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Selection and Identification of a Strain KT-10 Producing the Cathepsin B Inhibitor

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An actinomycetes, KT-10 isolated from ginseng field in Kyongpook, Korea was selected based on its ability to produce a lysosomal cathepsin B inhibitor. The inhibitor purified from the culture supernatant of the isolate KT-10 showed strong inhibitory effects against cathepsin B as well as against papain when the activities were measured using synthetic substrate, α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (CLN) or α-N-benzoyl-D,L-arginine 2-naphthylamide (BANA). The isolate KT-10 was identified as a species of *Streptomyces* based on its morphological characteristics and chemotaxonomic data. The TAXON program of Ward was used to identify *Streptomyces* sp. KT-10 as a strain of *Streptomyces luteogriseus* belong to cluster 18 of the genus *Streptomyces* with a Willcox probability 0.999388. The cathepsin B inhibitor was presumed to a novel material composed of a polyhydroxylamine.

The lysosomal cathepsin B-like proteinase play a important role in tumor cell invasion and metastasis (12). It seems reasonable to link the invasiveness of cells in their ability to secrete or induce secretions of hydrolases. These secretions of hydrolases are necessary for the degradation of barriers that these cells must cross (15). The best studied proteolytic enzymes which may be implicated in tumor cell invasion are the plasminogen activators, the collagenases, and the cathepsin B like proteinases. These enzymes have been isolated from tissues of many mammalian species, including the liver, the spleen, the preputial gland, the lung, the parathyroid gland, the brain, and the breasts. The degradation observed at the invasion front of a tumor is a clear argument in favour of the contribution of cathepsin B to tumor invasion (16).

Cathepsin B inhibitors have been reported to cause changes in malignant tumors (13). These changes were observed not only in the synthesis of the enzyme but also in the processing and subcellular localization of the enzyme and in the enzyme's regulation (7). Proteinase inhibitors of microbial origin, including leupeptin, estatin, antipain, and E-64, which have been used extensively, inhibit both thiol and serine proteinases (1). However, their inhibitory activities are not effective specifically against cathepsin B.

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Soil microorganisms were used to screen for a novel microbial cathepsin B inhibitor as a tumor curing agent. We isolated an actinomycetes strain, KT-10 from ginseng field and identified by a numerical identification procedure using TAXON program.

MATERIALS AND METHODS

Materials

Cathepsin B, α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (CLN), and α-*N*-benzoyl-D,L-arginine 2-naphthylamide (BANA) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Papain was supplied by Junsei Chemical Co. (Tokyo, Japan) and cellulose-coated TLC plate was supplied by the Merck Co. (Darmstadt, Germany). Fluorescence measurement was carried out by Hitachi U-2000 Spectrophotometer (Tokyo, Japan).

Microoganism and Culture Conditions

The microorganism used was isolated from ginseng field in Kyongpook, Korea using an actinomycetes isolation medium (yeast extract, 1 g; malt extract, 1 g; glucose, 1 g; agar, 15 g; per 1 liter, pH 7.3) supplemented with 50 μg/ml of nystatin to prevent fungal growth. The isolate was then transferred to a starch-casein-nitrate agar medium (starch, 10 g; KNO_3 2 g; K_2HPO_4, 2 g; NaCl, 2 g; casein, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 18 g; per 1 liter) and allowed to form spores. The spores were kept in a deep freezer at -70°C in a glycerol-nutrient broth (17). Cultures were made at 28°C for 4 days.

For the production of the proteinase inhibitor, the isolate KT-10 was cultured at 28°C for 4 days in a medium containing glucose 20 g, peptone 3 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $NaNO_3$ 0.5 g NaCl 0.5 g, per 1 liter, pH 7.0, and filtered through Whatman No. 2 to obtain culture filtrate. The culture filtrate was heated for 10 min at 80°C, and used for the cathepsin B inhibition assay.

Assay of Cathepsin B Activity

The assay was carried out using a modification of the Bajkowski *et al.* method (2). The cathepsin B (5 μg/ml) stock solution contained 30 mM dithiothreitol and 15 mM EDTA. The incubation buffer was made up of a 25 mM sodium acetate buffer, pH 5.2 and 1 mM EDTA. The substrate solution was prepared by dissolving 5.22 mM of α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (CLN) in dimethylsulfoxide and α-*N*-benzoyl-D,L-arginine 2-naphthylamide (BANA), 171 mM in *N*,*N*-dimethylformamide. The reaction mixture included 0.8 ml of incubation buffer, 50 μl of cathepsin B stock solution, 50 μl of substrate solution and 100 μl of an inhibitor solution. After prereaction for 15 min at 37°C, the changes in absorbance was monitored spectrophotofluoremetrically at 326 and 340 nm. Inhibition activity (%) was calculated by dividing the rate of absorbance change of the reaction containing the sample by that of the control;

$$\text{Inhibition activity (\%)} = (B\text{-}C)/(A\text{-}C) \times 100$$

where A is the rate of absorbance change of the control, B that of sample reaction, and C that of blank reaction without cathepsin B.

Chemotaxonomical, Morphological Characteristics

The cultural and morphological characteristics were determined by using media recommended by the International Streptomyces Project (ISP) (14). Cells was grown in a starch-casein-nitrate medium at 28°C for 3 days were harvested and washed three times with a physiological saline solution. The washed cells were disrupted by a French Press and the cell walls were separated from the lysates by centrifugation (11,000×*g* for 15 min). The suspended lysates were freeze dried for diaminopimelic acid and sugar analyses (18). To examine spore chain morphology, *Streptomyces* strain KT-10 was incubated at 28°C for 14 days onto an inorganic salts starch agar medium (ISP medium 4). Spore chain morphology of the strain KT-10 was examined using scanning electron microscopy (SEM) (Hitachi, Japan).

Identification of *Streptomyces* by the TAXON Program

Identification of the strain KT-10 was conducted using the TAXON program to determine the species of the strain KT-10 (5, 8, 12). TAXON is a computer program that identifies unknown strains by testing 41 unit characters, and analyzing the results numerically based on data for major *Streptomyces* clusters collected by Williams *et al.* (19).

RESULTS AND DISCUSSION

Selection of the Cathepsin B Inhibitor-producing Strain

The strain KT-10 isolated from a ginseng field was selected among a large number of *Streptomyces* isolates. The culture filtrate of the isolate inhibited over 90% of the proteinase activities of cathepsin B and papain at the conditions described in the Materials and Methods section.

Properties of the Cathepsin B Inhibitor

The culture supernatant was used to test the inhibitory effects on the proteinase activities of cathepsin B and papain using CLN and BANA. As shown in Fig. 1 and Fig. 2, the culture supernatant inhibited proteinase activities of cathepsin B on CLN as well as BANA. The inhibitory effect on cathepsin B was increased with reaction time as on papain, a thiol endopeptidase of plant origin (Fig. 3). CLN appeared to be better substrate for cathepsin B than BANA.

Chemotaxonomic and Morphological Characteristics

The cultural characteristics were determined by using the ISP media as shown in Table 1. The selected strain KT-10 formed a typical *Streptomyces* mycelium. Substrate mycelium on the glycerol-asparagine agar medium (ISP 5) was yellowish-brown, and diffusible pigments were detected (Table 3). Melanin was produced on the

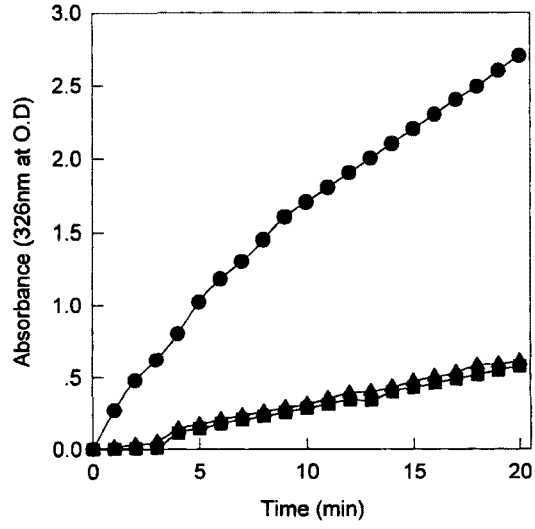

Fig. 1. Inhibition of proteinase activity of cathepsin B on CLN by culture filtrate of isolate KT-10.

●, control reaction with cathepsin B; ▲, reaction with the culture filtrate; ■, blank reaction without cathepsin B. The enzyme activity was measured at 37°C. Fluorescence measurement was made at 326 nm. CLN: α-*N*-benzyloxycarboyl-L-lysine *p*-nitrophenyl ester.

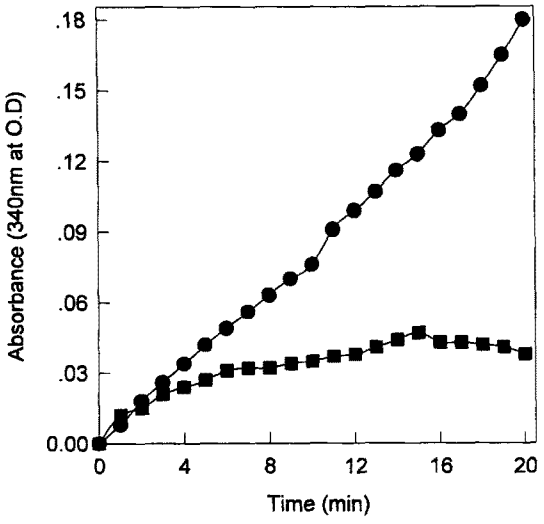

Fig. 2. Inhibition of proteinase activity of cathepsin B on BANA by culture filtrate of isolate KT-10.

●, control reaction; ■, reaction with the culture filtrate. The enzyme activity was measured at 37°C. Fluorescence was made at 340 nm. BANA: α-*N*-benzoyl-D,L-arginine 2-naphthylamide.

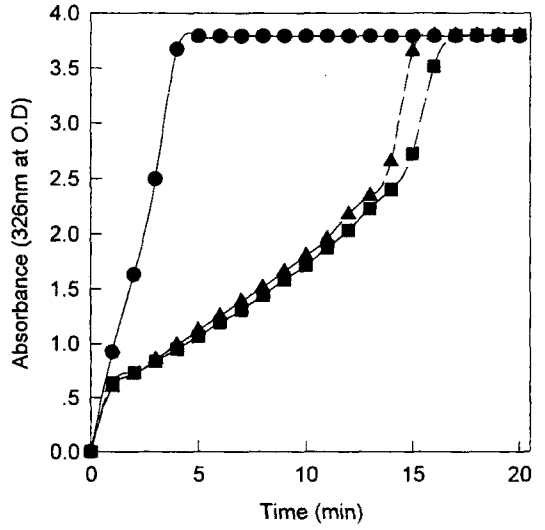

Fig. 3. Inhibition of papain on CLN by the culture filtrate of the isolate KT-10.

●, control reaction; ▲, reaction with the culture filtrate; ■, blank reaction without the enzyme. The enzyme activity was measured at 37°C Fluorescence was measured at 326 nm.

Table 1. Cultural characteristics of *Streptomyces* sp. KT-10.

Medium	Growth	Aerial mycelium	Soluble pigment
Trypton yeast extract broth (ISP 1)	M	brown	brown
Yeast malt extract agar (ISP 2)	G	dark brown	brown
Oatmeal agar (ISP 3)	G	white	pinkish pale brown
Inorganic salts starch agar (ISP 4)	M	grey	none
Glycerol asparagine agar (ISP 5)	M	dark brown	brown
Peptone-yeast extract iron agar (ISP 6)	P	black	black
Tyrosine agar (ISP 7)	P	black	black
Starch agar	M	pinkish grey	brown
Nutrient agar	M	brownish grey	brown
Potato dextrose agar	M	greyish brown	brown
Glucose peptone agar	M	brown	brown
Czapeck agar	G	dark brown	pinkish brown

G, good; M, moderate; P, poor. Media were employed by International Streptomyces Project (ISP). The strain was cultured in various kinds of media at 28°C for 14 days.

peptone yeast iron agar medium (ISP 6) and tyrosine agar medium (ISP 7) (Table 2). The color of the aerial spore mass developed on the surface of the inorganic salt starch agar (ISP 4) was grey (Table 2). The aerial mycelium turned to a rectiflexibile chain of spores and the spore surface ornamentation was smooth (Fig. 4). Vegetative hyphae, grown in a submerged culture using a Bennet medium, were extensively branched and fragmented. Cell wall hydrolysate contained *LL*-diaminopimelic acid. These results indicate that the isolate KT-10 has a wall chemotype I and peptidoglycan type A3. TLC analysis of whole-cell hydrolysates did not show any diagnostic sugar. We suggest that the whole-cell sugar pattern was type C. From the morphological and chemotaxonomical characterization, it was concluded that the isolate KT-10 belonged to the genus *Streptomyces*.

Table 2. Morphological characteristics of *Streptomyces* sp. KT-10.

Item	Characteristics
Colony surface	powdery
Spore chain	rectiflexibles
Spore surface ornamentation	smooth
Colony size	discrete
Other soluble pigment	none
Reverse side color	brown
Aerial mass color	brown
Melanin pigment	positive

Table 3. Taxonomic unit characters used in the identification of the *Streptomyces* sp. KT-10.

1. Morphology and pigmentation
   Spore chain morphology: rectiflexible (RFS) +, retinaculiaperti −, spirales − , verticillati (BIV) −
   Spore chain ornamentation: smooth (SMO) +, rugose (RUG) −
   Color of aerial spore mass: red (RED) −, grey (GRY) +, green (GRN) −
   Diffusible pigment: reverse red/orange (ROP) −, reverse yellow/brown (YBP) +
   Melanin production on PI agar (MPI) +
   Fragmentation of mycelium (FRG) −
2. Antimicrobial activity
   Bacillus subtilis (SUB) −, *Streptomyces murinus* (MUR) +, *Aspergillus niger* (NIG) +
3. Biochemical tests
   lectinase (LEC) −, pectin hydrolysis (PEC) −, Nitrate Reduction (NO3) −, H_2S Production (H2S) +
4. Degradation tests
   Xanthine (XAN) +, Allantoin (ALL) −, Arbutin (ARB) −
5. Antibiotic resistance
   Neomycin (50 μg/ml) (NEO) −, Rifampicin (50 μg/ml) (RIF) −
6. Growth test
   45°C (45C) −, NaCl (7%, w/v) (7NA) −, Sodium azide (0.01%, w/v) (01Z) −, Phenol (0.1%, w/v) (PHN) −
7. Compound as sole source of nitrogen (0.1%, w/v)
   DL-α-Aminobutyric acid (BUT) −, L-Histidine (HIS) −, L-Hydroxyproline (HYD) +
8. Organic compounds as sole source ot carbon
   D-Xylose (XYL) −, *meso*-Inositol (IND) +, Mannitol (MAN) +, L-Rhamnose (RMA) +, Raffinose (RAF) +, D-Fructose (FRU) −, Inulin (INN) +, Adonitol (ADO) +, Cellobiose (CEL) +

Characters in parenthesis is the code name for computer program. Symbols + and - represent positive and negative results, respectively.

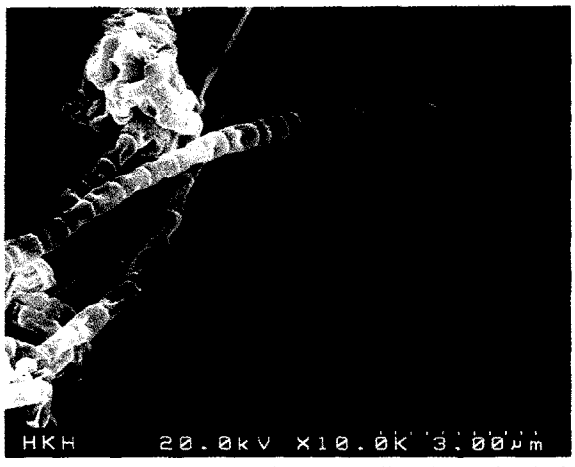

Fig. 4. Scanning electron micrograph (SEM) of the isolate *Streptomyces* sp. KT-10 cultured on inorganic salts starch agar (ISP4) medium for 14 days.

Identification of *Streptomyces* sp. Strain KT-10 Using the TAXON Program

Identification of *Streptomyces* sp. strain KT-10 was conducted based on 41 taxonomical characters using the TAXON program, and the *Streptomyces* probability matrices, based on 26 major clusters and 40 minor clusters, respectively (Table 4). The criteria adopted for a correct identification were: a Willcox probability of greater than 0.85, taxonomic distance within a 95% taxon radius and low score, and a % probability of strain further away greater than the score of other clusters.

A Willcox probability of *Streptomyces* sp. KT-10 to major cluster 18 (*S. cyaneus*) was 0.998490. It was much higher than the 0.000109, 0.000001 of Willcox probability to the taxon major cluster 33 (*S. chromogenus*) and 29 (*S. lydicus*), respectively (Table 5). A TAXON distance value (0.4788) for cluster 18 of the strain KT-10 was smaller than the major clusters 33 (0.5066), and 29 (0.4979) but larger than the 95% taxon radius score

Table 4. Identification of the *Streptomyces* sp. KT-10 to the major cluster of *Streptomyces* by TAXON program.

TAXON major cluster (centrotype mumber)	Taxonomic distance[a]	95% TAXON radius[b]	% Prob of strain further away[c]	Willcox probability[d]
18 (*S. cyaneus*)	0.4788	0.4276	0.1765	0.998490
29 (*S. lydicus*)	0.4979	0.3615	0.0000	0.000001
33 (*S. chromogenus*)	0.5066	0.3932	0.0001	0.000109

[a]This expresses the distance of an unknown strain from the centroid of the group with which it is being compared. [b]This represents the radius of taxonomic groups including 95% groups of Taxon *J*. [c]This indicates what percent of all the cluster groups in represented by strains in cluster groups outside the identified strain. [d]This is the likelihood of unknown strain (u) against Taxon J divided by the sum of the likelihood of u against all q taxa.

Table 5. Comparison of taxonomic scores among hypothetical median organism (HMO), centrotype, outer-most member strain (OMS), best matched organism (BMO) and the isolate.

Member of cluster 18	TAXON distance	95% TAXON radius	Probability of further away (%)	Willcox probability
HMO[a]	0.2445	0.4276	99.8221	0.999842
Centrotype[b] 592PE (*S. peruviensis*)	0.2718	0.4276	98.7261	0.999875
OMS[c] 042HA (*S. hawaiiensis*)	0.4454	0.4276	1.8417	0.962592
BMO[d] 483LT (*S. luteogriseus*)	0.4125	0.4276	10.2368	0.999388
Isolate KT-10	0.4788	0.4276	0.1765	0.998490

[a]This calculates the best identification scores achievable by entirely example of each group in a matrix. [b]This designated to determine overlap between groups in a matrix constructed using percentage positive values for character states. [c]This calculates the lowest identification scores achievable by entirely typical example of each group in a matrix. [d]This designated to the best matched identification scores at example of group in a matrix by the isolate strain.

(0.4276). Percent probability of strain further away from the strain KT-10 (0.1765) to the taxon major cluster 18 was distinctly greater than that of the other major clusters 29 (0.0000) and 33 (0.0001). Thus, the isolate KT-10 was placed in regions further away from the center of cluster 18. From these results, it was concluded that the isolate KT-10 is nearest the group cluster 18, and was identified as a member of cluster 18.

Major cluster 18, which is represented by *S. luteogriseus*, *S. peruviensis*, *S. hawaiiensis* etc, contains 36 *Streptomyces* strains. The Willcox probability of the hypothetical median organism (HMO) in cluster 18 was 0.999842. The centrotype 592PE (*S. peruviensis*) was 0.999875. The out-most member 042HA (*S. hawaiiensis*) was 0.962592. The best match strain (*S. luteogriseus*) was 0.999388 (Table 6). The taxonomic distance (0.4788) of isolate KT-10 was larger than the 95% taxon radius (0.4276). A 0.1765% distance of probability of further away took place which was lower than that of either centrotype (98.7261). By comparing with the Willcox probability of major clusters, it is possible to know in which major cluster the unidentified strain is included. The KT-

Table 6. Comparison of taxonomic unit characters among organisms in cluster 18 of *Streptomyces* and the S_{SM} value calculated by TAXON program.

Taxonomic unit characters (TAXON code)	Positive characters state (%)	KT – 10	HMO in cluster 18	Best matched strain *S. luteogriseus*	Centrotype *S. peruviensis*
RFS	5	+	–	–	–
RAS	31	–	–	–	–
SPI	28	–	+	+	+
BIV	1	–	–	–	–
SMO	93	+	–	+	–
RUG	1	–	–	–	–
RED	23	–	–	–	–
GRY	92	+	–	+	–
GRN	1	–	–	–	–
ROP	61	–	–	–	–
YBP	31	+	–	–	–
MPI	79	+	+	+	+
FRG	1	–	–	–	–
BUT	23	–	–	–	–
HIS	48	–	+	+	+
HYD	29	+	–	–	–
LEC	1	–	–	–	–
PEC	55	–	+	+	–
NO3	73	–	–	+	–
H2S	98	+	+	–	+
SUB	54	–	–	–	–
MUR	36	+	+	+	+
NIG	11	+	–	+	–
XAN	28	+	+	+	+
ALL	55	–	+	–	–
ARB	55	–	+	–	–
NEO	1	–	–	–	–

Table 6. Continued.

Taxonomic unit characters (TAXON code)	Positive characters state (%)	KT – 10	HMO in cluster 18	Best matched strain *S. luteogriseus*	Centrotype *S. peruviensis*
RIF	54	–	–	–	–
45C	24	–	–	–	–
7NA	81	–	–	–	–
01Z	61	–	–	–	–
PHN	36	–	+	–	–
XYL	98	–	+	–	+
INO	59	+	+	+	+
MAN	79	+	+	+	+
FRU	98	–	+	–	+
RHA	29	+	+	+	+
RAF	99	+	+	+	+
INU	28	+	+	+	+
ADO	48	+	+	+	+
CEL	59	+	+	+	+
Matching to KT – 10		41	27	33	31
Mismatching to KT – 10		0	14	8	10
S_{SM} value		100	65.85	80.48	75.61
Willcox Probability		0.998490	0.999842	0.999388	0.999875

HMO, Hypothetical Median Organism; S_{SM}, Simple match coefficient.

10 strain was best-matched to *S. luteogriseus*. Willcox probability of the isolate KT-10 to its best match strain *S. luteogriseus* was 0.999388 which was smaller than those to HMO and centrotype organism, but higher than those to outer-most member organism. Comparison of the taxonomic distance and % probability of strain further away, also revealed that the isolate KT-10 was taxonomically placed further away from the centrotype organism. From the simple matching coefficient (S_{SM}) analysis, the taxonomic unit characters of the isolate were compared with cluster 18. Simple matching cofficients (S_{SM}) of *Streptomyces* sp. KT-10 were calculated, based on the data of 41 unit characters (Table 7). The best matched *S. luteogriseus* strain appeared 80.4% S_{SM} similarity level among those of the 36 strains analysed. From these results, *Streptomyces* sp. KT-10 identified as a strain of *S. luteogriseus* belonging to the major cluster 18.

In conclusion, unit characters for numerical identification of the isolate KT-10 were analyzed. The isolate KT-10 and its best match strain *Streptomyces luteogriseus* were identical in 33 of 41 characters, for example, pigment production, antimicrobial activity, antibiotic resistance, growth test, utilization of carbon sources, etc. In contrast, they showed different reactions of pectin hydrolysis, nitrate reduction, H_2S production, or utilization of nitrogen sources.

Cathepsin B Inhibitor Produced by *S. luteogriseus* KT-10

The cathepsin B inhibitor produced by the *Streptomyces luteogriseus* KT-10 strain was purified and partially charaterized. ^{13}C NMR spectral data showed that the inhibitor does neither contain amino acids nor sugars, but it is a new polyhydroxylamine (Fig. 5).

The physico-chemical properties of the cathepsin B in-

Table 7. Physico-chemical properties of cathepsin B inhibitor produced from *Streptomyces luteogriseus* KT-10.

Appearance	White powder
Elemental analysis (%)	C; 31.427, H; 4.277, N; 5.767
UV	No detect
IRν_{max} cm^{-1}	788.7, 1041.3, 1288.4, 1398.9, 1463.3, 1638.2, 2940.5, 3399.9
Amino acid analysis	No detect
Solubility	
soluble	MeOH, H_2O, *n*-butanol, acetone
insoluble	chloroform, hexan, ethyl ether, toluene
Color reaction	
positive	ninhydrin, H_2SO_4, phosphomolybdic acid
negative	Ehrlich's regent, Pauly reagent, Sakaguchi, phthalic acid, DNS, aniline, molisch, fehling
Rf value[a]	0.9

[a]Silica gel TLC (Kieselgel 60 F_{254}, merck), solvent=MeOH : H_2O(7 : 3).

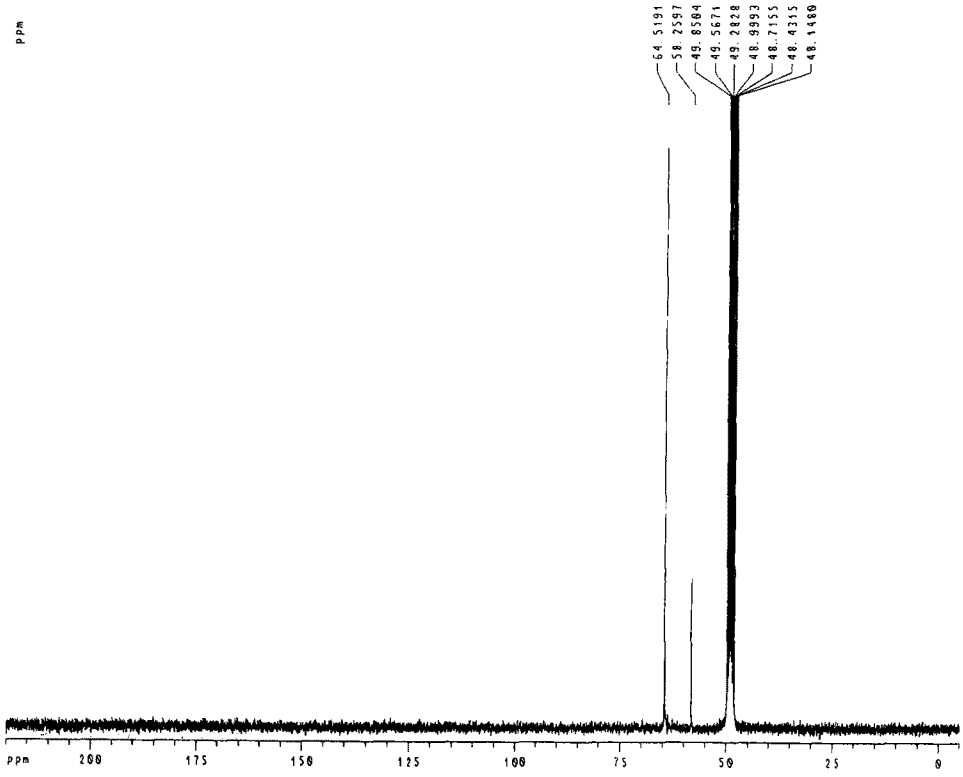

Fig. 5. ^{13}C-NMR spectrum of cathepsin B inhibitor in MeOH (75 MHz).

hibitor substance are summerized in Table 7. The elemental analysis showed that the inhibitor compound contains carbon (31.427%), hydrogen (4.277%), nitrogen (5.767%) and oxygen (58.529%). The inhibitor was soluble in methanol, water, *n*-butanol, and acetone but insoluble in chloroform, hexane, ethyl ether, and toluene. The inhibitor showed positive reactions to the ninhydrin, 5% H_2SO_4 and phosphomolybdic acid. The UV spectra showed no characteristic absorption bands. The IR spectra showed wide absorption bands at 3000~3600 cm^{-1} of OH group. The inhibitor was not composed of amino acids by amino acid analysis.

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