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TMC-95A, B, C, and D, Novel Proteasome Inhibitors Produced by *Apiospora montagnei* Sacc. TC 1093

Taxonomy, Production, Isolation, and Biological Activities

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In our course of screening for novel proteasome inhibitors, TMC-95A and its diastereomers, TMC-95B to D, were isolated from the fermentation broth of *Apiospora montagnei* Sacc. TC 1093. TMC-95A inhibited the chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl-peptide hydrolyzing (PGPH) activities of 20S proteasome with IC_{50} values of 5.4 nM, 200 nM, and 60 nM, respectively. TMC-95B inhibited these activities to the same extent as TMC-95A, while the inhibitory activities of TMC-95C and D were 20 to 150 times weaker than that of TMC-95A and B. TMC-95A did not inhibit m-calpain, cathepsin L, and trypsin at 30 μM, suggesting its high selectivity for proteasome. Taxonomy of the producing strain is also described.

All eukaryotic cells contain 20S proteasome, which is a large cylindrically shaped complex composed of a stack of four rings, each containing seven subunits, $\alpha_7\beta_7\beta_7\alpha_7$[1]. The 20S proteasome is a nucleophile hydrolase possessing active sites of *N*-terminal threonine residues of the β-subunits, and shows at least three distinct peptidase activities, cleaving peptide bonds on carboxyl side of hydrophobic, basic, and acidic amino acids, *i.e.* chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl-peptide hydrolyzing (PGPH) activities[1,2].

The 20S proteasome constitutes the catalytic core of 26S proteasome, which degrades ubiquitinated proteins in ATP dependent process. The ubiquitin-proteasome pathway is responsible for regulated degradation of many important cellular proteins including G1 cyclins, c-Fos, and p53[1]. This pathway also plays important roles in the activation of NF-κB as well as the processing of histocompatibility complex (MHC) class I ligands[3,4].

In a past decade, several proteasome inhibitors such as lactacystin, 3,4-dichloroisocoumarin, and substrate-related peptides have been discovered, and have contributed substantially to deciphering the functions of proteasome[4~6]. New types of proteasome inhibitor would facilitate the study of proteasome.

Recently, S. MEYER *et al.* have reported that anti-inflammatory properties of cyclosporin A might, at least in part, be due to inhibition of 20 proteasome and consequent suppression of NF-κB activation[7]. The suppressive effect of 20S proteasome inhibitors on the generation of peptides presented on MHC class I molecule has also been reported[4]. Thus, 20S proteasome might be a therapeutic target for inflammatory and autoimmune diseases.

As a result of screening for 20S proteasome inhibitors, we have found several compounds. We have reported on new members of epoxy-β-aminoketone group, TMC-86A, B and TMC-96[8,9]. Here we report on novel cyclic peptides, TMC-95A and its diastereomers, TMC-95B to D (Fig. 1). In this paper, we describe the taxonomy of producing strain, production, isolation, and biological activities of TMC-95s. The physico-chemical properties and structure elucidation of TMC-95s will be reported in another paper[10].

Results

Taxonomy

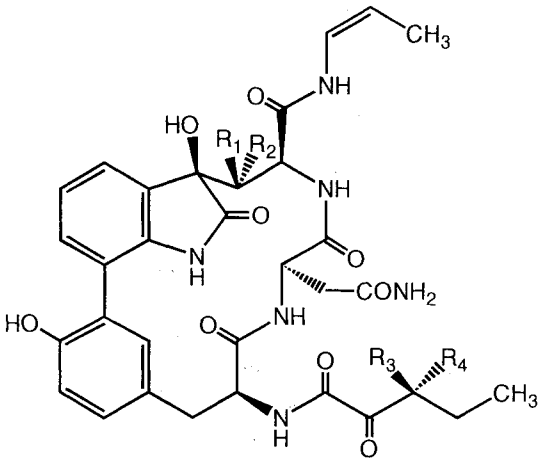
The producing strain TC 1093 was isolated from a soil sample from a bamboo forest in Kanagawa, Japan.

Colonies of the strain TC 1093 on LCA spread broadly and thinly, reaching 70 mm in diameter after 7 days at 25°C. Conidiation was induced under day light, affecting the colony color, brownish olive (Munsell 5Y5/4, tawny olive). Reverse was uncolored to pale brown. No diffusible pigment was observed. Colonies on oatmeal agar grew rapidly, reaching 70 mm in diameter after 7 days at 25°C. Surface was floccose to funiculose, showing medium gray (Munsell N6/0, battleship gray) when sporulated. Reverse was yellowish gray (Munsell 5Y7/2, oyster).

Conidiophore mother cells were flask-shaped, 5~9.5×2.5~4.0 μm, producing a conidiophore at the apex, 1~2 μm in width (Fig. 2). Conidia, basauxic, were born densely along the conidiophore, typically lenticular and dark brown with an equatorial hyaline band of germ slit, 6.0~7.5 μm (average 6.5~7.0 μm, SD 0.42) in diameter, with 4.5~5.5 μm in thickness (Fig. 2). A typical ellipsoidal, fusiform, or clavate conidia were also present, 7.0~13.5×4.5~6.5 μm.

Characteristic dark lenticular conidia with a germ slit at the equatorial ridge readily led us to place the producing strain in the genus *Arthrinium*. Based on the shape and size of conidia, and conidiophore morphology, TC 1093 was closely related to *Arthrinium* state of *Apiospora montagnei*. Although *Apiospora montagnei* has narrower conidiophores of 0.5 μm in width, the other important characteristics such as size of conidia and hyaline septa agreed well with those of TC 1093. The strain was therefore identified as *Arthrinium* state of *Apiospora montagnei* Sacc.

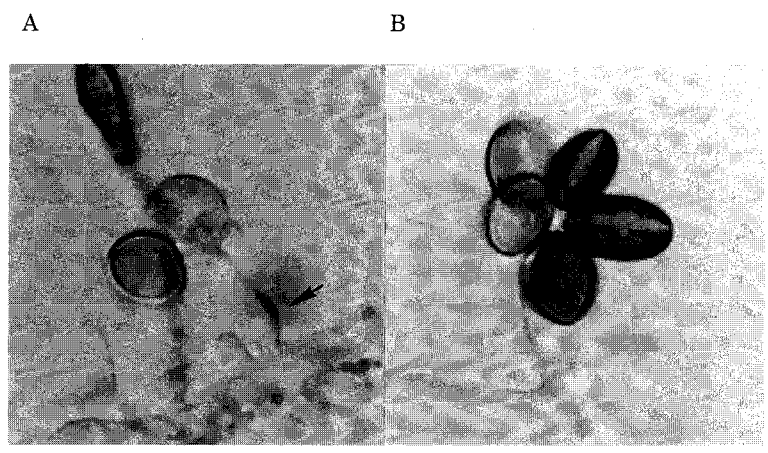
Fig. 1. Structures of TMC-95A to D.



	R_1	R_2	R_3	R_4
TMC-95A	H	OH	CH_3	H
TMC-95B	H	OH	H	CH_3
TMC-95C	OH	H	CH_3	H
TMC-95D	OH	H	H	CH_3

Fig. 2. Photomicrographs of producing strain, *Apiospora montagnei* Sacc. TC 1093.

Conidial structures of *Arthrinium* state.



A, conidiophore (arrow): B, lenticular conidia with germ slit

Bar represents 10 μm.

Table 1. Inhibitory activities of TMC-95A to D and ALLN against ChT-L, T-L, and PGPH activities of 20S proteasome.

Compound	In the presence or absence of 0.02 % SDS	IC_{50} (μM)		
		ChT-L	T-L	PGPH
TMC-95A	+ SDS	0.0054	0.20	0.060
	- SDS	0.012	1.5	6.7
TMC-95B	+ SDS	0.0087	0.49	0.060
TMC-95C	+ SDS	0.36	14	8.7
TMC-95D	+ SDS	0.27	9.3	3.3
ALLN	+ SDS	6.6	6.0	21

Isolation

The fermentation broth (29.5 liters) of *Apiospora montagnei* Sacc. TC 1093 was diluted with water (17.5 liters) and centrifuged to separate the mycelium. The broth was then applied to a Diaion HP-20 column (0.8 liters, Mitsubishi Chemical Industries). The column was washed with 10% aqueous acetone (3 liters) and developed with 60% aqueous acetone (3 liters). The fraction containing TMC-95s was extracted twice with 1-butanol (0.45 liters) after removing acetone under reduced pressure. Crude solid (2.76 g) containing TMC-95s was precipitated from the extract by addition of *n*-hexane. The crude solid was then applied to a silica gel column chromatography (Wakogel C-200, Wako Pure Chemical industries) and eluted with dichloromethane - methanol (9 : 1). The fractions containing TMC-95s (438 mg) were concentrated and further purified by a reversed phase preparative HPLC (column: YMC D-ODS-5-B, 30×250 mm) developed with 28% aqueous acetonitrile (flow rate: 25 ml/minute) to yield TMC-95A (87.8 mg, Rt: 30~32 minutes), TMC-95B (19.4 mg, Rt: 28~30 minutes), TMC-95C (11.3 mg, Rt: 36~38 minutes), and TMC-95D (4.2 mg, Rt: 34~35 minutes) as white powder, respectively.

Biological Activities

The inhibitory activities of TMC-95s against the 20S proteasome were assessed in the presence of 0.02% SDS (Table 1). SDS has been reported to activate 20S proteasome[2,11]. TMC-95A inhibited the ChT-L, T-L, and PGPH activities of 20S proteasome with IC_{50} values of 5.4 nM, 200 nM, and 60 nM, respectively. TMC-95B inhibited each of these activities to the same extent as TMC-95A, while the inhibitory activities of TMC-95C and D were 20 to 150 times weaker than that of TMC-95A and B. *N*-Acetyl-Leu-Leu-nLeu-CHO (ALLN), a known proteasome inhibitor, inhibited the ChT-L, T-L, and PGPH activities with IC_{50} values of 6.6 μM, 6.0 μM, and 21 μM, respectively, in this assay system. In the absence of SDS, TMC-95A showed weaker inhibitory activities than in the presence of 0.02% SDS, especially against the PGPH activity (Table 1). TMC-95A did not inhibit m-calpain, cathepsin L, and trypsin at 30 μM.

Fig. 3 shows the double reciprocal Lineweaver-Burk plot for the ChT-L activity of 20S proteasome in the presence or absence of TMC-95A. The appearance of this plot was characteristic of a competitive inhibitor. *Km* and *Ki* values were determined to be 42 μM and 2.3 nM, respectively. In this assay system, ALLN also showed characteristic appearance of a competitive inhibitor with *Ki* value of 6.5 μM (data not shown).

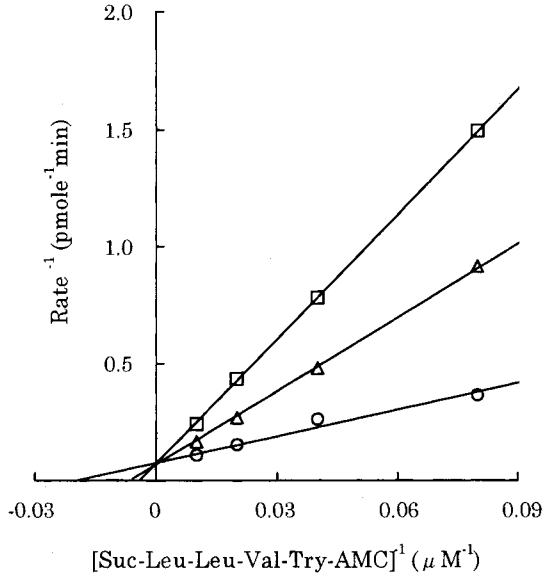
TMC-95A showed cytotoxic activities against HCT-116 human colon carcinoma cells and HL-60 human promyelocytic leukemia cells with IC_{50} values of 4.4 μM and 9.8 μM, respectively.

Discussion

In this study, TMC-95A and its diastereomers, TMC-95B to D, were discovered from the fermentation broth of *Apiospora montagnei* Sacc. TC 1093 as novel proteasome inhibitors. TMC-95A inhibited the ChT-L, T-L, and PGPH activities of 20S proteasome much stronger than ALLN. TMC-95A did not inhibit m-calpain, cathepsin L, and

Fig. 3. Double reciprocal Lineweaver-Burk plot for the ChT-L activity of 20S proteasome in the presence or absence of TMC-95A.

None (○), 5 nM TMC-95A (△), 10 nM TMC-95A (□).



Assay was carried out in the presence of 0.02% SDS. *Km* and *Ki* values were determined to be 42 μM and 2.3 nM, respectively.

trypsin at 30 μM. These results suggested that TMC-95A is a potent and specific 20S proteasome inhibitor. TMC-95A showed characteristic appearance of a competitive inhibitor with *Ki* value of 2.3 nM when analyzed by the double reciprocal Lineweaver-Burk plot for the ChT-L activity. However, this inhibitory mechanism and *Ki* value might be apparent, because the 20S proteasome is an enzyme with more than one active site[12].

TMC-95A and B inhibited 20S proteasome to a similar extent, while TMC-95C and D did much weaker than TMC-95 A nd B. Considering that TMC-95 C and D are easily converted into TMC-95A and B, respectively, in solution, the inhibitory activities of TMC-95C and D might be due to the contamination of TMC-95A and B. TMC-95A and B differ from TMC-95C and D in the stereochemistry at C-7, respectively. The stereochemistry at C-7 would be essential for their inhibitory activities.

TMC-95A is a specific proteasome inhibitor and does not share common structure with the previously reported proteasome inhibitors, lactacystin[13], 3,4-dichloro-isocoumarin, and substrate-related peptidyl inhibitors. Thus, TMC-95A would be a valuable tool to further the understanding of proteasomes.

20S proteasome constitutes the catalytic core of proteasome, which plays important roles in the activation of NF-κB and the processing of MHC class I ligands[3,4]. Therefore, TMC-95A and B are expected to ameliorate inflammatory and autoimmune diseases. Recently, it has been implied that the activation of ubiquitin-proteasome pathway and subsequent protein breakdown might be the major cause of rapid muscle wasting seen in many pathological states including cancer cachexia, diabetes, and sepsis[14]. Accordingly, TMC-95A and B might be useful in the treatment of muscle wasting in the pathological states.

Experimental

Materials

20S proteasome was isolated from THP.1 monocytic cells according to the method published previously[11]. Other enzymes, substrates and inhibitors were from commercial sources[8].

Taxonomic Studies

The strain TC 1093 was inoculated on Miura medium (LCA) and oatmeal agar, and incubated at 25°C for 7 days under fluorescent light. Color of colonies was described using Munsell system[15]. Taxonomic studies were based on ELLIS[16] and DOMSCH *et al.*[17]

Production of TMC-95s

Apiospora montagnei Sacc. TC 1093 was inoculated into three 500-ml Erlenmeyer flasks, each containing 70 ml of a medium composed of 1.0% glucose, 2.5% lactose, 1.0% wheat germ, 0.5% corn steep liquor, 0.5% NaCl, and 0.4% $CaCO_3$, adjusted at pH 6.5 before autoclaving. The inoculated flasks were incubated for 5 days at 27°C on a rotary shaker (220 rpm). The seed culture was transferred to a 30-liter jar fermentor containing 18 liters of a medium composed of 1.0% glucose, 5.0% dextrin, 1.0% wheat germ, 0.5% corn steep liquor, 0.5% NaCl, 0.4% $CaCO_3$, 0.1% deforming agent (CC-438; NIPPON OIL & FAT CO., LTD.), adjusted at pH 6.5 before autoclaving. The fermentation was carried out for 5 days at 27°C.

Enzyme Assays

The activities of enzymes were measured by using fluorescence substrates according to the method reported previously[8]. Briefly, the sample to be tested and enzyme were incubated for 5 minutes in reaction buffer. Reaction was initiated by addition of substrate except m-calpain. The reaction of m-calpain was initiated by addition of $CaCl_2$.

The progress of reaction was monitored fluorometrically ($EX_{380\,nm}/EM_{460\,nm}$). The following substrates were used for measuring the activities of enzymes indicated: Suc-Leu-Leu-Val-Tyr-MCA for m-calpain and the ChT-L activity of 20S proteasome, Boc-Leu-Arg-Arg-MCA for the T-L activity of 20S proteasome, Z-Leu-Leu-Glu-MCA for the PGPH activity of 20S proteasome, Z-Phe-Arg-MCA for cathepsin L, and Bz-Arg-MCA for trypsin.

Cytotoxic Assays

Cytotoxic activities were evaluated according to the methods described previously[18]. Briefly, cells were incubated with a test sample at 37°C for 72 hours in culture medium, and their viability was determined by the tetrazolium or neutral red assay method.

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