH-stat. The substrate emulsion (0.6 ml per assay) for porcine pancreatic lipase was prepared by the ultrasonication of triolein (30 mg/ml) in a solution containing 1 mM taurodeoxycholate, 9 mM taurocholate, 0.1 mM cholesterol, 1 mM lecithin, 15 mg/ml of bovine serum albumin, 2 mM Tris-HCl (pH 8), 100 mM NaCl, and 1 mM $CaCl_2$. This emulsion composition was chosen so that the test could be done, as close as possible, to *in vivo* conditions. After the addition of the test compound or vehicle alone into 8 mM Tris-HCl, the pH was adjusted to 8.0, and porcine pancreatic lipase was added. Then, the reaction was started by the addition of the substrate emulsion. The amount of lipolytic activity was adjusted to result in the generation of 0.2 to 0.3 μmol fatty acid/ml per minute. The activity of other lipases was measured by the same method as that done for pancreatic lipase.

The mode of action of panclicins A, B, C, D, and E was studied by an excess dilution method. The compound (2 μl) dissolved in DMSO was preincubated with 10 μl of the substrate emulsion and a 20-fold excess of lipase for 45 minutes at room temperature. Then, it was diluted with 220 μl of the lipase solution containing 0.85% NaCl, 1% bovine serum albumin, and 2 mM Tris-HCl (pH 8). The residual lipase activity was measured as described above. By the excess dilution method, the compound was diluted 3,630-fold and normally did not exhibit inhibitory activity against the pancreatic lipase at the concentrations used.

Biological Activity of Panclicins A, B, C, D, and E

Panclicins A, B, C, D, and E dose-dependently inhibited the hydrolysis of triolein to fatty acids by porcine pancreatic lipase with IC_{50}s of 2.9, 2.6, 0.62, 0.66, and 0.89 μM, respectively (Table 4). The panclicins were categorized based on their structures. One group consisted of alanine-type compounds, panclicins A

Table 4. Inhibition of pancreatic lipase *in vitro* by panclicins and THL.

Name	Structure	IC_{50} (μM)[a]	Ratio[b]
Pancilicin A		2.9	2.4
Pancilicin B		2.6	2.2
Pancilicin C		0.62	0.52
Pancilicin D		0.66	0.55
Pancilicin E		0.89	0.74
THL		1.2	1

[a] IC_{50} values present a mean of 4 experiments.
[b] Ratio = IC_{50} (test compound)/IC_{50} (THL).

and B (alanine moiety in place of leucine in THL) and the other, glycine-type compounds, panclicins C, D, and E (glycine moiety in place of leucine in THL). The inhibitory activity of the alanine-type compounds (panclicins A and B) was two to threefold weaker than that of THL. On the other hand, the inhibitory activity of the glycine-type compounds (panclicins C, D, and E) was twofold stronger than that of THL. Panclicins A and C have an *iso*-alkyl side chain, and panclicin E has a longer alkyl side chain, however, the type of the alkyl side chain had by the compounds did not affect their inhibitory activities.

The inhibitory activities of panclicins A, B, C, D, and E for other lipases are shown in Table 5, and are compared with THL. Post-heparin plasma lipases contain lipoprotein lipase and hepatic lipase[13]. Panclicins A, B, C, D, and E also potently inhibited plasma lipases with IC_{50}s of 1.0, 1.2, 0.29, 0.25, and 0.15 μM, respectively. The inhibitory profiles of these compounds were almost the same as those for the porcine pancreatic lipase. Panclicins A and B inhibited plasma lipases with the same potency as THL. Panclicins C, D, and E had a three to sixfold greater inhibitory activity than THL.

Table 5. Inhibitory activity of panclicins for various lipases.

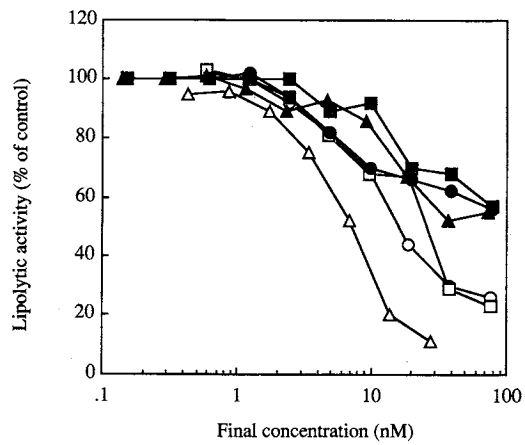
Compound	IC_{50} (μM) for:			
	Post-heparin rat plasma[a]	*Rhizopus arrthizus*[b]	*Pseudomonas* sp.[b]	*Chromobacterium viscosum*[b]
Panclicin A	1.0	0.21	0.81	1.12
Panclicin B	1.2	0.33	0.44	0.72
Panclicin C	0.29	0.062	0.26	0.14
Panclicin D	0.25	0.11	0.24	0.15
Panclicin E	0.15	0.14	0.19	0.12
THL	0.87	0.62	0.11	0.13

[a] Post-heparin plasma contains lipoprotein lipase and hepatic lipase.
[b] Purchased from Sigma Chemical Company.

Other lipases listed in Table 5 are bacterial and fungal lipases. Panclicins A, B, C, D, and E inhibited bacterial and fungal lipases with profiles similar to those for porcine pancreatic lipase.

The mode of action of panclicins A, B, C, D, and E was studied by the excess dilution method. The results are shown in Fig. 3. Panclicins A, B, C, D, E, and THL, up to the maximum concentration of the compounds tested, did not inhibit porcine pancreatic lipase if preincubation was omitted. When pancreatic lipase was preincubated with the compounds for 45 minutes at room temperature, panclicins A, B, C, D, E, and THL inhibited the porcine pancreatic lipase even at the maximum dilution. From these results, it was shown that panclicins A, B, C, D, and E inhibit the pancreatic lipase irreversibly. Boronic acid derivatives, which are known to be the reversible inhibitors of lipases[14], did not inhibit pancreatic lipase even when the preincubation step was included (data not shown). THL was shown to possess the most potent irreversibility. The activity ranking order of panclicins A, B, C, D, E, and THL was as follows; THL $\geq$ panclicins A and B > panclicins C, D, and E, which was not consistent with that shown with the standard assay (without preincubation).

Fig. 3. Relative irreversibility. Experiments were performed as described in the "Experimental" except for the addition of the indicated amounts of panclicin A (○), B (□), C (●), D (■), E (▲), and THL (△).



Discussion

In our search for pancreatic lipase inhibitors with the use of microbial origin, we discovered panclicins A, B, C, D, and E with novel structures from *Streptomyces* sp. NR 0619. They are the analogues of THL. The panclicins were categorized into two groups, based on their amino acid moiety described above. Panclicins B and D also have an *iso*-alkyl side chain, however, the inhibitory activity of panclicin B was almost the same as that of panclicin A, and the inhibitory activity of panclicin D was the same as that of panclicin C. Furthermore, panclicin E has a longer alkyl side chain than panclicin C does, but its inhibitory activity was the same. And the same profile of inhibitory activity against other lipases was shown

by panclicins. Therefore, it is considered that the inhibitory activity of panclicins A, B, C, D, and E is attributed to the amino acid moiety of the compounds, not to the *iso*-alkyl side chains nor the length of the side chains. From the study of the mode of action of panclicins A, B, C, D, and E, they were characterized to be irreversible inhibitors of pancreatic lipase, similar to THL. However, the activity of the irreversible inhibition of panclicins C, D, and E were not as potent as that of THL, although they were two to three times more potent than THL in the standard assay. It is possible that the irreversible inhibitions of the compounds are correlated with the hydrophobicity of the amino acid moiety of the compounds since pancreatic lipase acts at the surface of lipid droplets[1]; however, more evidence is required to draw a firm conclusion on the extent of the inhibitory activities of panclicins A, B, C, D, E, and THL.

References

1) SÉMÉRIVA, M. & P. DESNUELLE: Pancreatic lipase and colipase. An example of heterogeneous biocatalysis. Advances in Enzymology & Relative Areas of Molecular Biology 48: 319~370, 1979
2) DECARO, J.; M. BOUDOUARD, J. BONICEL, A. GUIDONI, P. DESNUELLE & M. ROVERY: Porcine pancreatic lipase. Completion of the primary structure. Biochimica et Biophysica Acta 671: 129~138, 1981
3) BORGSTRÖM, B.: Digestion and absorption of lipid. Int. Rev. Physiol. 12: Gastrointest. Physiol. II: 305~323, 1977
4) WEIBEL, E. K.; P. HADVÁRY, E. HOCHULI, E. KUPFER & H. LENGSFELD: Lipstatin, an inhibitor of pancreatic lipase, produced by *Streptomyces toxytoricini*. 1. producing organism, fermentation, isolation and biological activity. J. Antibiotics 40: 1082~1085, 1987
5) HOCHULI, E.; E. KUPFER, R. MAURER, W. MEISTER, Y. MERCADAL & K. SCHMIST: Lipstatin, an inhibitor of pancreatic lipase, produced by *Streptomyces toxytoricini*. 2. Chemistry and structure elucidation. J. Antibiotics 40: 1086~1091, 1987
6) HOGAN, S.; A. FLEURY, P. HADVÁRY, H. LENGSFELD, M. K. MEIER, J. TRISCARI & A. C. SULLIVAN: Studies on the antiobesity activity of tetrahydrolipstatin, a potent and selective inhibitor of pancreatic lipase. Int. J. of Obesity 11, Suppl. 3: 35~42, 1987
7) BORGSTRÖM, B.: Mode of action of tetrahydrolipstatin, a derivative of the naturally occurring lipase inhibitor lipstatin. Biochimica et Biophysica Acta 962: 308~316, 1988
8) HADVÁRY, P.; H. LENGSFELD & H. WOLFER: Inhibition of pancreatic lipase *in vitro* by the covalent inhibitor tetrahydrolipstatin. Biochem. J. 256: 357~361, 1988
9) FERNANDEZ, E. & B. BORGSTRÖM: Effect of tetrahydrolipstatin, a lipase inhibitor on absorption of fat from the intestine of the rat. Biochimica et Biophysica Acta 1001: 249~255, 1989
10) HADVÁRY, P.; W. SIDLER, W. MEISTER, W. VETTER & H. WOLFER: The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase. J. Biol. Chem. 266: 2021~2027, 1991
11) YOSHINARI, K.; M. AOKI, T. OHTSUKA, N. NAKAYAMA, Y. ITEZONO, M. MUTOH, J. WATANABE & K. YOKOSE: Panclicins, novel pancreatic lipase inhibitors. II. Structural elucidation. J. Antibiotics 47: 1376~1384, 1994
12) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
13) NILSSON-EHLE, P. & MICHAEL C. SCHOTZ: A stable, radioactive substrate emulsion for assay of lipoprotein lipase. J. Lipid Res. 17: 536~541, 1976
14) CHARLES, W. GARNER: Boronic acid inhibitors of porcine pancreatic lipase. J. Biol. Chem. 255: 5064~5068, 1980