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TMC-86A, B and TMC-96, New Proteasome Inhibitors from *Streptomyces* sp. TC 1084 and *Saccharothrix* sp. TC 1094

I. Taxonomy, Fermentation, Isolation, and Biological Activities

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TMC-86A, B and TMC-96, new 20S proteasome inhibitors with an epoxy-β-aminoketone moiety, were isolated from the fermentation broth of *Streptomyces* sp. TC 1084 and *Saccharothrix* sp. TC 1094, respectively. TMC-86A, B and TMC-96 inhibited the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities of 20S proteasome with the following IC_{50} values: TMC-86A, 5.1 μM and 3.7 μM; TMC-86B, 1.1 μM and 31 μM; TMC-96, 2.9 μM and 3.5 μM, respectively. TMC-86A, B and TMC-96 exhibited the weak inhibitory activity against the trypsin-like activity of 20S proteasome with IC_{50} values of 51 μM, 250 μM, and 36 μM, respectively. They did not inhibit m-calpain, cathepsin L, and trypsin at 100 μM, suggesting their high specificity for proteasome. Taxonomy of the producing strains is also described.

Proteasomal proteolysis is a major extralysosomal proteolytic system in eucaryotes. This proteolysis is involved in the degradation of many important cellular proteins such as oncogene products, cyclins, and transcriptional factors[1]. The catalytic core of proteasome is 20S proteasome, whose structure resembles a cylinder of four stacked rings, each ring being composed of seven subunits, $\alpha_7\beta_7\beta_7\alpha_7$[1]. The 20S proteasome has *N*-terminal threonine residues of the β-subunits as its active sites. It has at least three distinct peptidase activities, cleaving peptide bonds on carboxyl side of hydrophobic, basic, and acidic amino acids, designated as chymotrypsine-like (ChT-L), trypsin-like (T-L), and peptidylglutamylpeptide hydrolyzing (PGPH) activities[1,2].

Recently, proteasomal proteolysis has been shown to play critical roles in the activation of NF-κB, which has been observed in a variety of inflammatory diseases[3]. Proteasomal proteolysis has also been implied to contribute to the muscle wasting seen in many pathological states including cancer cachexia, diabetes, and sepsis[4]. Thus, 20S proteasome inhibitors might have utilities in the treatment of inflammatory diseases and pathological muscle wasting.

In the course of our screening for 20S proteasome inhibitors, we found TMC-86A, B and TMC-96 (Fig. 1), new members of the epoxy-β-aminoketone group, from the fermentation broth of actinomycete strains. In this paper, we describe the taxonomy of producing strains, fermentation, isolation, and biological activities of TMC-86A, B and TMC-96. The physico-chemical properties and structure elucidation of TMC-86A, B and TMC-96 will be reported in another paper[5].

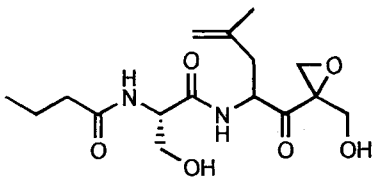
Results

Taxonomy

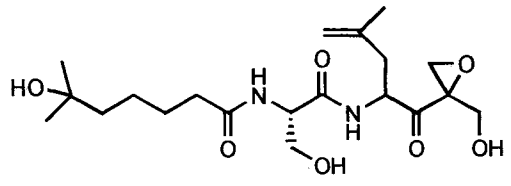
TMC-86A, B Producing Strain

The cultural characteristics of the strain TC 1084 are summarized in Table 1. The substrate mycelia developed well and were irregularly branched. Each spore chain, which was spiral type, had 10 to 50 or more spores per chain. The spores were cylindrical, with size of 0.5~0.7× 0.7~0.9 μm, and the surface was rugose (Fig. 2A). Fragmentation of substrate mycelia, sporangia, or motile

Fig. 1. Structures of TMC-86A, B, TMC-96, and eponemycin.



TMC-86A



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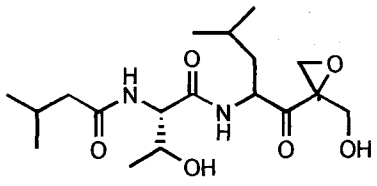
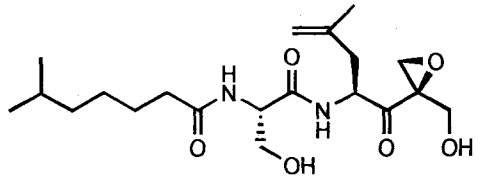


TMC-96



Eponemycin

Table 1. Cultural characteristics of strains TC 1084 and TC1094.

Medium		TC 1084	TC1094
Yeast extract-malt extract agar (ISP No.2)	G:	Good	Good
	AM:	Moderate, Light gray (0-18-0)*	Abundant, Pale orange (4-19-2)
	R:	Pale yellowish brown (6-17-3)	Pale brown (5-17-3)
	S:	None	Pinkish white (1-19-1)
Oatmeal agar (ISP No.3)	G:	Good	Good
	AM:	Abundant, Grayish black (0-11-0)	Abundant, Pale orange(5-19-2)
	R:	Pale yellowish brown (6-17-2)	Pale orange (5-19-3)
	S:	None	Pinkish white (1-19-1)
Inorganic salts-starch agar (ISP No.4)	G:	Good	Poor
	AM:	Abundant, Light gray (0-18-0)	Poor, Brownish white(5-19-1)
	R:	Pale yellowish brown (6-18-2)	Pale orange (5-19-2)
	S:	None	None
Glycerol-asparagine agar (ISP No.5)	G:	Good	Moderate
	AM:	Moderate, Gray (0-14-0)	Moderate, Pale orange (5-19-2)
	R:	Pale yellowish brown (6-17-3)	Dull orange (5-18-4)
	S:	None	None
Peptone-yeast extract iron agar (ISP No.6)	G:	None	Poor
	AM:	None	None
	R:	None	Dull orange (5-17-4)
	S:	None	None
Tyrosine agar (ISP No.7)	G:	Good	Good
	AM:	Abundant, Gray (0-14-0)	Abundant, Pale orange (5-19-3)
	R:	Grayish yellow brown (6-16-2)	Dull orange (5-17-4)
	S:	None	Pale yellow orange (6-19-2)

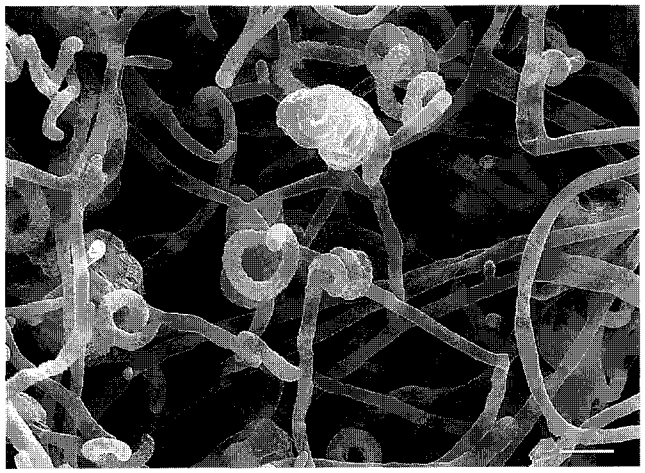
* Color codes from the Guide to Color Standard

Abbreviation: G, Growth; AM, Aerial mycelium; R, Reverse side color; S, Soluble pigment

Fig. 2. Scanning electron micrographs of producing strains, *Streptomyces* sp. TC 1084 and *Saccharothrix* sp. TC 1094, grown on yeast extract - malt extract agar for 10 days at 27°C.

Bar represents 2 μm.

A. *Streptomyces* sp. TC 1084



B. *Saccharothrix* sp. TC 1094

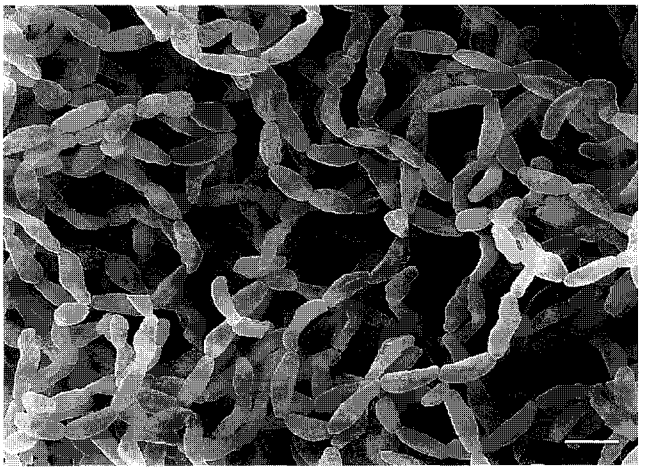


Table 2. Physiological properties of strains TC 1084 and TC1094.

Characteristic	TC 1084	TC 1094
Temperature range for growth (ISP No. 2)	25~32°C	25~37°C
Optimum temperature for growth (ISP No. 2)	27~30°C	25~32°C
Formation of melanoid pigment		
ISP No.6	−	−
ISP No.7	−	−
Liquefaction of gelatin	−	+
Coagulation of milk	−	−
Peptonization of milk	−	−
Hydrolysis of starch	+	+
Decomposition of cellulose	−	−
Reduction of nitrate	−	−
NaCl tolerance (ISP No. 2)	2%	2%
Utilization of carbon source		
L-Arabinose	+	+
D-Fructose	−	+
D-Glucose	+	+
Inositol	+	−
D-Mannitol	+	+
Raffinose	+	+
L-Rhamnose	+	+
Sucrose	−	+
D-Xylose	+	+

+, Positive; −, negative.

spore were not observed.

The physiological properties and the utilization of carbon sources are summarized in Table 2. Analysis of the whole-cell hydrolysates showed the presence of LL-diaminopimelic acid, indicating that the cell wall belongs to type I. On the basis of these morphological and chemotaxonomic characteristics, strain TC 1084 was assigned to the genus *Streptomyces*.

TMC-96 Producing Strain

The cultural characteristics of the strain TC 1094 are summarized in Table 1. The substrate mycelia developed well and were irregularly branched, sometimes showing a zigzag appearance on glycerol-asparagine agar. Aerial mycelia were well developed, long, straight to wavy, and branched, which then divided into a number of smooth surface spindle shaped spores, 0.6~1.1×1.8~2.6 μm (Fig. 2B). Sporangium or motile spores were not observed.

The physiological properties and the utilization of carbon sources are summarized in Table 2. The utilization of carbon sources was tested by growth on carbon utilization medium (ISP No. 9) supplemented with 0.01% yeast extract. Analysis of the whole-cell hydrolysates showed the presence of *meso*-diaminopimelic acid and contained galactose, mannose, rhamnose and ribose. This indicates that the cell wall of this strain belongs to type III, and the whole cell sugar pattern to type C. On the basis of these morphological and chemotaxonomic characteristics, strain TC 1094 was assigned to the genus *Saccharothrix*.

Fermentation

Time courses of the fermentation are shown in Fig. 3. The activity was found after 48 hours of cultivation and reached maximum after 72 hours (TC 1084) (Fig. 3A) or 96 hours (TC 1094) (Fig. 3B) fermentation.

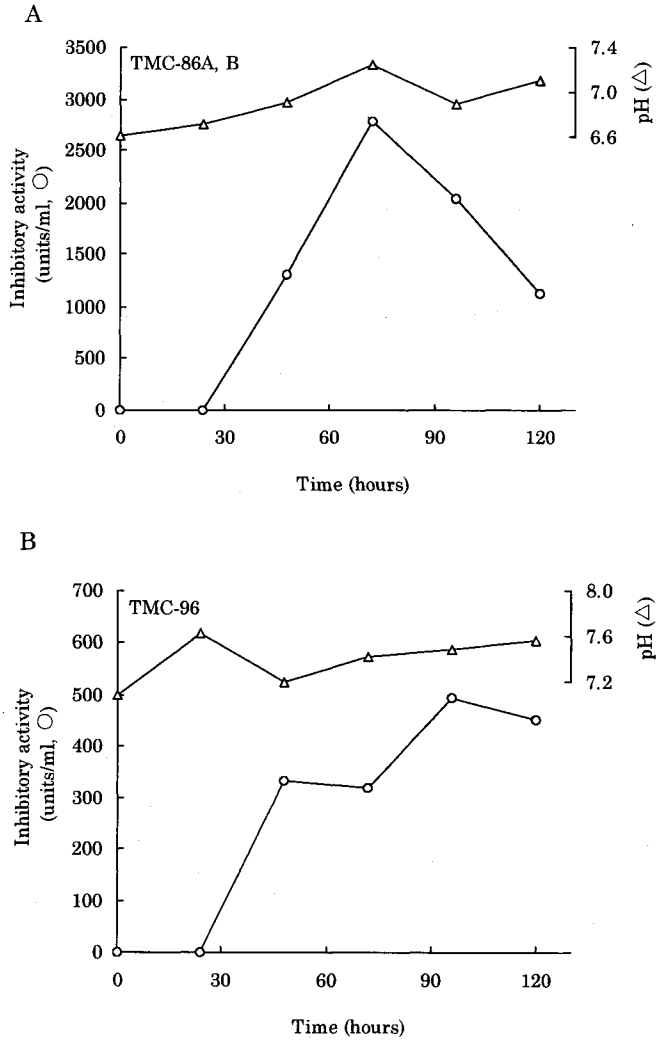
Isolation

After solvent partition and column chromatography, the inhibitory activity contained in each fraction was assessed by the enzyme assay.

TMC-86A, B

The fermentation broth (31 liters) of strain TC 1084 was extracted with 1-butanol (15 liters). The extract was concentrated and subjected to a silica gel column chromatography developed with a mixture of dichloromethane - methanol (92 : 8). Evaporation of the eluate gave two fractions containing semi-pure TMC-86A (1.21 g) and B (935 mg). The fraction containing TMC-86A was purified by a medium-pressure reverse-phase silica gel chromatography (MPLC, YMC ODS A60) with acetonitrile - water (18 : 82). The active fractions were concentrated and rechromatographed on a preparative HPLC (YMC D-ODS-5B) with acetonitrile - water (16 : 84). Evaporation of the eluate gave pure TMC-86A (28.1 mg) as colorless oil. The fraction containing TMC-86B was similarly processed by the MPLC and the HPLC, followed by a Sephadex LH-20 column chromatography with methanol. After concentration of the active fractions, pure TMC-86B (86.5 mg) was obtained as white powder.

Fig. 3. Time course of fermentation of TMC-86s by *Streptomyces* sp. TC 1084 and TMC-96 by *Saccharothrix* sp. TC 1094.



Inhibitory activities were determined as described under Experimental.

TMC-96

The fermentation broth (36 liters) of strain TC 1094 was extracted with 1-butanol (18 liters) and the extract was concentrated to dryness. The extract was suspended in water (200 ml) and extracted twice with ethyl acetate (200 ml). The extract was concentrated and the residual solid was dissolved in 90% aqueous methanol (200 ml), and partitioned against *n*-hexane (200 ml). The aqueous

methanol phase was evaporated to yield a crude solid (14.5 g), which was subjected to a silica gel column chromatography developed with a mixture of *n*-hexane and acetone (2 : 1). Fractions containing TMC-96 were evaporated (2.44 g) and further purified by a MPLC (YMC ODS A60) with acetonitrile - water (10 : 90 and 25 : 75). The active fractions were concentrated and re-chromatographed on a preparative HPLC (YMC D-ODS-5B) with acetonitrile - water (23 : 77), followed by a Sephadex LH-20 column chromatography with dichloromethane - methanol (1 : 1). After concentration of the active fractions, pure TMC-96 was obtained as colorless stickly solid (111 mg).

Biological Activities

As shown in Table 3, TMC-86A, B and TMC-96 inhibited the ChT-L, T-L, and PGPH activities of 20S proteasome with the following IC_{50} values: for TMC-86A, 5.1 μM, 51 μM, and 3.7 μM; for TMC-86B, 1.1 μM, 250 μM, and 31 μM; and for TMC-96, 2.9 μM, 36 μM, and 3.5 μM, respectively. *N*-Acetyl-Leu-Leu-nLeu-CHO (ALLN), a known proteasome inhibitor, inhibited the ChT-L, T-L, and PGPH activities of 20S proteasome with IC_{50} values of 6.6 μM, 6.0 μM, and 21 μM, respectively. TMC-86A, B and TMC-96 did not inhibit m-calpain, cathepsin L, and trypsin at 100 μM.

Table 3. Inhibitory effects of TMC-86A, B, TMC-96, ALLN, and eponemycin on the ChT-L, T-L, and PGPH activities of 20S proteasome in the presence of 0.02% SDS.

Compound	IC_{50} (μM)		
	ChT-L	T-L	PGPH
TMC-86A	5.1	51	3.7
TMC-86B	1.1	250	31
TMC-96	2.9	36	3.5
ALLN	6.6	6.0	21
Eponemycin	1.1	7.4	5.4

The structures of TMC-86A, B and TMC-96 are similar to that of eponemycin[6], an anti-angiogenic compound. This similarity led us to study the effects of eponemycin on 20S proteasome. Eponemycin was found to inhibit the ChT-L, T-L, and PGPH activities of 20S proteasome with IC_{50} values of 1.1 μM, 7.4 μM, and 5.4 μM, respectively.

The cytotoxic activities of TMC-86A, B and TMC-96 against several tumor cell lines are summarized in Table 4. All compounds showed strong cytotoxicity for the various tumor cell lines tested.

Discussion

In this study, we isolated TMC-86A, B and TMC-96, new members of the epoxy-β-aminoketone group, as inhibitors of 20S proteasome from *Streptomyces* sp. TC 1084 and *Saccharothrix* sp. TC 1094, respectively. TMC-86A and TMC-96 inhibited the ChT-L and PGPH activities of 20S proteasome with IC_{50} values of 2.9~5.1 μM. TMC-86B also inhibited the ChT-L activity of 20S proteasome with IC_{50} values of 1.1 μM. TMC-86A, B and TMC-96 did

Table 4. Cytotoxicities of TMC-86A, B and TMC-96 against tumor cells *in vitro*.

Cell line	IC_{50} (μM)		
	TMC-86A	TMC-86B	TMC-96
HCT-116 human colon carcinoma	0.22	0.21	0.22
HeLa S3 human epitheloid carcinoma	0.23	0.75	0.21
SK-BR-3 human breast adenocarcinoma	0.27	0.65	0.32
WiDr human colon adenocarcinoma	0.25	0.53	0.27
HL-60 human promyelocytic leukemia	0.20	0.43	0.24
B-16 murine melanoma	0.22	0.20	0.20
P388D1 murine lymphoid neoplasm	0.22	0.21	0.22

not inhibit m-calpain, cathepsin L, and trypsin at 100 μM. These results suggested that they were specific 20S proteasome inhibitors.

TMC-86A, B and eponemycin exhibited characteristic inhibitory profiles against the three proteolytic activities of 20S proteasome as depicted by the following rank orders: TMC-86A, ChT-L=PGPH>T-L; TMC-86B, ChT-L> PGPH>T-L; eponemycin, ChT-L≧PGPH=T-L. They share a chemical structure with a 1,2-epoxy-2-hydroxymethyl-4-serylamino-6-methylhept-6-ene-3-one, and differ only in an alkanoyl moiety[6]. The structure of the alkanoyl moiety might be important for determining their inhibitory profiles.

Eponemycin has been reported as an anti-angiogenic compound[6]. Our result on inhibition of eponemycin against 20S proteasome suggested that its anti-angiogenic activity might depend on the inhibition of proteasome. Recently, SIN *et al.* have reported that an eponemycin derivative interacts with two 23~25 kDa proteins in human endothelial cells[7]. Therefore, these 23~25 kDa proteins would be subunits of 20S proteasome.

Proteasome is responsible for the activation of TNF-α as well as the processing of major histocompatibility complex (MHC) class I ligands[3,8]. Proteasome also contributes to the muscle wasting seen in many pathological states[4]. The catalytic core of proteasome is 20S proteasome. Thus, TMC-86A, B and TMC-96 might be useful in the treatment of inflammatory diseases, autoimmune diseases, and pathological muscle wasting.

Experimental

Materials

20S proteasome was purified from THP.1 monocytic cells according to the method described previously[9]. Eponemycin was chemically synthesized as described previously[10]. Other proteinases, substrates, and inhibitors were purchased from the following sources: m-calpain from Nacalai Tesque Inc.; cathepsin L from Calbiochem Corp.; trypsin from Sigma Chemical Co.; Suc-Leu-Leu-Val-Tyr-MCA, *Z*-Phe-Arg-MCA, Bz-Arg-MCA, Boc-Leu-Arg-Arg-MCA, and *Z*-Leu-Leu-Glu-MCA from Peptide Institute Inc.; *N*-Acetyl-Leu-Leu-nLeu-CHO from Boehringer Mannheim Inc.

Taxonomic Studies

Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[11]. Carbohydrate utilization was investigated using the procedure of PRIDHAM and GOTTLIEB[12]. The substrate and aerial mass color was assigned using the Guide to Color Standard, 1954 (Japan Color Research institute). Morphological characteristics were observed with a scanning electron microscope (Hitachi S-4200). 2,6-Diaminopimelic acid in whole-cell hydrolysates was analyzed by the method of BECKER *et al.*[13] as modified by HASEGAWA *et al.*[14] Sugars in the whole-cell hydrolysates were determined by the method of STANECK and ROBERTS[15].

Fermentation

The fermentation medium for the strain TC 1084 was composed of 0.5% glucose, 2.0% glycerol, 2.0% soluble starch, 1.5% wheat germ, 0.2% Polypeptone, 0.2% yeast extract, 0.5% corn steep liquor, and 0.3% $CaCO_3$, and was adjusted at pH 7.0 before autoclaving. The fermentation medium for the strain TC 1094 was composed of 0.5% glucose, 2.0% glycerol, 2.0% dextrin, 1.5% rape-seed meal, 0.5% corn steep liquor, 0.2% Polypeptone, 0.2% yeast extract, and 0.3% $CaCO_3$, and was adjusted at pH 6.5 before autoclaving

The inoculum for strains TC 1084 or TC 1094 was prepared in a 500-ml Erlenmeyer flask containing 70 ml of the respective fermentation medium and incubated for 5 days (TC 1084) or 3 days (TC 1094) at 27°C on a rotary shaker (220 rpm). One milliliter of the seed culture was transferred to a 500-ml Erlenmeyer flask containing 70 ml of the same medium and incubated for 4 days at 27°C on a rotary shaker (220 rpm). Two hundred fifty milliliters of the culture (TC 1084) or five hundred forty milliliters of the culture (TC 1094) was transferred to a 30-liter jar fermentor containing 18 liters of the fermentation medium and 18 g of a deforming agent (CC-438). The fermentation of both strains was carried out at 27°C with agitation of 200 rpm and aeration at 10 liters per minute. Ten milliliters of the fermentation broth was extracted with 1-butanol (5 ml) every 24 hours, and the extract was concentrated to dryness. The inhibitory activity of the extracts was assessed by the enzyme assay. One unit of inhibitory activity was defined as the amount that inhibited the PGPH activity of 20S proteasome by 50%.

Enzyme Assays

The activities of enzymes were measured by monitoring the increase in fluorescence ($EX_{380\,nm}/EM_{460\,nm}$) that accompanies cleavage of 7-amino-4-methylcoumarin (AMC) from substrates[9,16]. One unit of enzyme was defined as the amount of enzyme that catalyzed 1 pmole of AMC formation per minute under the assay conditions.

Incubation and reaction temperature were maintained at 37°C.

20S proteasome: The 190 μl of reaction mixture contained 10 units of 20S proteasome, 100 mM Tris buffer (pH 8.0), 1 mM dithiothreitol, 0.02% SDS, and the sample to be tested, and was incubated for 5 minutes. The reaction was initiated by addition of 10 μl of substrate (2 mM in DMSO), and then monitored for 15 minutes. The ChT-L, T-L, and PGPH activities of 20S proteasome were measured by using Suc-Leu-Leu-Val-Tyr-MCA, Boc-Leu-Arg-Arg-MCA, and *Z*-Leu-Leu-Glu-MCA, respectively.

m-Calpain: The 190 μl of reaction mixture contained 10 units of m-calpain, 200 μM Suc-Leu-Leu-Val-Tyr-MCA, 100 mM Tris buffer (pH 7.4), 5 mM 2-mercaptoethanol, and the sample to be tested, and was incubated for 5 minutes. The reaction was initiated by addition of 10 μl of $CaCl_2$ (35 mM), and then monitored for 5 minutes.

Cathepsin L: The 190 μl of reaction mixture contained 10 units of cathepsin L, 100 mM acetate buffer (pH 5.5), 1 mM EDTA, 8 mM cysteine, and the sample to be tested, and was incubated for 5 minutes. The reaction was initiated by addition of 10 μl of *Z*-Phe-Arg-MCA (2 mM in DMSO), and then monitored for 15 minutes.

Trypsin: The 190 μl of reaction mixture contained 10 units of trypsin, 100 mM Tris buffer (pH 7.4), and the sample to be tested, and was incubated for 5 minutes. The reaction was initiated by addition of 10 μl of Bz-Arg-MCA (2 mM in DMSO), and then monitored for 15 minutes.

Cytotoxic Assays

Cytotoxic activities against various cell types were evaluated according to the methods described previously[17]. Briefly, cells were incubated with a test sample at 37°C for 72 hours in following medium; HCT-116 and SK-BR-3: complete McCoy's 5A supplemented with 10% fetal bovine serum, HeLa S3 and B-16: complete MEM-E supplemented with 10 % fetal bovine serum, WiDr: complete MEM-E supplemented with 1% essential amino acid solution and 10% fetal bovine serum, HL-60: complete RPMI-1640 supplemented with 20% fetal bovine serum, P388D1: complete PRMI-1640 supplemented with 5% fetal bovine serum. Their viability was determined by the tetrazolium or neutral red assay method and IC_{50} values were calculated.

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