Thesis for the Degree of Master of Science

Purification and Characterization of Cathepsin B Inhibitors from a marine *Pseudomonas* sp. strain PB01

by

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Department of Chemistry

The Graduate School

Pukyong National University

February 2008

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마린 *Pseudomonas* sp. PB01 로부터 Cathepsin B 저해제의 정제 및 특성

Advisor : Prof. Se-Kwon Kim

by

Hoang Le Thu Van

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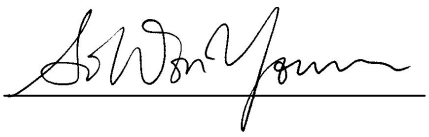

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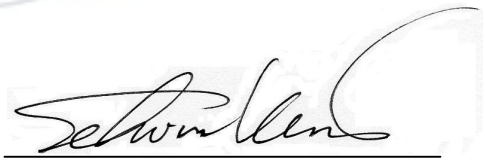

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Abstract

A novel cathepsin B inhibitor-producing microorganism was identified based on its 16S rRNA sequence as *Pseudomonas* sp. strain PB01 (accession no. EU126129). Before isolating inhibitors, enzyme inhibitors production was investigated under various culture conditions, including temperature, initial medium pH, and shaking speed. Additionally kinetic study revealed that the inhibitor production paralleled with cell growth. From the culture broth, two cathepsin B inhibitors were isolated, which structural characterizations were elucidated as phthalates. Both of them inhibited cathepsin B dose dependently with IC_{50} of 0.42 and 0.38 mM, respectively. Kinetic analyses showed that dibutyl phthalate and di-(2-ethylhexyl) phthalate acted in noncompetitive manners, which were agreeable with their characteristics. Furthermore, both of them not only caused inactivation of the pericellular but also intracellular cathepsin B of murine melanoma cell with no acute cytotoxicity.

Keywords: Cathepsin B inhibitor, *Pseudomonas* sp. PB01, phthalate

Table of contents

List of Tables

List of Figures

Lists of Abbreviations

^{13}C NMR	^{13}Carbon Nuclear Magnetic Resonance
1D NMR	One Dimensional Nuclear magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
2D NMR	Two Dimensional Nuclear magnetic Resonance
ACN	Acetonitrile
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarisation Transfer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
Em	Emission
Ex	Excitation
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HPLC	High Performance Liquid Chromatography
IC_{50}	50% Inhibitory Concentration
IR	Infra Red
Ki	Inhibitory Constant
LREIMS	Low Resolution Electron Impact Mass Spectroscopy
MMP	Matrix Metalloprotease
mRNA	Messenger Ribonucleic Acid
OD	Optical Density

PCR	Polymerase Chain Reaction	
Vmax	Maximal	Velocity

Introduction

Lysosomal cysteine proteases, generally known as the cathepsins, were discovered in the first haft of the 20th century. Cathepsin B (EC 3.4.22.1) is one of the most studied among them (Fig. 1). Although there are more than one mRNA species, cathepsin B is encoded by a single gene, with transcripts varying in size depending on the length of the untranslated regions. Briefly, preprocathepsin B is synthesized on the rough endoplasmic reticulum and transported to the Golgi apparatus. And then the mannose-6-phosphate signal is assembled which helps procathepsin B transport to an acidic compartment. Procathepsin B is blocked by a propeptide running through the active site cleft in the orientation opposite to that of a substrate (Cygler et al., 1996; Turk et al., 1996). Finally, the mature cathepsin B is attained by removing the propeptide (Mort and Buttle, 1997). Beside that, it also stabilizes the enzyme at neutral to alkaline pH and protects the body from damage by inappropriate proteolysis. Removal of the propeptide *in vitro* can be facilitated either by activation by other proteases or by autocatalytic activation at acidic pH (Turk et al., 2001).

Mature cathepsin B exists in two forms, a single-chain form and a double-chain form. Both of them are enzymatically active. Mature single-chain cathepsin B consists of 254 amino acid and is ~ 30 kDa in size (Sly and Fisher, 1982). The double-chain form consists of a heavy chain of ~ 25 kDa and a light chain of ~ 5 kDa (Ritonja, 1985; Sly and Fisher, 1982). The double-chain form is derived from the single-chain form as a result of enzyme cleavages between residues 47 and 50 with the loss of dipeptide (Sly and Fisher, 1982). The protein consists of two domains, one of them is composed mostly of β sheet and another contains several α helices and a small β sheet structure (Cygler et al., 1996).

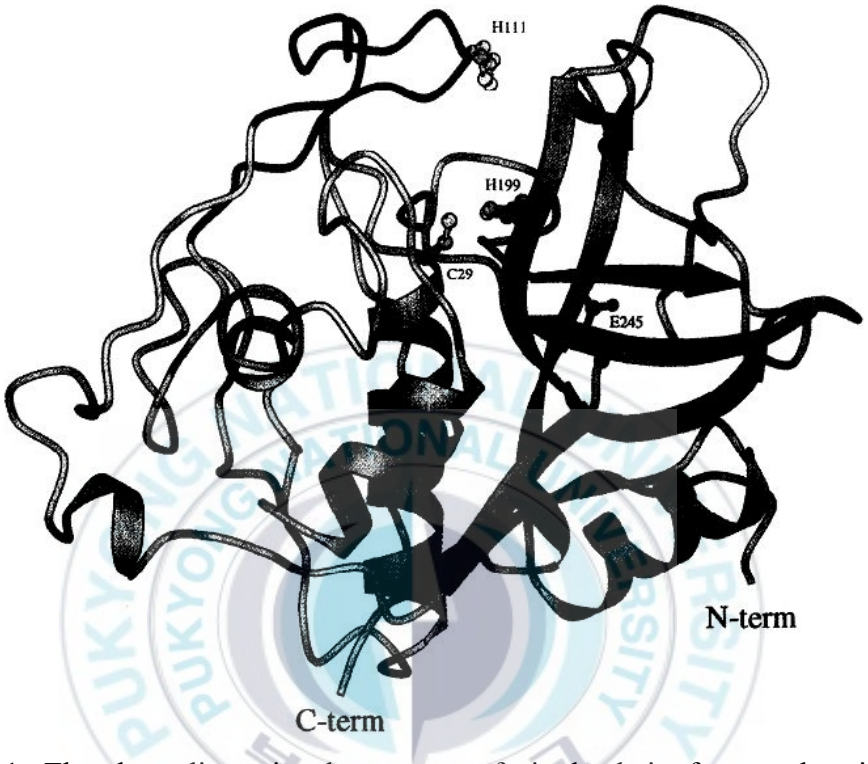


Figure 1. The three-dimensional structure of single-chain form cathepsin B (Mort and Buttle, 1997).

The active-site triad, Cys29-His199-Asn219, and the substrate-binding site are located at the interface between two domains. Cleavage of the substrate peptide bond is mediated by nucleophilic attack by S^- from Cys29 on the carbonyl carbon atom, followed by proton donation from His199 (Lee et al., 2004). Cathepsin B acts as an endopeptidase, cleaving internal peptide bonds, and favours a large hydrophobic side-chain in the substrate, two residues *N*-terminal to the scissile bond (Fig. 2). It preferentially cleaves -Arg-Arg-|-Xaa bonds in small molecule substrates. Interestingly, cathepsin B is a unique cysteine member showing exopeptidase activity, which can remove dipeptides from the *C*-terminus of proteins and peptides, due to the presence of the occluding loop in its structure (Mort and Buttle, 1997). The loop partially blocks the end of the active-site cleft and positions a positively charged imidazole group of a histidine residue (His111) to accept the negative charge at the *C*-terminus of the substrate. Cathepsin B is less effective endopeptidase than some other members of the papain family, probably because of the energy cost of altering the conformation of the occluding loop (Cygler et al., 1996). Similar to other lysosomal cysteine proteases, cathepsin B is optimally active in the slightly acidic, reducing milieu found in lysosome. Conversely, the endopeptidase activity has a pH optimum around 7.4 (Khour et al., 1991).

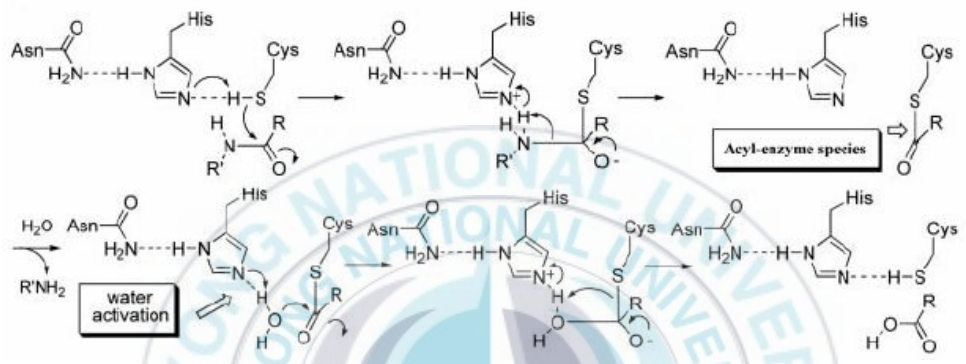


Figure 2. Proposed hydrolytic mechanism of cathepsin B (Lee et al., 2004).

Cathepsin B plays an important role in intracellular protein processing. In certain situations it, moreover, may also involve in hormone activation (Friedrichs et al., 2003), antigens formation in immune response (Reyes et al., 1991), and mechanism of calcium homeostasis (Mort et al., 1998). In normal cells, cathepsin B is regulated at every biosynthesis level including transcription, post-transcriptional processing, translation, post-translational processing and trafficking, to attain its normal functions in cell metabolism (Skrzydlewska et al., 2005). Over-expression and mislocation in cell membrane of cathepsin B, however, have contributed to several pathological processes (Leung-Toung et al., 2002) such as cancer (Szpaderska and Frankfater, 2001) and neurogenerative disorders (Petanceska et al., 1994). In tumor cells, dysregulation of one or more protein levels would result in increased mRNA and protein expression, increased activity, and altered intracellular distribution.

Among the lysosomal cysteine proteases, cathepsin B has been the most extensively investigated due to its important role in cancer progression. Cathepsin B mRNA or protein is often present in higher amount in malignant tumors than in normal tissues or benign tissues. The fact that cathepsin B is upregulated by many mechanisms, suggests this does not occur by chance. Tumour cells express transcript variants for cathepsins B, arising from the use of alternative promoters and alternative splicing (Berquin et al., 1995; Yan and Sloane, 2003). A shorter cathepsin B transcript is also more efficiently translated and could be responsible for the overexpressing of cathepsin B in human breast, colon and prostate carcinomas, gliomas and melanoma. At transcriptional level, cathepsin B can be regulated by the binding of Sp1 transcription factor to GC boxes in gliomas. Transcription factor ETS1 which is expressed in invasive

tumors, also regulates transcription of cathepsin B and other key components of the malignant phenotype, for example, the proteases MMP1, MMP3 and MMP9, uPA (Konduri et al., 2001).

People believe that only extracellular proteases function in cancer and that cysteine cathepsins do within intracellular vesicles. Actually cathepsin B can be secreted as a proenzyme and activated extracellularly although until now, it is the only cathepsin member that has been localized to a membrane microdomain. Fusion of lysosomes with the plasma membrane may allow the release of active cathepsin B by osteoclasts and tumor cells (Mort and Buttle, 1997). The secretion of procathepsin B can occur from cells that do not exhibit an increase in mRNA levels, indicating that this secretion is probably due to altered intracellular trafficking and distribution of this enzyme (Sloane et al., 1994). Moreover, it has been found that cathepsin B expression often increases specifically at the invasive edge of tumor cells. Granules containing cathepsin B in normal tissues are localized perinuclear, while during tumor progression they move to the inner basal surface of plasma membrane (Hazen et al., 2000). This is facilitating the degradation of the surrounding extracellular matrix (ECM) in tumor progression, because tumor cell invasion involves local proteolysis.

Cathepsin B can affect ECM directly causing its proteolytic degradation or indirectly via activation or amplification of other ECM-degrading proteases (Fig. 3). The acidified milieus surrounding tumor cells facilitate the secretion of precursors to active forms that degrade the protein components of basement membranes and the interstitial connective matrix including laminin, fibronectin, elastin, and various types of collagen (Buck et al., 1992).

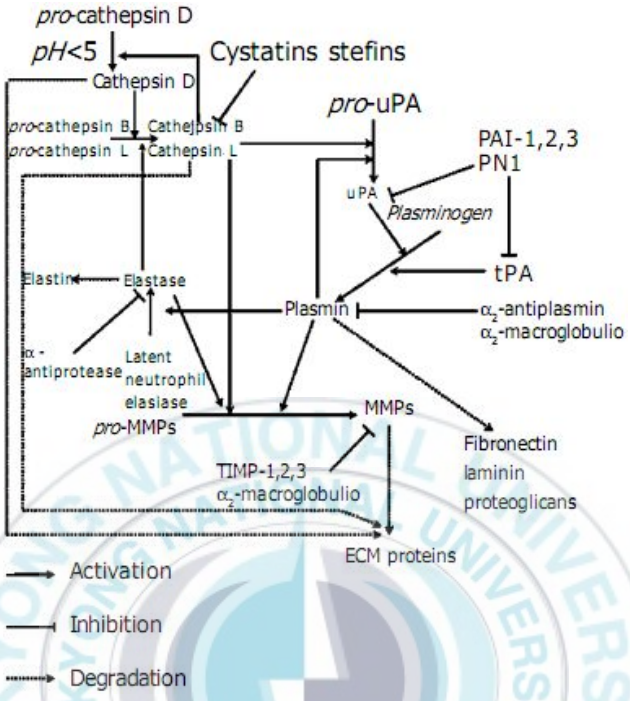


Figure 3. Regulation of ECM proteolysis (Skrzydlewska et al., 2005).

Besides cancer diseases, cathepsin B have been implied to several other disorders especially in inflammatory diseases, such as inflammatory myopathies, rheumatoid arthritis, and periodontitis (Berdowska, 2004). Similar in cancer, the overexpression of cathepsin B may degrade some extracellular peptide components, such as connective tissues components in rheumatoid arthritis, which damages to body. The relation of illegal cathepsin B and many diseases (Table 1) mostly relates to the mislocation and the quite broad pH ranging form 4.0-8.5 of cathepsin B even though it belongs to lysosomal.

Table 1. The involvement of cathepsin B in human diseases.

Type	Disease
Cancer	Breast cancer, Ovarian cancer, Thyroid cancer, Prostate cancer, Bladder cancer, Colorectal cancer, Gastric cancer, Melanoma, Pancreatic cancer, Liver cancer, Lung cancer, Brain tumor, Head and Neck cancer, Kidney cancer.
Other diseases	Inflammatory myopathies, sarcoidosis Alzheimer's, Down disease, Guillain–barre syndrome and multiple sclerosis, Rheumatoid arthritis, Osteoarthritis, Periodontitis, Asthma, Gaucher disease.

Proteolytic activity of cathepsins B is inhibited by endogenous inhibitors cystatin family dividing into cystatins, stefins, and kininogens. Distribution of particular inhibitors in organisms is different. For instance, stefins A and B exist mainly in tissues whereas cystatin C and kininogen do in blood plasma and other body fluid. The activity and the concentration of these inhibitors are changed in different pathological conditions including cancer (Bervar et al., 2003). A decrease of cystatin concentration results in the increase of cathepsin activity, thus enhancing the pathological process.

Cathepsin B inhibitors have been reported to cause changes in malignant tumors (Rozhin et al., 1990). The inhibition of cathepsin B has also been showed to decrease the severity of joint inflammation and to reduce the destruction of particular tissues in the rat model of antigen adjuvant-induced arthritis. Cathepsin B inhibitors are therefore expected to be useful for the treatment of inflammatory joint disease, invasion of cancer, and other diseases related to cathepsin B disorders. Together with some endogenous inhibitors, there are some low molecular weight natural and chemical synthesis inhibitors, i.e., leupeptin, E-64, tokaramide A, CA030, and CA 074 (Otto and Schirmeister, 1997). Therefore, searching for new inhibitors of CatB, especially reversible nonpeptidal inhibitors, should be given increased.

Marine organisms, including microorganisms represent as treasure sources for therapeutic natural products. Moreover, for industrial purpose, microorganisms are preferable to other natural sources, such as sea animal, or algae, etc. due to some advantages in geographic, seasonal viability, and especially in large scale production. Protease inhibitors have been reported to be produced by actinomyces (Jensen et al., 2005; Imada, 2005) and some fungi

(Shimada and Matsushima, 1969). Until now, pure cultures of microorganisms capable of producing cathepsin B inhibitors have been documented only from fungi such as *Aspergillus japonicus* (Hanada et al., 1978), *Aspergillus oryzae* O-1018 (FERM P-15834) (Yamada et al., 1998), and *Streptomyces* spp. (Maeda et al., 1971). Continuing this current trend, we were screening for isolating a marine microorganism which showed potential in producing cathepsin B inhibitors. So far, most of the cathepsin B inhibitors have lower *in vivo* activities than *in vitro* ones. Tumor cell lines with abundant pericellular (cell surface and secreted) cathepsin B would seem appropriate for using in these models in order to test the efficicacy of cathepsin B inhibitors against growth and metastasis *in vivo*. Cathepsin B activity, for example, was unaffected by CA074 and E64; both are potent inactivators in solution, but are negatively charged, and do not enter cells readily (Wilcox and Mason, 1992), although *in vitro* effects have been reported (Towatari et al., 1991). Therefore, pericellular cathepsin B inhibition was studied in order to assess *in vivo* potential of the isolated compounds.

Experimental Procedures

1. Materials

Three hundred marine microorganisms were provided by Korea Research Institute of Bioscience and Biotechnology, Korea, and kept in liquid media during transportation. Cathepsin B, REDTaq^{TM} DNA Polymerase, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Carbobenzoxy-L-Arginyl-L-Arginine 4-Methyl-Coumary-7-Amide (Z-Arg-Arg-MCA) was purchased from Bachem AG (Switzerland). PCR primers FC27 and RC1492 were purchased from Bioneer Inc. (USA). DNA recovery-TAKARA kit and 200 bp DNA Ladder 3410A were purchased from Takara Bio Inc. (Japan). Murine melanoma cell, B16-F10 (ATCC CRL 6475) was obtained from American Type of Culture Collection (Manassas, VA, USA). Dulbecco's modification of Eagle's medium-Ham's F-12 (DMEM-F12), Trypsin-EDTA, penicillin/streptomycin, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Other chemicals and reagents used were of analytical grade.

2. Methods

2.1. Microorganism culture conditions

Initially, the basal medium [10 g/l glucose, 1 g/l beef extract, 2 g/l peptone, 1 g/l yeast extract, and 20 g/l agar in case of solid medium] was used and prepared with 60% of sea water. Before experiment, each microorganism was activated by

two successive transfers in liquid medium and one to slant culture. The microorganism was stored at -80 ^{o}C in the same medium with 20% glycerol stock. The effects of culture conditions were investigated including initial pH, temperature, and shaking condition. Additionally, kinetic of inhibitor production was also carried for 70 h. The inocula were prepared by transferring a loopful of the activated microorganisms to 50 ml medium in 250-ml Erlenmeyer flasks, shaking at 200 rpm, 28 ^{o}C for 24 h. 4 ml of culture broth was transferred and inoculated under same condition and was used for studying the culture condition. The growth of microorganism was determined by measuring the optical density at 620 nm by GENios® microplate reader (Tecan Austria GmbH, Austria).

2.2. Cathepsin B inhibitory assay

Cathepsin B inhibitory assay was performed in 96-well black plate by the method of Barrett et al. (1982) with some modifications. Each well contains 12.5 µl of a 0.4 M sodium potassium phosphate buffer (pH 6.0) containing 8 mM dithiothreitol (DTT) and 4 mM EDTA, 12.5 µl of 400 µunits/ml cathepsin B solution and 12.5 µl of sample solution (dissolved in methanol). After 10 min at 37 ^{o}C, 12.5 µl of 20 µM Z-Arg-Arg-MCA in water was added to start the reaction. The reaction was stopped after 20 min by 50 µl of 100 mM sodium monochloroacetate in 100 mM sodium acetate (pH 4.3). The fluorescence of MCA released was measured at Ex 360 nm and Em 465 nm with GENios® microplate reader. Control test was run with methanol during purification procedure. The percentage of inhibition was calculated by following equation:

$$\mathrm{I}\ (\%) = \frac{Control - Test}{Control - Blank} \times 100\%$$

2.3. Isolation of a marine-derived microorganism genomic DNA

Culture was grown in 15 ml of basal medium in 50-ml conical tube for 4 days and then centrifuged at 4,000 rpm for 5 min. The pellet was resuspended in 700 µl of 10 mM Tris-Cl–1 mM EDTA (pH 7.5) (TE) containing 20 mg of lysozyme/ml and 20 mg of RNase A/ml and incubated at 37 °C for 1 h. Following incubation, 150 µl of 0.5 M EDTA, 150 µl of TE containing 5 mg of proteinase K/ml, and 100 µl of 10% SDS were added and incubated at 37 °C for 1 h. The tube was mixed by inversion after the addition of 100 µl of 5 M NaCl, and heated in a 65 °C water bath for 10 min. Cellular debris was removed by centrifugation 8,000 rpm for 5 min, and the supernatant solution was transferred to a new Eppendorf tube. Proteins and lipids were removed by the addition of 0.3 volume of phenol-chloroform, and centrifuged at 12,500 rpm for 5 min. DNA in the aqueous phase was precipitated with an equal volume of 100% ethanol and kept 15 min at -20 ^{o}C, and then centrifuged at 12,000 rpm for 5 min to remove ethanol. Finally DNA genome was redissolved in 200 µl of TE for immediate use or storage at 20 °C.

2.4. PCR amplification of the 16S rRNA gene

The 16S rRNA gene was amplified from genomic DNA obtained from microorganism using forward primer FC27 (5'-AGAGTTTGATCCTGGCTCAG-3'), reverse primer RC1492 (5'-TACGGCTACCTTGTTACGACTT-3') and REDTaq^{TM} DNA Polymerase kit. The reaction mixture was carried out in 50 µl of final volume containing 1 µl DNA genome, 2.5 µl Taq DNA polymerase, 5 µl 10X PCR buffer, 1 µl dNTP, 1 µl of each primer, and 38.5 µl of PCR water. The PCR reaction was carried out

by T1 Thermocycle (Biometra, Germany). Amplicon was obtained with a PCR cycling program of 80 ^{o}C for 5 min, 30 cycles at 94 ^{o}C for 30 s, a 55 ^{o}C for 30 s and a 72 ^{o}C for 60 s; and a final 7-minute extension at 72 ^{o}C. The amplified product was visualized by electrophoresis separation on 2% agarose gel stained with ethidium bromide.

2.5. DNA sequencing and data analysis

PCR fragments were purified by using Easy trap-Glass power for DNA recovery. Three volumes of NaI solution was added and incubated at 55 ^{o}C for 5 min. Then 5 µl of glass bead was put in the mixture. After 5 min at room temperature, the mixture was centrifuged at 5,000 rpm for 1 min. The glass bead was collected and washed three times by 1 ml of washing solution. At final step, 20 µl of distilled water was added and incubated at 55 ^{o}C for 5 min. 16S rDNA supernatant was collected by centrifuged at 5,000 rpm for 1 min. Total 1400 nucleotide sequence of the 16S rRNA gene was sequenced with the same primers by SolGent Co., Ltd. (Korea). Sequence then was aligned by using ClustalX software (version 1.8) and edited by using BioEdit software. The nearly complete 16S rRNA gene sequence was used to search the GenBank database to determine relative phylogenetic positions. Phylogenetic tree was constructed by generating a complete alignment of 16S rRNA gene sequences of selected members in GenBank by using MEGA software bootstrap values (from 1,000 resamplings) (Tamura et al., 2007).

2.6. Extraction and purification of inhibitors

Total 20 liters of culture broth of the chosen microorganism was extracted with EtOAc (1:1) to provide a crude extract which was subjected to silica gel column chromatography (Merck 70-230 mesh, 75g, Ø 2.5 x 40) and successively eluted with *n*-hexan to EtOAc. Eluted fractions were analyzed by thin layer chromatography (TLC). Eluted fractions with the same Rf values were pooled after TLC analysis. Enzyme inhibitory activity was obtained in *n*-Hexan-EtOAc (10:1) fraction, which was purified by HPLC using a Waters 600 controller equipped with a 2487 dual wavelength detector and Alltima C18 5u column (10 mm i.d. x 25 cm; Alltech, USA) with a linear gradient of acetonitrile (0-100%), flow rate 2.0 ml/min, and UV detection at 220 nm.

2.7. Spectroscopic measurements

NMR spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer, ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks: δ_H 7.62 and δ_C 77.31 for $CDCl_3$. Electron Impact Mass Spectrometric (EIMS) data was measured on a JEOL JMX-700 spectrometer. Optical rotations were determined on a Pekerin Elmer model 341 polarimeter in MeOH. IR spectra were recorded on Fourier transform IR spectrophotometer (Bucker FT-IR model IFS-88 spectrometer).

2.8. Determination of the inhibition pattern

Different concentrations of the isolated compounds were added to each reaction mixture with constant concentration of cathepsin B. Two different concentration of each inhibitor and substrate Z-Arg-Arg-MCA at varying

concentrations (20, 30, 40 μM) were used during kinetic analysis. The inhibition modes were determined by Lineweaver-Burk plots. The inhibition constant (*Ki*) were determined by the secondary plots (intercept on vertical axis of Lineweaver Burk plots against inhibitor concentrations) (Dixon and Webb, 1979).

2.9. Cytotoxic assessment using MTT assay

B16-F10 cells were cultured and maintained in DMEM-F12 supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 10% FBS and maintained at 37 °C under a humidified atmosphere with 5% CO_2. Cytotoxic levels of the inhibitors on B16-F10 cells were measured using MTT method as described by Hansen et al. (1989). Cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of inhibitors. After 24 h of incubation, cells were rewashed and 100 μl of MTT (1 mg/ml) was added and incubated for 4 h. Finally, DMSO (100 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using a GENios® microplate reader. Relative cell viability was determined by the amount of MTT converted into formazan salt. The percentage of viable cells compared to the control was calculated as a percentage (OD of treated cells / OD of blank x 100) and dose response curves were developed.

2.10. Fluorescent microplate assay

This assay was employed to measure pericellular cathepsin B activity using the fluorogenic substrate Z-Arg-Arg-MCA (Hulkower et al., 2000). B16-F10 cells were plated in 96-well plates and grown a density of 5×10^3 cells/well. The growth medium was then aspirated and the monolayer washed with 0.1 ml Dulbecco's $NaCl/P_i$. The cells were then incubated with 90 μl of pericellular assay buffer (PAB; Hank's balanced salt solution containing 2 mM L-cysteine, pH 7.0) at 37 ^oC. After 30 min, 10 μl of 200 μM Z-Arg-Arg-MCA was added in the presence or absence of inhibitors. The cells were incubated for an additional

20 min at 37 ^{o}C. The fluorescence of the MCA was measured at Ex 360 nm and Em 465 nm by GENios® microplate reader.

2.11. Analysis of the effects of two isolated phthalates on intracellular cathepsin B on tumor cell

The ability of isolated samples to affect intracellular cathepsin B was tested with B16-F10 cells (Navab et al., 1997). Confluent cultures of B16-F10 cells in serum free DMEM-F12 medium were exposed to different concentrations of samples. After 4 hours, the medium was removed and the cell layers were washed twice in PBS buffer pH 7.2, and lysed in a 1% Triton X-100 solution containing 50 mM sodium acetate and 1 mM EDTA, pH 6.0, and frozen and thawed. Cell debris was removed by centrifugation 5,000 rpm in 5 min and the supernatants were assayed with Z-Arg-Arg-MCA by cathepsin B assay described previously.

2.12. Statistical analysis

Data were presented as mean ± standard error of the mean (n=3). Student's t-test was used to determine the level of significance. P value < 0.05 was considered significant.

Results and Discussions

1. Screening of marine microorganism culture broths for cathepsin B inhibitor

In our screening, all culture broths were applied for enzyme inhibitory assay and the medium without culture was used as control (Table 2). Ten strains among 300 marine microorganisms were chosen because they showed cathepsin B inhibitory activities. G010387 isolated from Daegu stood out as a good

candidate according to enzyme inhibitory result. Therefore G010387 was selected for further purification.

Table 2. List of marine microorganism strains which showed cathepsin B inhibitory activities.

Group	Strain	I (%)	Sampling site	Sampling date
A	G010383	12.25	Daegu	2001
	G010387	43.02		
	G010397	10.87		
	G010417	33.21		
B	G010510	11.32	Dosan	2001
	G010511	30.43		
	G010517	24.75		
	G010533	16.59		
C	G021058	16.05	SongKungMi	2002
	G021083	33.05		

2. Identification of a marine microorganism

Colonies on solid media were circular, opaque, and low convex. The PCR products were appeared at 1,400 Nucleotide position in comparison to DNA Ladder bands (Fig. 4). The nearly completed 16S rRNA gene sequence was submitted to the GenBank database (accession no. EU126129). G010387 was named as *Pseudomonas* sp. strain PB01. Due to the result of the alignment of 16S rRNA on GenBank, there were 100 16S rRNA gene sequences having high similarity with that of strain PB01. The 16S rRNA sequence identity between strain PB01 and P.

fluorescens (accession number AY622219) was approximately 99%. These values fall within the range of 16S rRNA gene sequence similarities (93.7–99.9%) reported for species of the genus Pseudomonas (Mort and Buttle, 1997). Furthermore, strain PB01 was conformed to the genus *Pseudomonas* with a high bootstrap value based on an analysis of the phylogenetic tree (Fig. 5). Culture characteristic and analysis of the 16S rRNA gene of strain PB01 suggested that this strain could belong to *Pseudomonas* species. Hitherto relatively few species originating from marine environments are classified within *Pseudomonas* species including *Pseudomonas stutzeri* (Baumann et al., 1983), *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes* (Palleroni, 1984), some strains of *Pseudomonas balearica* (Bennasar et al., 1996) from marine sediments, *Pseudomonas alcaliphila* (Yumoto et al., 2001) from seawaters, and *Pseudomonas pachastrellae* sp. nov. from sponge (Romanenko et al., 2005). Strain PB01 is the first marine *Pseudomonas* species used for isolating cathepsin B inhibitor.

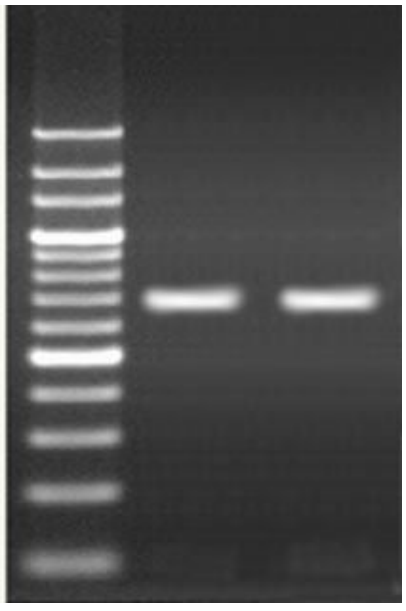


Figure 4. The 16S rRNA genes from a single isolated colony by primers FC27 and RC1492. PCR reaction was carried out by Sigma REDTaq^{TM} kit: (A) 200 bp DNA Ladder - Takana 3410A; (B) Strain PB01; (C) *P. aeruginosa* ATCC 27853.

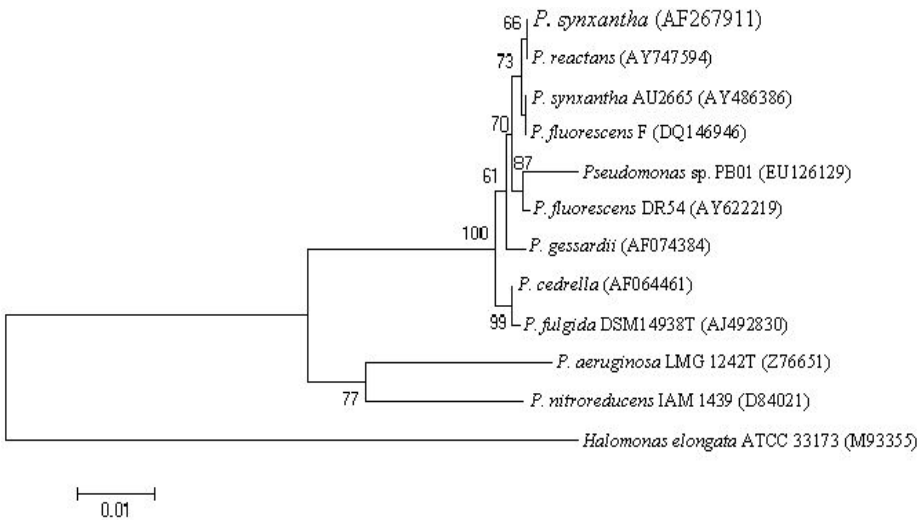


Figure 5. Phylogenetic relationship of *Pseudomonas* sp. strain PB01 and some related *Pseudomonas* species on the basis of 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbor-joining method. Numbers indicate bootstrap values greater than 60%. Bar, 0.01 *Knuc* units.

3. Effects of culture conditions on cathepsin B inhibitor production

Since it was first time to employ strain PB01 for such activity, it is important to set up some culture conditions. That is not only useful to support for microorganism growth, but also to predict the origin of isolated compounds.

3.1. Effects of initial medium pH, temperature, and shaking speed on cathepsin B inhibitor production

The initial pH of medium was adjusted to various values 4.0-10.0 by sterile 1.0 N NaOH or 1.0 N HCl solution. Cultivation was carried at 28 ^{o}C and with shaking speed at 200 rpm for 24 h. As showed in Fig. 6, strain PB01 could grow well in a broad pH and the optimum are at pH from 6.0 to 7.0. Further increase of the initial pH over 7.0 resulted in the slightly reduced biomass and activity. The initial pH however does not affect too much on the growth and without causing major changes in production.

Growth temperature is another critical parameter that needs to be controlled. Cultivation was conducted at pH 6.6 and with shaking speed at 200 rpm after 24 h. As showed in Fig. 7, strain PB01 grew well at moderate temperature from 25 ^{o}C to 28 ^{o}C which is comparable to many kinds of *Pseudomonas* sp. that can grow relatively at higher temperature. It may be because strain PB01 was originally isolated from normal marine source. The difference of biomass between 28 ^{o}C and 40 ^{o}C was 2.5 times, and of inhibitory activity was 5 times. The inhibitory activity reached the highest value of 48.02% at 28 ^{o}C.

As an aerobic microorganism, the suitable shaking speed during cultivation of strain BP01 was also tested at 0-200 rpm at initial pH 6.6 and 28 ^{o}C. Even though strain PB01 is not so strictly aerobic, the increasing oxygen supply during culture favors for the growth and inhibitor production (Fig. 8). It was noted that with a shaking speed of 200 rpm, the inhibitory activity was 47.52%, which was the highest among the various shaking speeds tested and was higher 7 times that showed in static culture.

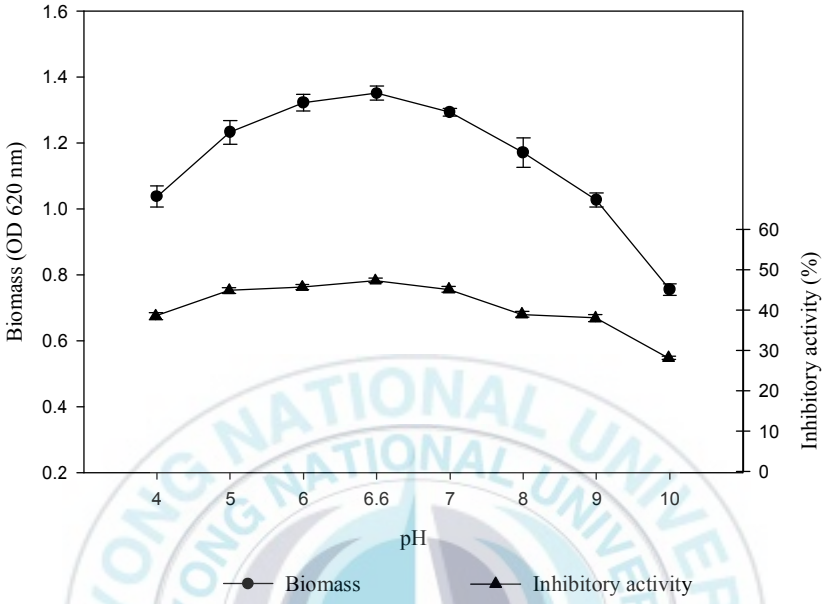


Figure 6. Correlation between growth and inhibitory activity of strain PB01 with various initial pHs. The cultivation was carried out at 28 ^{o}C and 200 rpm for 24 hours.

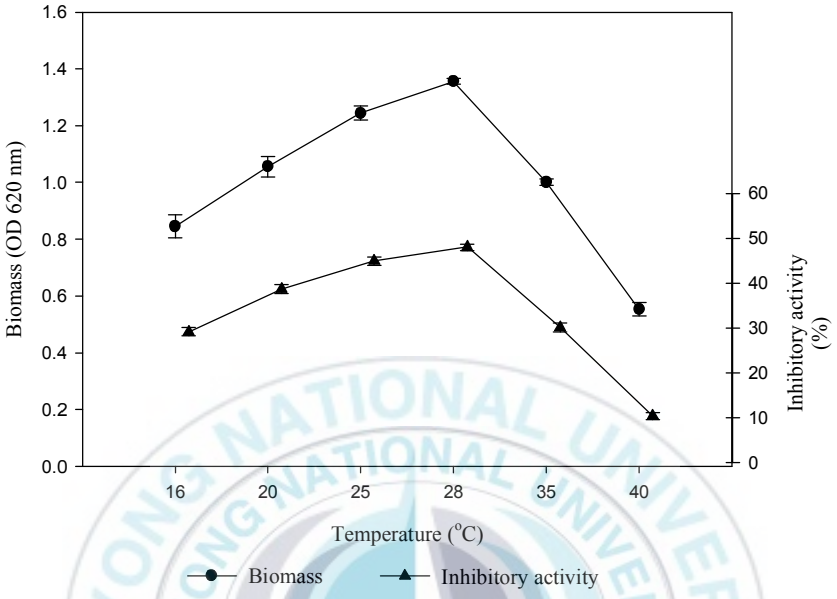


Figure 7. Correlation between growth and inhibitory activity of strain PB01 at different cultivation temperatures. The cultivation was carried out at pH 6.6 and 200 rpm for 24 hours.

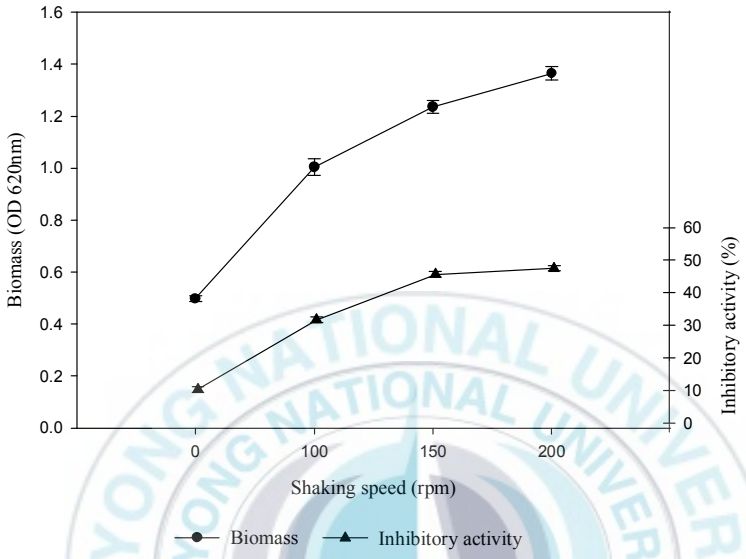


Figure 8. Correlation between growth and inhibitory activity of strain PB01 at different shaking conditions. The cultivation was carried out at pH 6.6 and 28 ^{o}C rpm for 24 hours.

3.2. Growth of strain PB01 and kinetics of inhibitor production according to cultivation time

A typical time course of growth, cathepsin B inhibitory activity, and pH variation were performed in 70 h follow the above results and showed in Fig. 9. These results figured out that the increase or decrease of inhibitory activity was accompanied with biomass for each tested condition. After a short lag period 2 h, biomass of strain PB01 increased rapidly and entered the stationary phase after 20-24 h of cultivation. During culture time, the pH of the medium declined rapidly from 6.6 at the beginning to 4.86 after 25 h. The cathepsin B inhibitory activity paralleled with the cell growth at a constant rate up to the end of the log phase. This is unusual for secondary metabolites (Bu'Lock, 1961) and may be closer to the so-called growth-associated type (Jr. Garden, 1959). Hence the inhibitors may be products from the growing stage and useful for microorganism growth.

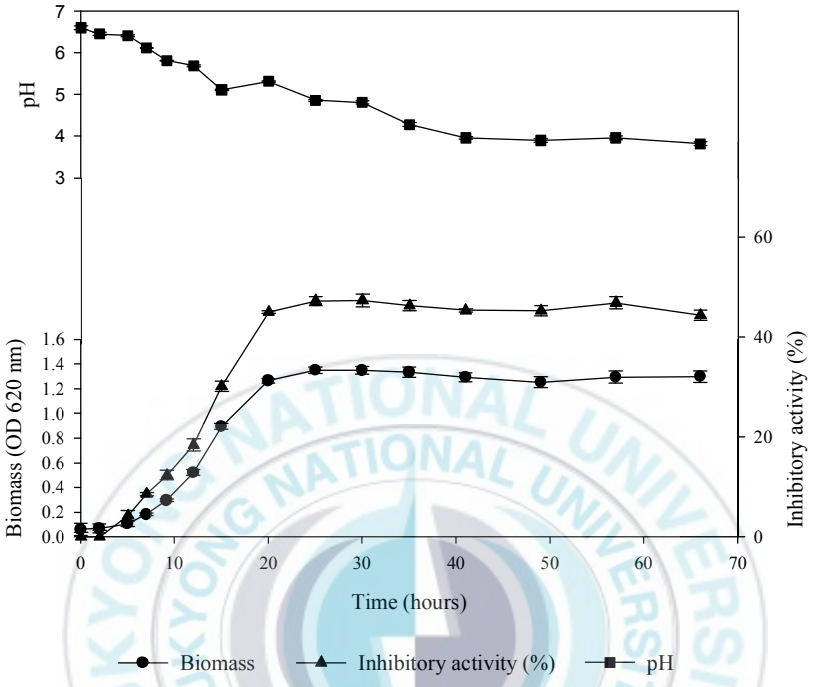


Figure 9. Time course of growth, cathepsin B inhibitory activity, and pH variation. The cultivation was carried out at 28 °C, pH 6.6, and 200 rpm.

4. Isolation and purification of active compounds from *Pseudomonas* sp. PB 01 culture broth

Total 20 liters of culture broth was extracted with EtOAc to provide a crude extract (2.8 g) which was subjected to silica gel column chromatography and eluted with *n*-hexan to EtOAc (*n*-hexan:EtOAc: 100, 80, 60, 50, 0%) (Fig.10). Solvent volume differed from each ratio and depended on TLC result. Out of 70 collected fractions, 8 major fractions were combined based on the similarity of TLC pattern. All fractions dissolved well in methanol solution at concentration 1 mg/ml, which was easy to assess inhibitory activity at their actual potential. Enzyme inhibitory activity was observed in fraction A (55.7 mg).

The potent A fraction was purified by HPLC runs on an Alltima C18 column and successively eluted with a linear gradient of acetonitrile (0-100%) at flow rate 2 ml/min. Interestingly, both separated fractions from HPLC showed inhibitory activities. Final purification was carried out using same HPLC condition for each fraction. After all, two active compounds dibutyl phthalate (**1**) (15 mg, 0.54% yield based on wet weight, t_R 36.05 min), and di-(2-ethylhexyl) phthalate (**2**) (20 mg, 0.71% yield based on wet weight, t_R 53.44 min) were obtained (Fig. 11).

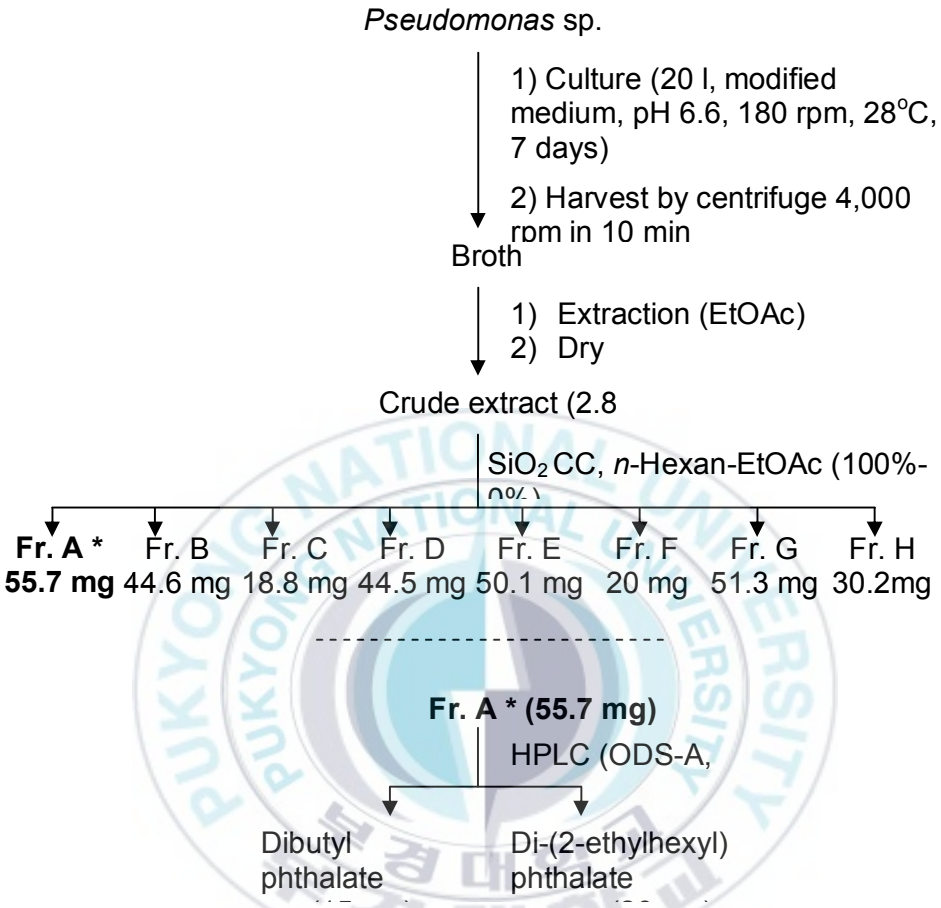


Figure 10. Purification procedure of cathepsin B inhibitors from marine *Pseudomonas* sp. PB01.

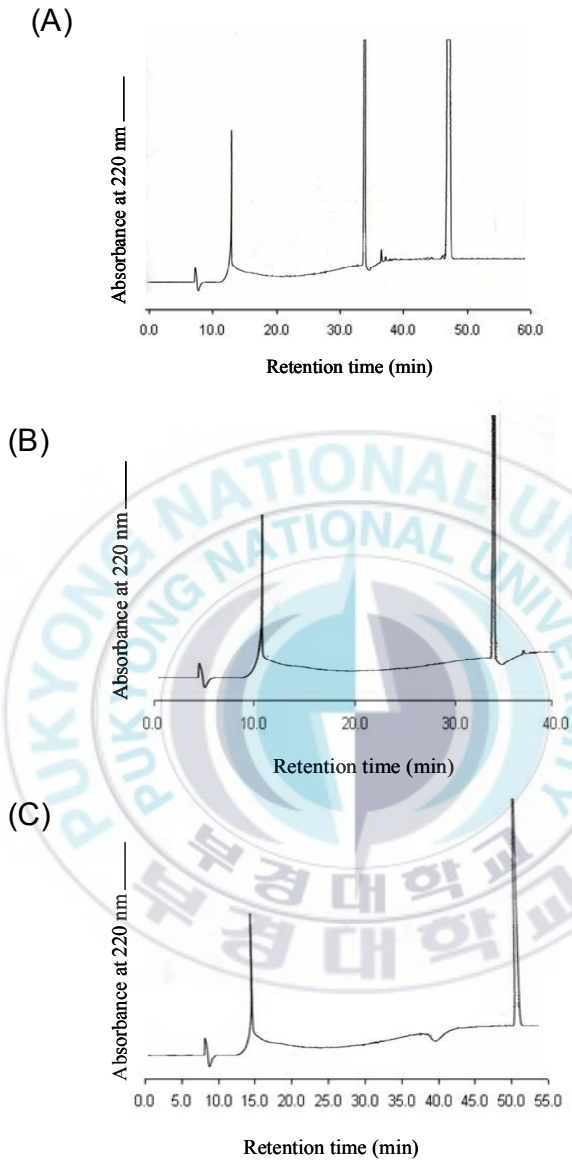


Figure 11. Purification of cathepsin B inhibitors by HPLC running on Alltima C18 column with a linear gradient of acetonitrile at flow rate 2ml/min, 220 nm. (A) Fraction A was separated into two fractions. (B) HPLC chromatogram of dibutyl phthalate. (C) HPLC chromatogram of di-(2-ethylhexyl) phthalate.

5. Elucidation of chemical structure of two isolated compounds

Compound (**1**) was isolated as colorless oil, which had an IR spectrum [aromatic system (1600, 1579, 1465, 740 cm^{-1}) and ester moiety (1724 cm^{-1}, 1271 cm^{-1})] indicating carbonyl and phenyl functional groups. The molecular formula of (**1**) was provided by LREIMS (positive ion), which gave an $[M+H]^+$ ion peak at 279, which analyzed for $C_{16}H_{22}O_4$ on the basis of ^1H and ^{13}C NMR data as well. Moreover, LREIMS showed phthalate base peak at *m/z* 149 for the characterization of phthalate ester with lateral chains bigger than two carbons (El-naggar, 1997). Analysis of the ^1H, ^{13}C, DEPT, and HMQC NMR data of (**1**) indicated the presence of two methyl groups, six methylene units (two O-bearing methylene), four sp^2 aromatic carbons, two sp^2 quaternary carbons, and two carbonyl carbons. The ^1H NMR spectrum showed a characteristic AA'BB' system at δ 7.47 (2H, dd, *J*= 9.22, 2.19 Hz), 7.25 (2H, dd, *J*= 8.78, 2.63 Hz), as well as ^{13}C NMR data at δ 131.6 (s), 127.9 (d), 130.0 (d). These data accounted for all required the compound to have di *ortho*-substituted aromatic ring. The partial structure of butane moiety was interpreted mainly by COSY NMR data. These data described above was suggested compound (**1**) as dibutyl phthalate. The structure and key correlations from COSY spectrum of compound (**1**) were described in Fig. 26.

Compound (**2**) was isolated as an optically inactive oil, $[\alpha]_D^{20}$ 0° (*c* 0.5, MeOH). Its molecular formula was provided by LREIMS (positive ion), which gave an ion peak $[M+H]^+$ at 391, which analyzed for $C_{24}H_{38}O_4$ on the basis of ^1H and ^{13}C NMR data as well. The IR spectrum revealed the existence of alkane (2924 cm^{-1}), aromatic system (1602, 1575, 1485, 741 cm^{-1}), and ester moiety (1723 cm^{-1} and 1267 cm^{-1}). The six degrees of unsaturation in this formula could be

partially accounted for by one benzene ring and two carbonyl units, which was agreeable to LREIMS ion peak *m/z* 149. Analysis of the ^{1}H, ^{13}C, DEPT and HMQC NMR data of (**2**) indicated the presence of four methyl groups, ten methylene units (two O-bearing methylene), four sp^2 aromatic carbons, two sp^2 quaternary carbons, two alkane methane units, and two carbonyl carbons. The ^{1}H NMR spectrum showed a characteristic AA'BB' system at δ 7.67 (2H, dd, *J*= 9.15, 2.56 Hz), 7.47 (2H, dd, *J*= 8.78, 2.19 Hz), as well as ^{13}C NMR data at δ 132.3 (s), 128.5 (d), 130.7 (d). These data accounted for all required the compound to have di *ortho*-substituted aromatic ring. The connections of di-substituted ester moieties attached to the aromatic quaternary carbons, were elucidated by the long-range coupling between δ_H 7.67 and δ_C 167.5 (s). The signals from HMBC and COSY NMR spectra clearly elucidated the partial structure of alkane moieties. The HMBC correlation between δ_H 1.65 (m) and δ_C 67.8 (t) finally completed the plane structure of compound (**2**) as di-(2-ethylhexyl) phthalate. The two symmetric chiral carbons (*R,* C-2', 2") led to the molecular symmetry, which was supported by the optical rotation data $[\alpha]_D^{20}$ 0° (*c* 0.5, MeOH). The structure and key correlations from HMBC and COSY spectra of compound (**2**) were described in Fig. 26.

Dibutyl phthalate (1): colorless oil, IR (neat) ν_{max} 2924, 1723, 1600, 1577, 1488, 1462, 1379, 1267, 1118, 1071, 1037, 956, 741, 702 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 3; LREIMS *m/z* (rel. int.) 279 $[M+H]^+$ (0.51), 167 (118), 149 (100).

Di-(2-ethylhexyl) phthalate (2): yellowish oil, $[\alpha]_D^{20}$ 0° (*c* 0.5, MeOH), IR (neat) ν_{max} 2924, 1723, 1600, 1577, 1488, 1462, 1379, 1267, 1118, 1071, 1037, 956, 741, 702 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz,

$CDCl_3$) data, see Table 3; LREIMS *m/z* (rel. int.) 391 $[M+H]^+$ (50), 279 (56), 167 (81), 149 (100), 113 (47), 83 (24), 57 (66).

Dialkyl phthalate, including dibutyl phthalate and di-(2-ethylhexyl) phthalate are used as plasticizers in variety of industrial products and thought to pollute environment. However both of them were also considered as natural products, which were already mentioned in many papers. For instance some dialkyl phthalate have been isolated from terrestrial and marine organisms such as *Aloe vera* (Lee et al., 2000), marine algae (Chen, 2004; Noguchi et al., 1979; Sastry and Rao, 1995; Stefanov et al., 1988), and fungal and bacterial culture broths (Al-Bari et al.; 2005, Amade et al., 1994; Roy et al., 2006). In order to conform our compounds were from *Pseudomonas* sp. PB01, the culture broth of another marine microorganism, together with pure medium, was investigated. As expected, these compounds did not appear in any other culture broths, except *Pseudomonas* sp. PB01 one (data not showed). As a result of that, dibutyl phthalate and di-(2-ethylhexyl) phthalate isolated from *Pseudomonas* sp. PB01 must be biogenetic products of this marine microorganism, because they have not been contaminated from environment. Roles of phthalate esters are unclear. They may be stored in the cell membrane and affect the flexibility of the cell (Chen, 2004).

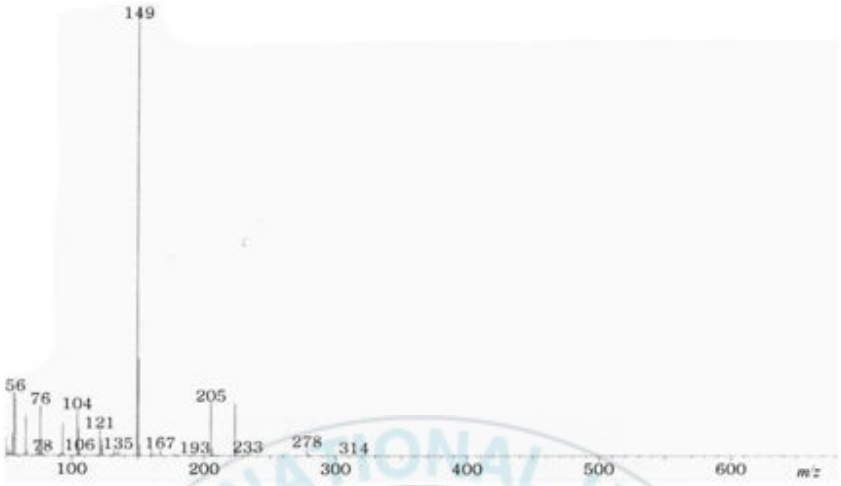


Figure 12. Electron Impact Mass Spectrometric of Dibutyl phthalate (**1**)

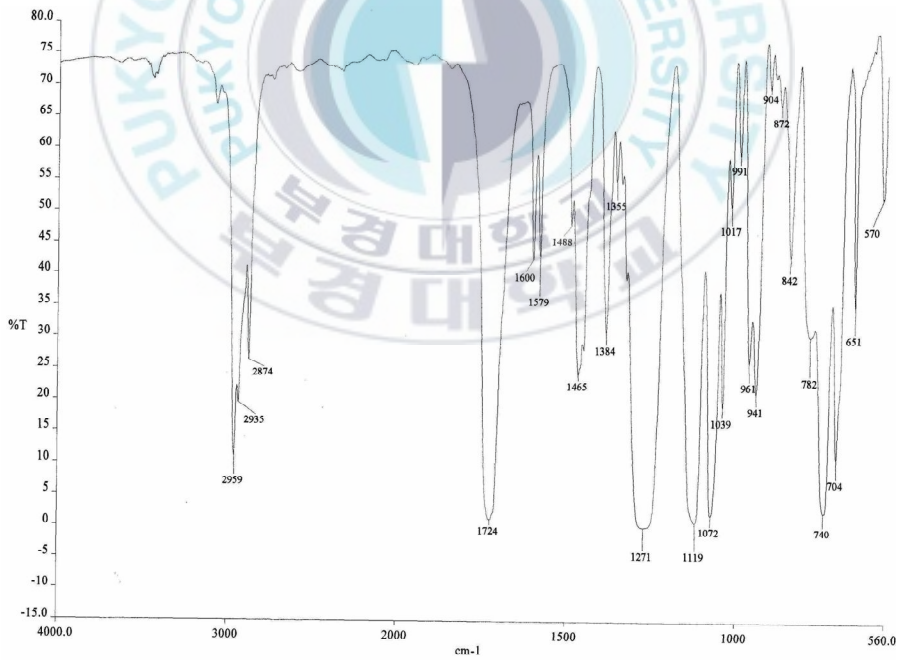


Figure 13. IR spectrum of Dibutyl phthalate (**1**)

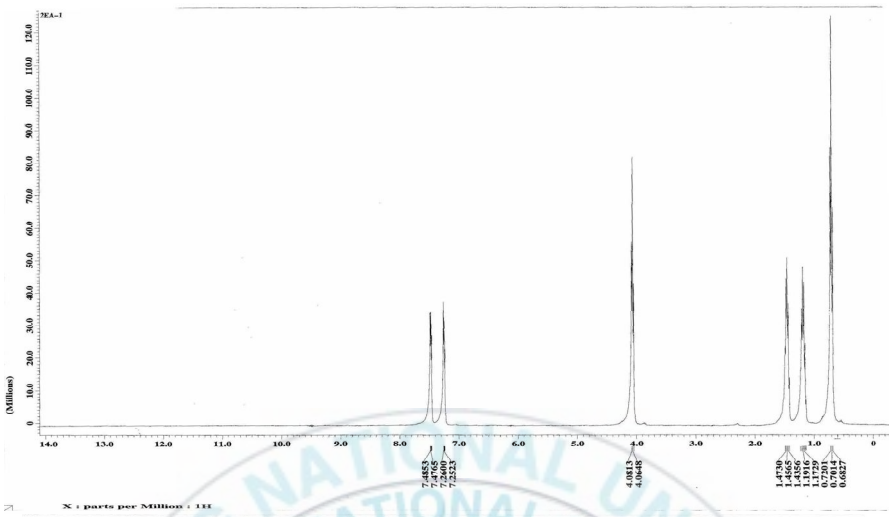


Figure 14. ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of Dibutyl phthalate (**1**)

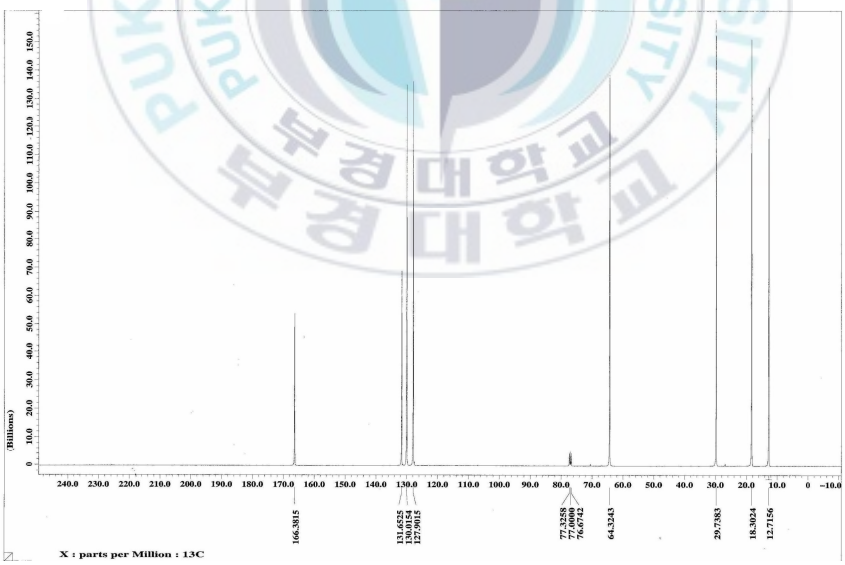


Figure 15. ^{13}C NMR (400 MHz, $CDCl_3$) spectrum of Dibutyl phthalate (**1**)

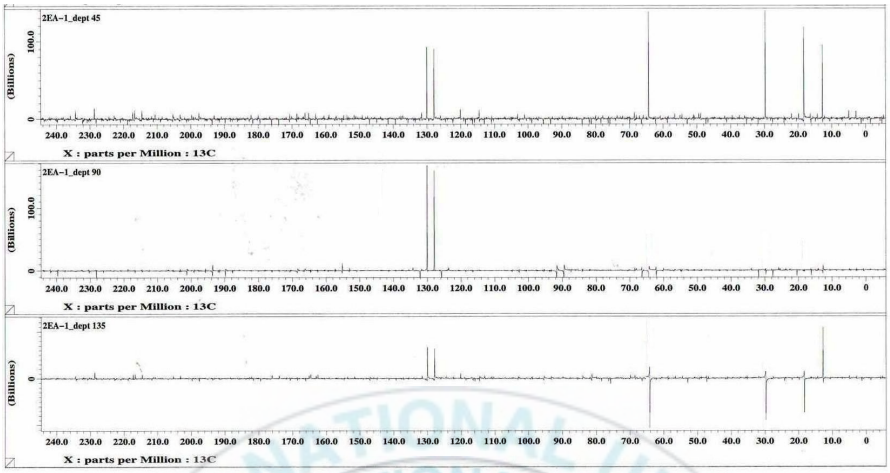


Figure 16. DEPT (45^{o}, 90^{o}, 135^{o}) spectra of Dibutyl phthalate (**1**)

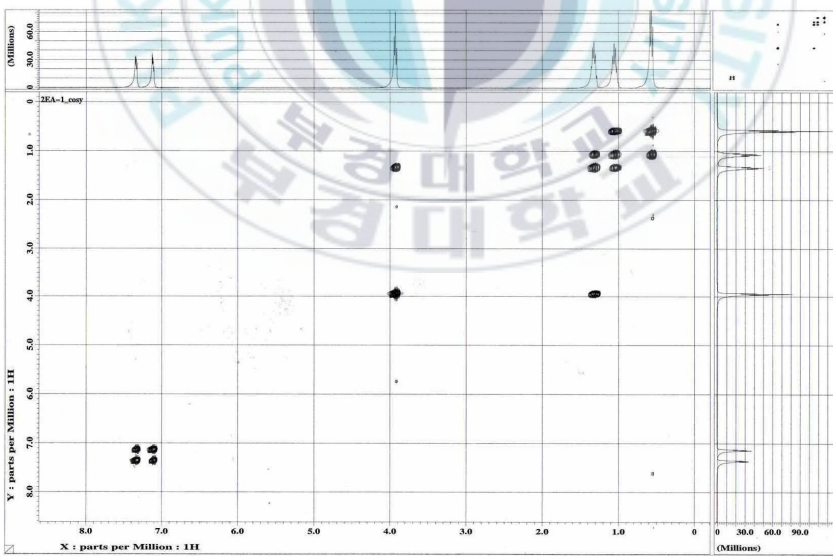


Figure 17. COSY spectrum in $CDCl_3$ of Dibutyl phthalate (**1**)

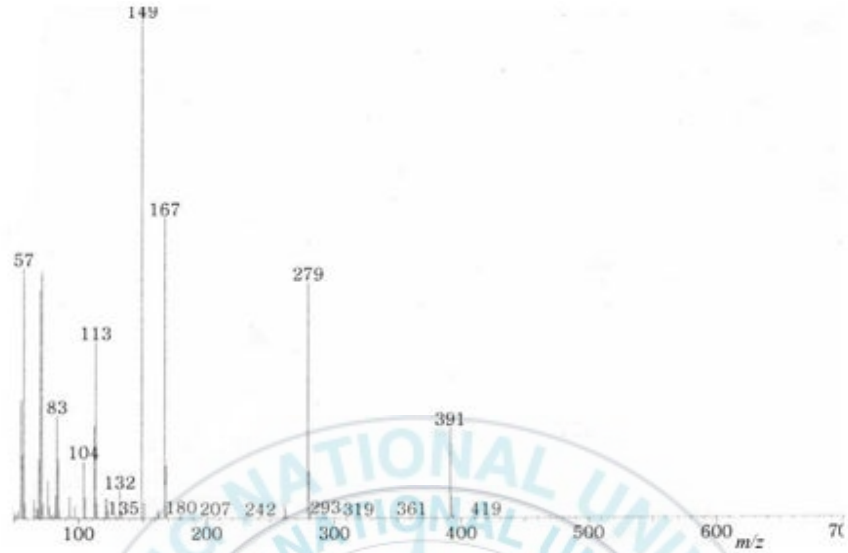


Figure 18. Electron Impact Mass Spectrometric of Di-(2-ethylhexyl) phthalate (**2**)

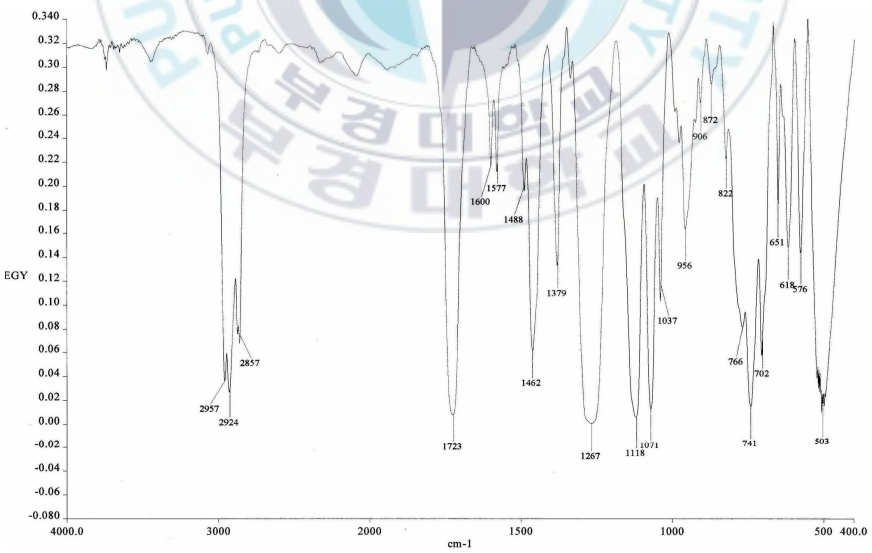


Figure 19. IR spectrum of Di-(2-ethylhexyl) phthalate (**2**)

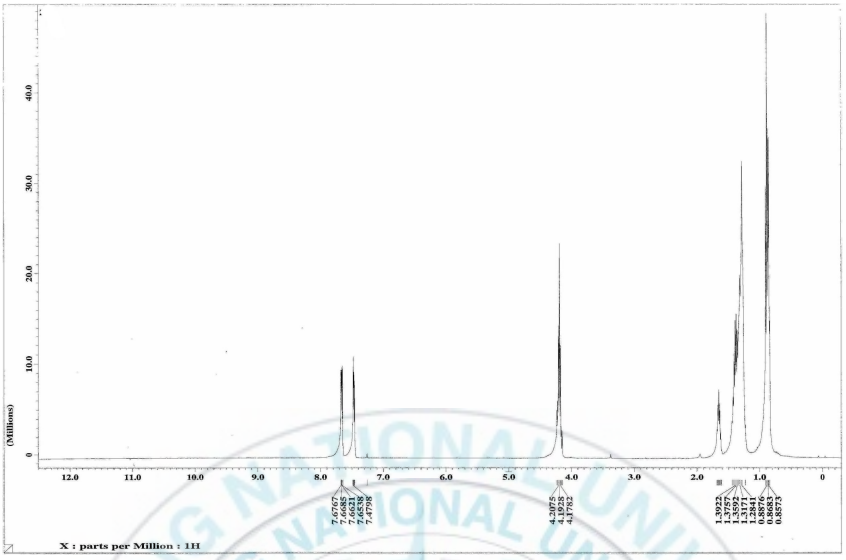


Figure 20. ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of Di-(2-ethylhexyl) phthalate (**2**)

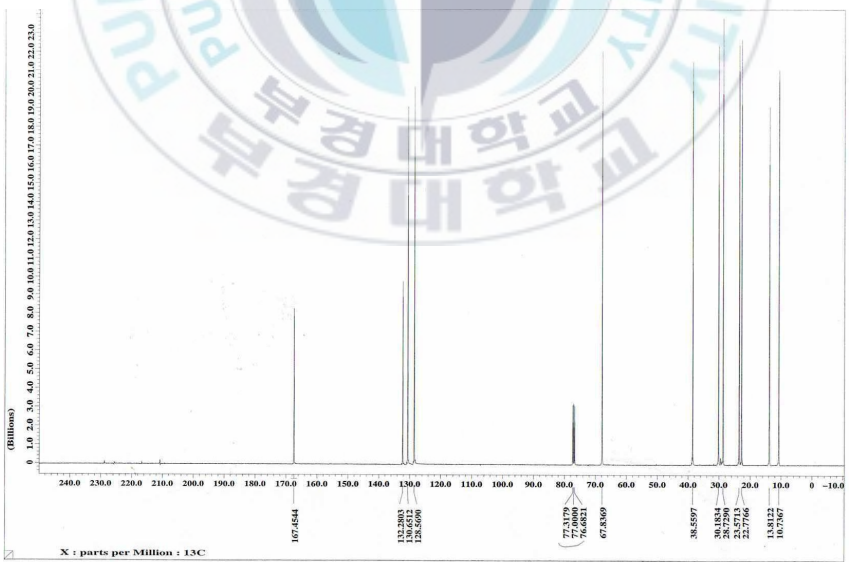


Figure 21. ^{13}C NMR (400 MHz, $CDCl_3$) spectrum of Di-(2-ethylhexyl) phthalate (**2**)

Figure 22. DEPT (45°, 90°, 135°) spectra of Di-(2-ethylhexyl) phthalate (**2**)

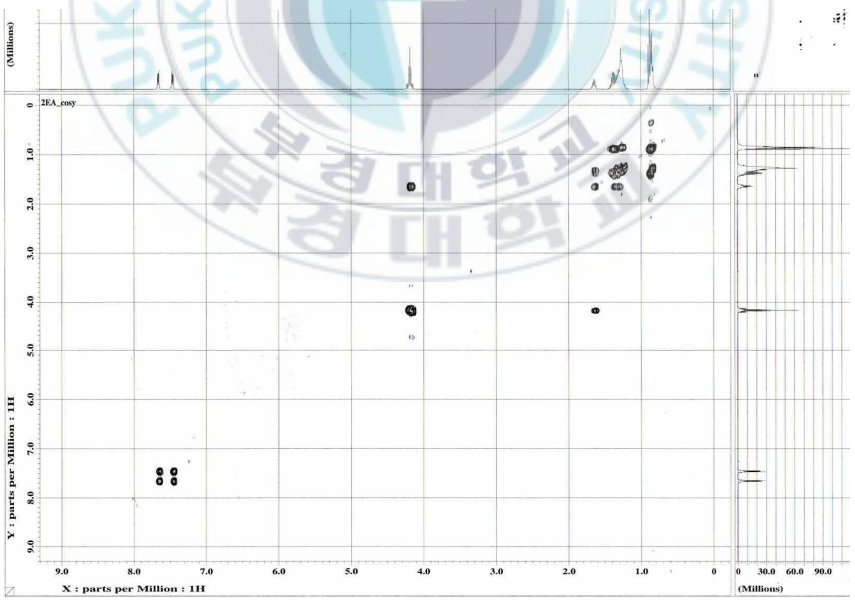


Figure 23. COSY spectrum in $CDCl_3$ of Di-(2-ethylhexyl) phthalate (**2**)

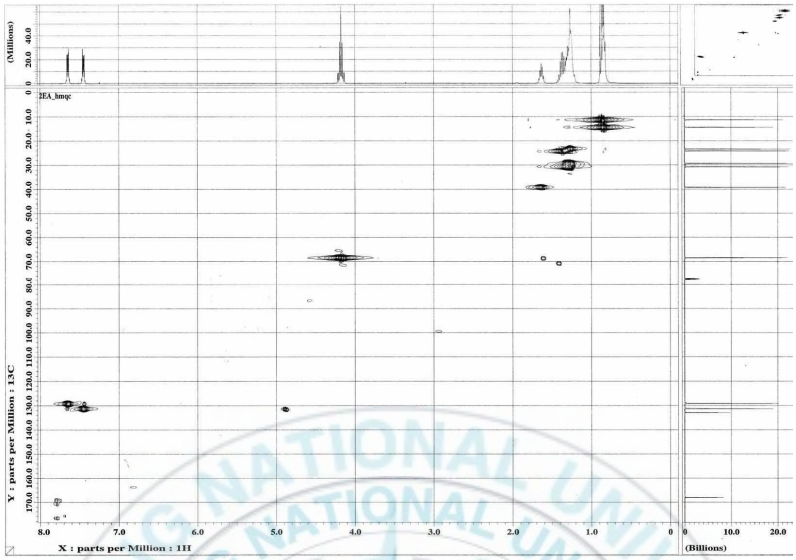


Figure 24. HMQC spectrum in $CDCl_3$ of Di-(2-ethylhexyl) phthalate (**2**)

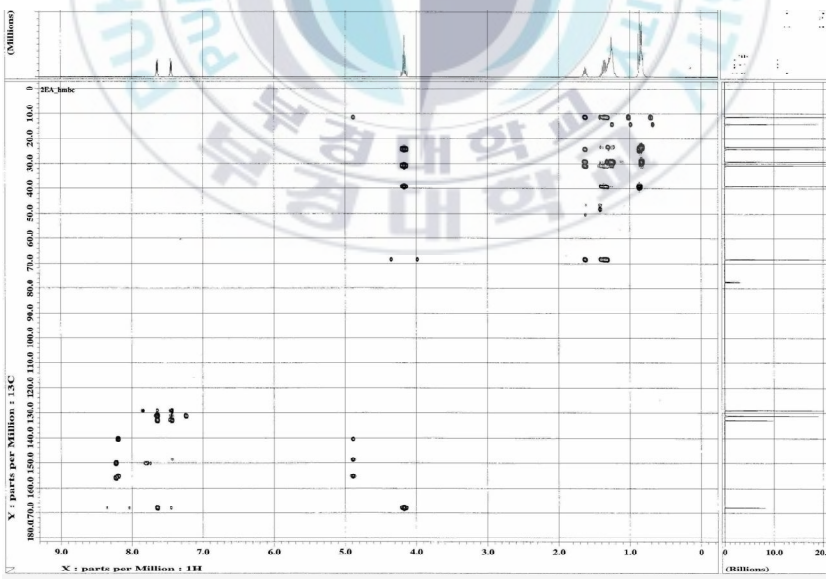
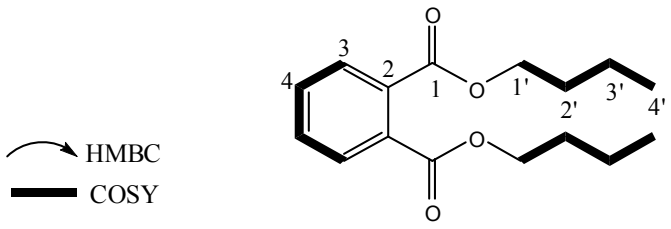


Figure 25. HMBC spectrum in $CDCl_3$ of Di-(2-ethylhexyl) phthalate (**2**)

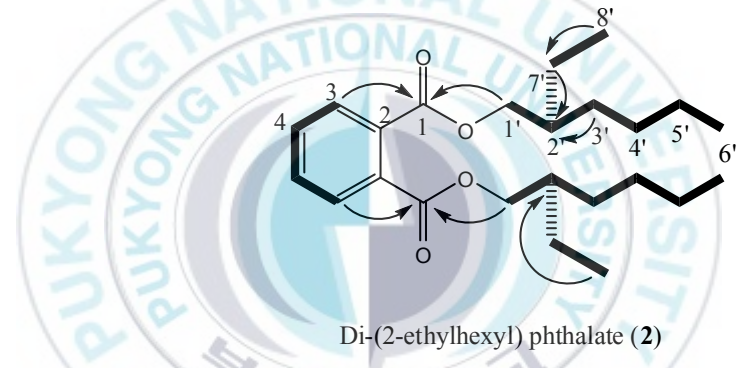
Table 3. NMR data of dibutyl phthalate (**1**) and di-(2-ethylhexyl) phthalate (**2**)

C#	Dibutyl phthalate		Di-(2-ethylhexyl) phthalate	
	δ_C (mult)	δ_H (mult, *J*)	δ_C (mult)	δ_H (mult, *J*)
1	166.4 (s)		167.5 (s)	
2	131.6 (s)		132.3 (s)	
3	127.9 (d)	7.47 (dd, 9.22, 2.19)	128.5 (d)	7.67 (dd, 9.15, 2.56)
4	130.0 (d)	7.25 (dd, 8.78, 2.63)	130.7 (d)	7.47 (dd, 8.78, 2.19)
1’	64.3 (t)	4.06 (t, 6.59)	67.8 (t)	4.20 (t, 5.85)
2’	29.7 (t)	1.45 (tt, 6.59, 8.34)	38.6 (d)	1.65 (m)
3’	18.3 (t)	1.19 (q, 7.47)	30.2 (t)	1.28 (m)
4’	12.7 (q)	0.70 (t, 7.47)	28.7 (t)	1.28 (m)
5’			22.8 (t)	1.28 (m)
6’			13.8 (q)	0.86 (m)
7’			23.6 (t)	1.39 (m)
8’			10.7 (q)	0.90 (m)

Recorded in $CDCl_3$ at 400 MHz (^1H) and 100 MHz (^{13}C)



Dibutyl phthalate (**1**)



Di-(2-ethylhexyl) phthalate (**2**)

Figure 26. Chemical structures of dibutyl phthalate (**1**) and di-(2-ethylhexyl) phthalate (**2**)

6. Cathepsin B inhibitory activities of two isolated phthalates

Both isolated phthalates showed inhibitory activities on cathepsin B. In kinetic studies of the hydrolysis of *Z*-Arg-Arg-MCA into MCA, inhibition against cathepsin B activity was found to be dose dependent. Various concentrations of dibutyl phthalate and di-(2-ethylhexyl) phthalate were tested with IC_{50} of 0.42 mM and 0.38 mM, respectively (Fig. 27). Several cathepsin B inhibitor peptides have been reported and available as commercial products such as E-64, CA030, and leupeptine. However they generally showed poor pharmacokinetic properties (Falgueyret et al., 2001). Moreover the non-peptide inhibitors can be more stable because they are less likely to be degraded by peptidases in cell.

Inhibitory activities of isolated compounds correlated with the carbon number of alkyl groups. A similar correlation has been reported by Ohyama (1977) about the inhibitory activities of phthalate on glucose 6-phosphate dehydrogenase. The inhibition strength depended on the carbon number of the alkyl groups of phthalate esters; the longer the chains of alkyl groups, the more potent the inhibition. They concluded that hydrophobic bonding is of major importance in the affinity of these compounds.

Fig. 28 illustrated the inhibition kinetics of dibutyl phthalate and di-(2-ethylhexyl) phthalate analyzed by Lineweaver-Burk plots. For each plot, three lines obtained from uninhibited enzyme and two different concentrations of one inhibitor. The results indicated that dibutyl phthalate and di-(2-ethylhexyl) phthalate inhibited cathepsin B in a non-competitive fashion. The *Ki* values of dibutyl phthalate and di-(2-ethylhexyl) phthalate were found as 0.64 mM and 0.42 mM, respectively. These values may vary with enzyme concentration and

pre-incubation time. This observation is agreeable with the structural features of our compounds as absence of hydrogen-donors. Cysteine proteases inhibitors reported to date rely on covalent attachment of an electrophilic peptide-derived ligand to the active site thiol to achieve potent enzyme inhibitions (Thurmond et al., 2004). Obviously, the inhibitors are not binding to the same site as substrate, so the inhibition cannot be overcome by raising substrate concentration. Extracellular matrix degradation activity of cathepsin B is unexpected, therefore, such kind of mechanism is advantage for cathepsin B inhibition pattern.

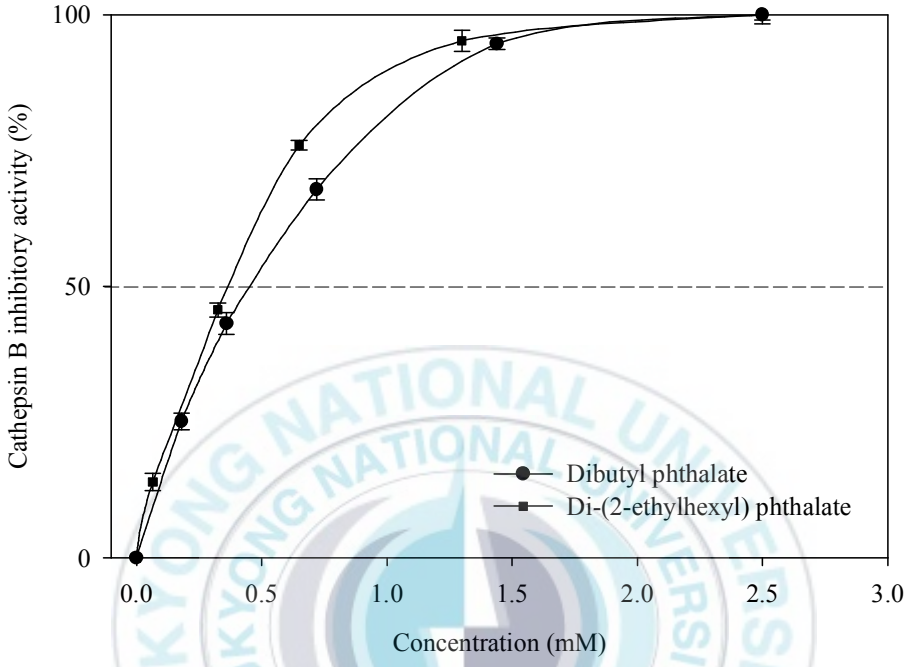


Figure 27. Dose-dependent inhibitions of cathepsin B by dibutyl phthalate and di-(2-ethylhexyl) phthalate. The assays were performed in 96-well plate as described in method section.

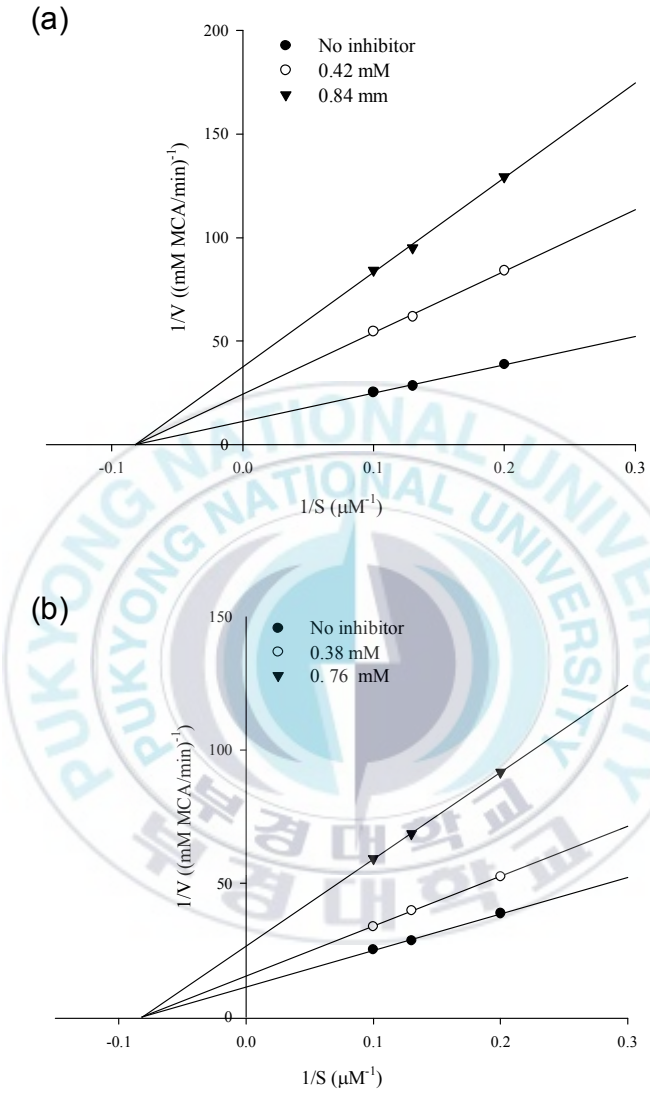


Figure 28. Lineweaver-Burk plots of cathepsin B in the presence of phthalates. (a) Concentration of dibutyl phthalate: (●), 0 mM; (O), 0.42 mM; (▲), 0.84 mM. (b) Concentration of di-(2-ethylhexyl) phthalate: (●), 0 mM; (O), 0.38 mM; (▲), 0.76 mM. S: Z-Arg-Arg-MCA concentration; V: MCA release.

7. Effects of two isolated phthalates on cell viability

The amount of cathepsin B released varies greatly with cell types and culture conditions. It has been shown to be increased response to malignant progression in melanoma B16 cells. Before carrying the assay, the cytotoxic effect of dibutyl phthalate and di-(2-ethylhexyl) phthalate were investigated (Fig. 29). MTT is a tetrazolium salt that is oxidized by mitochondrial dehydrogenases in living cells to give a dark blue formazan product. Damaged or dead cells show reduced or no dehydrogenase activity. At a concentration of 1000 μM, the low cytotoxicities of dibutyl phthalate and di-(2-ethylhexyl) phthalate on B16-F10 were 9.45% and 7.34%, respectively. Since the difference between treated and control group were not too significant, our compounds could be used for next cellular assay.

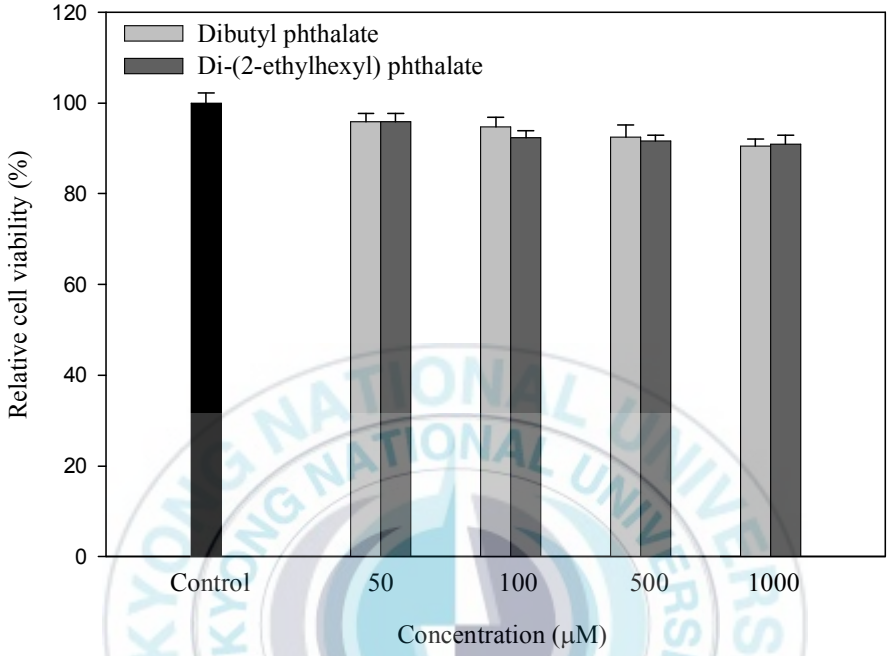


Figure 29. Dose-dependent effects of cathepsin B by dibutyl phthalate and di-(2-ethylhexyl) phthalate on B16-F10 cells. Cells were treated with different concentrations of inhibitors. Control groups: the groups that were not treated samples.

8. Cathepsin B inhibitory activities of two isolated phthalates on metastatic potential cell

The effects of two isolated phthalates as cathepsin B inhibitors were evaluated with two models extracellular and intracellular B16-F10 cells. Depending on the amount of active cathepsin B, fluorescence conjugated substrates are differentially hydrolyzed. Metastatic B16-F10 cells release not only cathepsin B but also other cysteine proteases, such as cathepsin L. In order to prevent other proteases activities, specific substrate for cathepsin B was used. Pericellular cathepsin B activity was inhibited by both dibutyl phthalate and di-(2-ethylhexyl) phthalate with potency similar to that observed against purified cathepsin B, with IC_{50} values of 0.23 and 0.14 mM, respectively (Fig. 30).

In cell lysis assay, obviously cathepsin B expression in intracellular is higher than in extracellular milieu. The cathepsin B inhibitory activities of dibutyl phthalate and di-(2-ethylhexyl) phthalate at the concentration 100 μM were 32.86 and 42.14%, respectively (Fig. 31). Since the enzyme inhibition depends on many factors, such as enzyme concentration, incubation time, or some other unknown mechanisms related to cell. Hence it is simply impossible to compare equivalently the inhibitory activities of phthalates in different experimental models. Therefore we concluded that dibutyl phthalate and di-(2-ethylhexyl) phthalate are also possible to effect on intracellular cathepsin B activity.

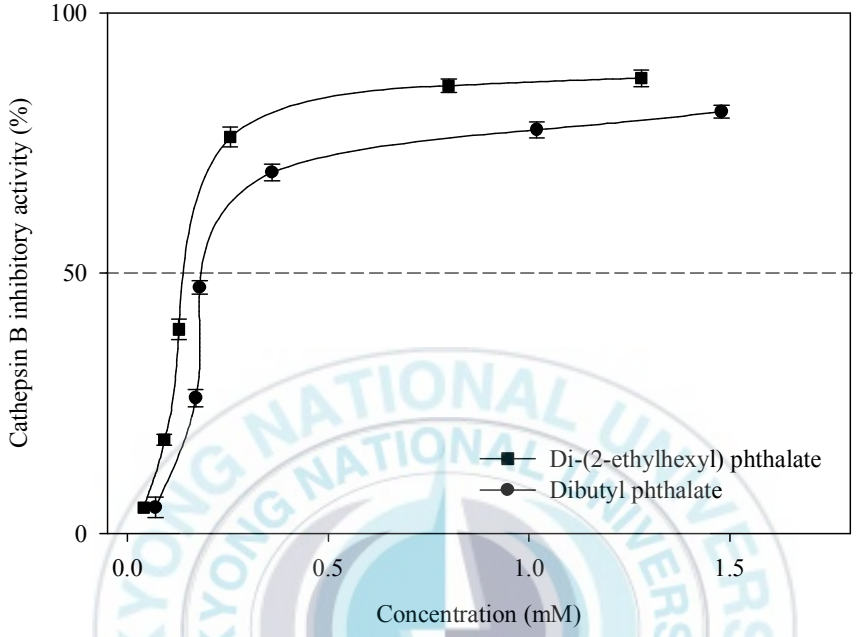


Figure 30. Dose dependent inhibitions of dibutyl phthalate and di-(2-ethylhexyl) phthalate on pericellular cathepsin B. The assays were performed in 96-well plate as described in method section.

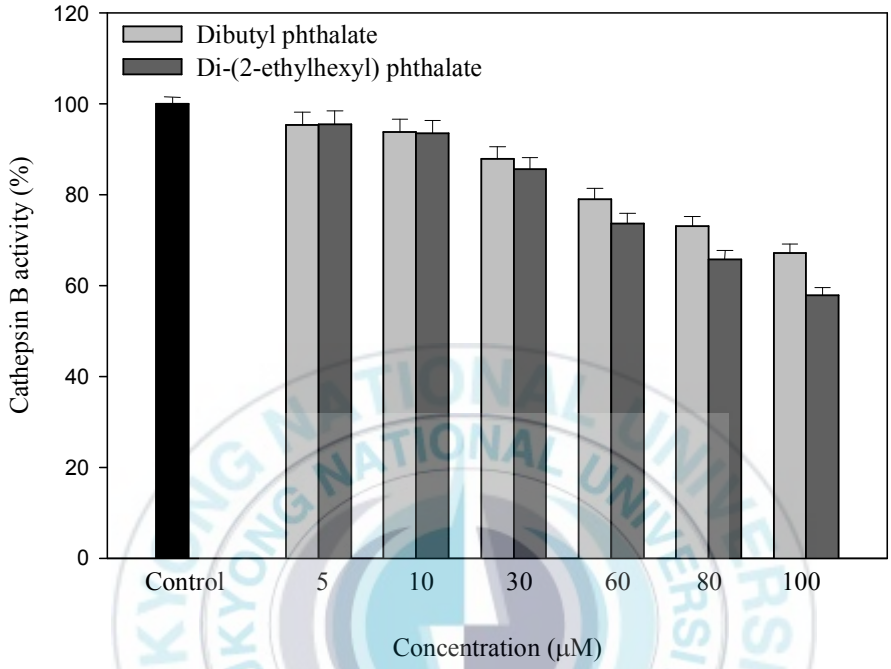


Figure 31. Effects of dibutyl phthalate and di-(2-ethylhexyl) phthalate on intracellular cathepsin B activity. The assays were performed in 96-well plate as described in method section.

Conclusion

Pseudomonas sp. PB01 (GenBank accession no. EU126129) was selected as the best strain through the preliminary screening of cathepsin B inhibitors among 300 marine microorganisms. The enzyme inhibitor production was investigated under various culture conditions. Additionally kinetic study revealed that inhibitor production paralleled with cell growth. The ethyl acetate extract of microorganism culture broth was purified by silica gel column chromatography and HPLC successively. Structural characterizations of purified compounds were determined by EIMS, IR, 1D, and 2D NMR which revealed that our compounds are dibutyl phthalate and di-(2-ethylhexyl) phthalate. Both of them were dose-dependent cathepsin B inhibitions with IC_{50} of 0.42 and 0.38 mM, respectively. Kinetic analyses showed that dibutyl phthalate and di-(2-ethylhexyl) phthalate acted in noncompetitive manners, which were agreeable with their characteristics. Furthermore, both of them caused inactivation of the pericellular and intracellular cathepsin B of murine melanoma cell with no acute cytotoxicity. The discovery of small non-peptide compounds that exhibit inhibitory effects on cathepsin B without relying upon covalent attachment to the active site thiol represents new candidates for the development of small-molecule therapeutics inhibiting this important proteinase target.

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