TMC-52A to D, Novel Cysteine Proteinase Inhibitors, Produced by *Gliocladium* sp.

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New cysteine proteinase inhibitors, TMC-52A, B, C, and D, were isolated from the fungal fermentation broth. On the basis of a taxonomical study, the producing strain, F-2665, was characterized as *Gliocladium* sp. Spectroscopic analyses and chemical degradation have shown TMC-52A to D to be epoxysuccinyl peptides. TMC-52A to D strongly inhibited cysteine proteinases, in paticular, cathepsin L with IC_{50} values of 13 nM, 10 nM, 10 nM, and 6 nM, respectively.

Cathepsin L, a lysosomal cysteine proteinase, has been involved in the pathogenesis of bone disorders. KAKEGAWA *et al.* indicated that a cathepsin L specific inhibitor, PLCLI, completely inhibited bone resorption by osteoclasts[1]. Thus cathepsin L specific inhibitors are potential medicines for osteoporosis and rheumatoid arthritis.

During our screening for cathepsin L inhibitors, new microbial metabolites have been isolated from a fungal strain F-2665 and were designated as TMC-52A to D. In this paper, we describe the taxonomy of the producing organism, structural studies, and biological properties of these new inhibitors.

Materials and Methods

Materials

The following proteinases and their substrates were purchased from the sources indicated: cathepsin L from Calbiochem Corp.; cathepsin B, trypsin, thermolysin, cathepsin D and hemoglobin from Sigma Chemical Co.; papain and casein from Wako Pure Chemical Industries Ltd.; m-calpain and μ-calpain from Nacalai Tesque Inc.; carbobenzoxy-L-phenylalanyl-L-arginine 4-methyl-coumaryl-7-amide (*Z*-Phe-Arg-MCA), benzoyl-L-arginine 4-methyl-coumaryl-7-amide (Bz-Arg-MCA) and succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methyl-coumaryl-7-amide (Suc-Lys-Lys-Val-Tyr-MCA) from Peptide Institute Inc.

Production Organism and Taxonomical Characterization

The producing fungal strain, F-2665, was isolated from a rotten leaf of *Phalaenopsis* sp., a cultivated orchid, collected in Warabi-shi, Saitama, Japan.

The following agar media were employed for taxonomic characterization. One-fifth concentration malt extract agar (1/5 MA) contained malt extract 0.2 g, yeast extract 0.02 g, soytone 0.02 g, glucose 0.2 g, agar 20 g in 1 liter of distilled water. Cornmeal agar (CMA) consisted of 17 g of cornmeal agar powder (Nissui) in 1 liter of distilled water. Malt extract agar (MEA) contained malt extract 20 g, peptone 1 g, glucose 20 g, agar 20 g in 1 liter of distilled water. Oatmeal agar (OA) was prepared from 23 g of ISP No. 3 agar powder (Nippon Pharmaceutical Co.) in 1 liter of distilled water. Miura medium (LCA) consisted of glucose 1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, KCl 0.2 g, $NaNO_3$ 2 g, yeast extract 0.2 g, agar 13 g in 1 liter of distilled water.

Four standard sized Petri dishes (90 mm) were used; each for 1/5MA, CMA, MEA, OA or LCA. Each plate was inoculated at three points, equidistant from the edge of the plate and each other. The plates were incubated at 25°C. All plates were observed after 7 days. Further observation was made after 14 or more days.

Fermentation

A loopful of spores of *Gliocladium* sp. F-2665 was inoculated into 500-ml Erlenmeyer flasks containing

70 ml of a seed medium (glucose 0.5%, glycerol 2.0%, soybean meal 2.0%, yeast extract 0.2%, $CaCO_3$ 0.4% and NaCl 0.25%, pH 6.5) and incubated on a rotary shaker at 220 rpm at 27°C for 3 days. Then 300 ml of the seed culture was transferred into a 50-liter jar fermenter containing 30 liters of a production medium (glucose 0.5%, glycerol 10%, fish meal 2.0%, yeast extract 0.2%, $CaCO_3$ 0.4% and NaCl 0.25%, pH 6.5). The fermentation was carried out at 27°C for 5 days with agitation of 200~600 rpm and aeration of 15 liters/minute to keep the dissolved oxygen concentration at 10% of saturation. The production of the inhibitors was monitored by cathepsin L inhibition.

Enzyme Assays

The activities of all enzymes except thermolysin and cathepsin D were determined on the basis of the amount of 7-amino-4-methylcoumarin (AMC) formed during incubation for 10 minutes at 37°C by monitoring the fluorescence intensity with Fluoroskan II (Labosystems)[2]. One unit of the enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of AMC per minute under the assay conditions.

Cathepsin B and L: The reaction mixture (total volume, 100 μl) consisted of 50 μM *Z*-Phe-Arg-MCA, 8 mM cysteine, 5 mM DTT, 2 mM EDTA, 40 mM Tris buffer (pH 5.5), and 40 ng/ml of cathepsin L or 30 U/ml of cathepsin B.

Papain: The assay conditions were the same as described above except for buffer pH (pH 8.0) and enzyme concentration (1 mg/ml).

Calpains: The assay mixture (total volume, 100 μl) consisted of 500 μM Suc-Lys-Lys-Val-Tyr-MCA, 2 mM $CaCl_2$, 5 mM 2-mercaptoethenol, 40 mM Tris buffer (pH 7.5), and 0.1 mg/ml of m-calpain or 0.01 mg/ml of μ-calpain.

Trypsin: The assay mixture (total volume, 100 μl) consisted of 300 μM Bz-Arg-MCA, 5 mM DTT, 2 mM EDTA, 40 mM Tris buffer (pH 8.0), and 1 μg/ml of trypsin.

Thermolysin and cathepsin D activities were determined on the basis of the amount of TCA-soluble amino acids produced during incubation for 1 hour at 37°C by measuring A_{280}[3].

Thermolysin: The assay mixture (total volume, 5 ml) consisted of 5 mg/ml of casein, 5 mM DTT, 40 mM Tris (pH7.5), and 5 μg/ml of thermolysin.

Cathepsin D: The assay mixture (total volume, 5 ml) consisted of 6.25 mg/ml of acid denatured hemoglobin, 100 mM sodium acetate buffer (pH 3.5), and 0.1 U/ml of cathepsin D.

Results

Taxonomy

Colonies on 1/5MA reached 17~18 mm after 7 days at 25°C. These were plain forming a ring-like conidial area and were cream in color (Munsell 2.5Y9/2). The hyphae were colorless. No exudate or pigment in agar was observed. The reverse was yellowish gray (Munsell 5Y8/1).

Colonies on CMA attained a diameter of 18~20 mm after 7 days, showing ivory coloration (Munsell 10YR8/2). Ring-like conidiation was observed also within 7 days.

Colonies on MEA grew somewhat slowly attaining 15 mm in diameter after 7 days (Fig. 1).

Colonies on OA reached 22~23 mm after 7 days showing pale yellowish brown pigmentation. Abundant conidia of a slightly darker shade were produced in a ring-like zone.

Colonies on LCA reached 22~23 mm after 7 days showing pale brown surfaces with pale yellowish brown reverses.

Abundant sporulation in a ring-like manner somewhat resembled sporodochial structures. However, no setae or synnema or other distinct conidiomata were observed.

Conidiophores were erect and branched several times to show a candelabrum-like structure. Phialides were born almost in parallel at the apex of the branching and were cylindrical, 8.0~13.5 × 2.0~4.0 mm (average 10.5 × 2.5 mm). Phialoconidia were cylindrical to ellipsoidal with a truncate base, 4.0~5.5 × 2.0~2.5 mm (average 4.5 × 2.5 mm, SD: 0.40 × 0.14) and were produced in a short chain but soon forming a large slimy mass with conidia from adjacent phialdes. The length to width ratio of the conidia was 1.71~2.32 (average 1.90) (Fig. 1).

On the basis of phialides born almost in parallel from their bearer and hyaline 1-celled conidia in slimy mass, the producing strain, F-2665, was considered most closely related to the genus *Gliocladium*[4].

Isolation

The 27 liters of broth filtrate was absorbed on Diaion HP-20 and the active fractions were eluted with 50% aqueous MeOH. The active eluate was evaporated in vacuo to give a crude solid (16.2 g). The solid was dissolved in 25 ml of water and chromatographed on a reverses phase ODS (YMC GEL A60) column developed with a mixture of acetonitrile and 10 mM pyridine-formic

Fig. 1. Cultural and morphological characteristics of *Gliocladium* sp.

A, colonies on MEA after 7 days at 25°C. B, conidial structure, bar represents 10 μm.

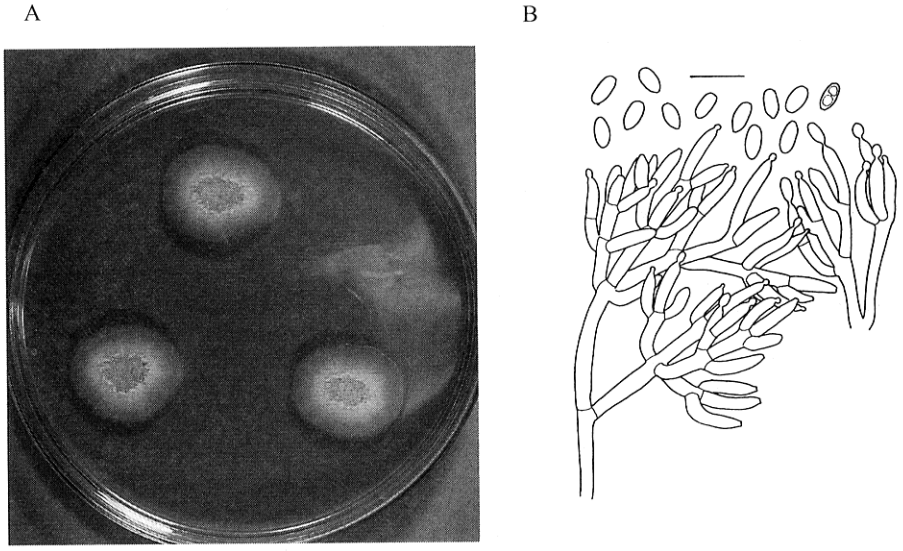


Table 1. Physico-chemical properties of TMC-52A, B, C, and D.

	TMC-52A	TMC-52B	TMC-52C	TMC-52D
Formula	$C_{20}H_{30}N_4O_6$	$C_{20}H_{30}N_4O_6$	$C_{20}H_{30}N_4O_5$	$C_{20}H_{30}N_4O_5$
$[\alpha]_D^{24}$ (H_2O)	+22°(*c* 0.4)	+22°(*c* 0.4)	+17°(*c* 0.5)	+11°(*c* 0.5)
HR-FAB-MS (m/z, MH^+)				
Found	423.2251	423.2218	407.2310	407.2288
Calcd	423.2244	423.2244	407.2295	407.2295
UV λ_{max} nm (ε) in H_2O	275 (1,200) 281 (1,000, sh)	275 (1,200) 281 (1,000, sh)	251 (180) 257 (210) 263 (170)	251 (180) 257 (210) 263 (170)
IR ν_{max} (KBr) cm^{-1}	1660,1600,1520, 1450, 1380,1250	1660,1600,1520, 1450, 1380,1250	1660,1600,1520, 1455, 1380,1250	1660,1600,1520, 1460, 1380,1250
HPLC Rt (minute)[a]	2.04	2.54	5.19	7.08

[a] Column: YMC AM-301-3 (4.6 mm i.d. × 100 mm, ODS, 3 μm), mobile phase: CH_3CN - 10 mM KH_2PO_4, pH 3.5 (5:95), detection: UV absorption at 210 nm.

acid buffer, pH 3.5 (5:95) to yield 4 fractions containing semi-pure TMC-52A, B, C, and D, respectively. The active fractions were re-chromatographed on the ODS column eluted with a mixture of acetonitrile and 15 mM pyridine - formic acid buffer, pH 3.5 (3.5:96.5 for TMC-52A and B, 5:95 for TMC-52C and D). The active fractions were further purified on a Sephadex LH-20 column eluted with 50% aqueous MeOH. Evaporation of the active elute gave pure TMC-52A (158 mg), TMC-52B (205 mg), TMC-52C (112 mg), and TMC-52D (115 mg).

Physico-chemical Properties

Each component of TMC-52 was obtained as white powder and was soluble in water, dimethyl sulfoxide and lower alcohols but insoluble in chloroform and ethyl-acetate. Each showed positive reaction with ninhydrin reagent. The other physico-chemical properties of TMC-52 components are summarized in Table 1.

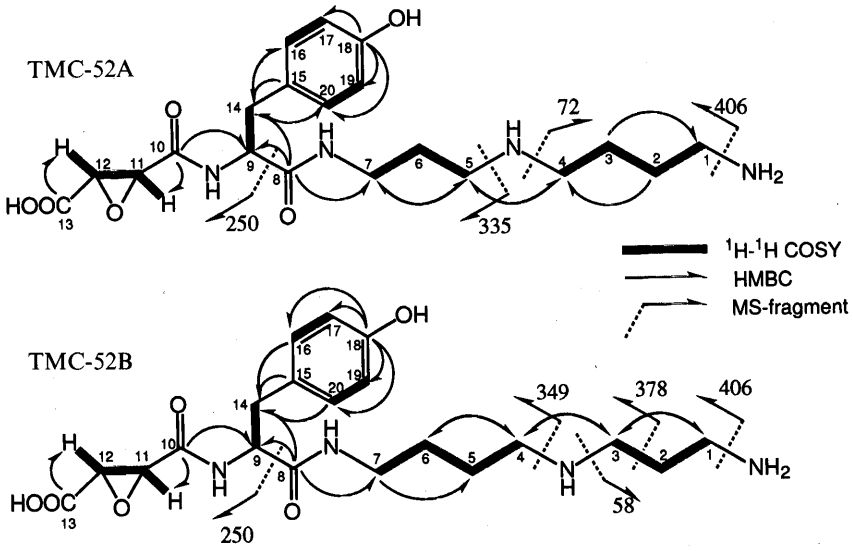
The molecular formulae of TMC-52 components were determined on the basis of HR-FAB-MS and NMR data. The ^1H and ^{13}C NMR data of TMC-52 components are

Table 2. ^{1}H and ^{13}C NMR data of TMC-52A, B, C, and D (D_2O).

Position	TMC-52A		TMC-52B		TMC-52C		TMC-52D	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	41.6 (t)	3.06 (m)	39.4 (t)	3.11 (m)	41.6 (t)	3.05 (m)	39.6 (t)	3.12 (m)
2	26.8 (t)	1.76 (m)	26.6 (t)	2.09 (m)	26.7 (t)	1.78 (m)	26.6 (t)	2.08 (m)
3	25.5 (t)	1.76 (m)	47.2 (t)	3.14 (m)	25.5 (t)	1.75 (m)	47.2 (t)	3.15 (m)
4	49.7 (t)	3.03 (m)	50.1 (t)	3.03 (m)	49.7 (t)	3.02 (m)	50.1 (t)	3.09 (m)
5	47.7 (t)	2.85 (t, 7.6)	25.6 (t)	1.54 (m)	47.7 (t)	2.89 (t, 7.6)	25.6 (t)	1.57 (m)
6	28.2 (t)	1.80 (m)	28.2 (t)	1.46 (m)	28.2 (t)	1.80 (m)	28.2 (t)	1.48 (m)
7	38.8 (t)	3.22 (t, 6.5)	41.2 (t)	3.14 (m)	38.8 (t)	3.22 (m)	41.2 (t)	3.15 (m)
8	176.0 (s)		175.5 (s)		176.0 (s)		175.4 (s)	
9	58.4 (d)	4.47 (t, 7.8)	58.4 (d)	4.46 (t, 7.8)	58.2 (d)	4.54 (t, 7.8)	58.2 (d)	4.54 (t, 7.8)
10	172.3 (s)		172.3 (s)		172.4 (s)		172.3 (s)	
11	55.6 (d)	3.55 (d, 2.0)	55.6 (d)	3.54 (d, 2.2)	55.6 (d)	3.52 (d, 2.1)	55.6 (d)	3.52 (d, 2.1)
12	57.2 (d)	3.30 (d, 2.0)	57.2 (d)	3.29 (d, 2.2)	57.2 (d)	3.26 (d, 2.1)	57.2 (d)	3.24 (d, 2.1)
13	176.4 (s)		176.4 (s)		176.4 (s)		176.3 (s)	
14	38.7 (t)	3.01 (m)	38.9 (t)	3.01 (m)	39.5 (t)	3.10 (m)	39.4 (t)	3.07 (m)
15	130.8 (s)		130.9 (s)		139.0 (s)		139.1 (s)	
16, 20	133.4 (d)	7.16 (d, 8.6)	133.4 (d)	7.15 (d, 8.5)	132.0 (d)	7.30 (m)	132.0 (d)	7.29 (m)
17, 19	118.4 (d)	6.88 (d, 8.6)	118.4 (d)	6.88 (d, 8.5)	131.7 (d)	7.40 (m)	131.6 (d)	7.40 (m)
18	157.4 (s)		157.4 (s)		130.1 (d)	7.37 (m)	130.1 (d)	7.36 (m)

Chemical shifts in ppm from sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal standard.

Fig. 2. Structures of TMC-52A as elucidated by NMR and MS data.

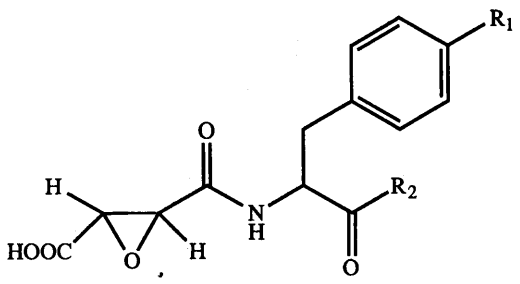


shown in Table 2.

Structure Determination

The ^{13}C NMR spectra of both TMC-52A and B displayed 20 signals composed of $CH_2 \times 8$, $>CH- \times 3$, $CH= \times 4$, $>C= \times 2$ and carbonyl C $\times 3$. The structures of TMC-52A and B were elucidated by NMR studies involving ^{1}H-^{1}H COSY, HMQC and HMBC, and ESI-MS fragmentation (Fig. 2). TMC-52A and B exhibited very similar spectral properties, differing slightly in the alkyl amine region. The sequence of the spermidine moiety was found to be transposed. The *trans* configuration of the epoxysuccinate moiety of TMC-52A or B was assigned on the basis of the coupling constant between H-11 and H-12 ($J_{H-H}=2.0$ Hz). The stereochemistry of C-9 was determined to be *S* by chiral TLC

Fig. 3. Structures of TMC-52A, B, C, D, and cathestatins A and B.



	R_1	R_2
TMC-52 A	OH	$-NH(CH_2)_3NH(CH_2)_4NH_2$
TMC-52 B	OH	$-NH(CH_2)_4NH(CH_2)_3NH_2$
TMC-52 C	H	$-NH(CH_2)_3NH(CH_2)_4NH_2$
TMC-52 D	H	$-NH(CH_2)_4NH(CH_2)_3NH_2$
Cathestatin A	H	$-NH(CH_2)_4NH_2$
Cathestatin B	OH	$-NH(CH_2)_4NH_2$

Table 3. Inhibitory effect of TMC-52A, B, C, and D on several proteinases.

Enzyme	IC_{50} (μM)			
	TMC-52A	TMC-52B	TMC-52C	TMC-52D
Cysteine proteinases				
Cathepsin L	0.013	0.01	0.01	0.006
Cathepsin B	0.32	0.2	0.46	0.28
Papain	0.044	0.007	0.088	0.049
m-Calpain	52	37	78	93
μ-Calpain	64	44	92	90
Serine proteinase				
Trypsin	>100	>100	>100	>100
Metalloproteinase				
Thermolysin	>100	>100	>100	>100
Aspartic proteinase				
Cathepsin D	>100	>100	>100	>100

analysis of the acid hydrolysate.

The molecular formula of TMC-52C differs from that of TMC-52A by an oxygen. The NMR data of TMC-52C correspond well to those of TMC-52A, except for the lack of a phenolic hydroxy group. Thus, the tyrosine moiety in TMC-52A was replaced by phenylalanine in TMC-52C. Similarly, TMC-52D was revealed to be the phenylalanine analog of TMC-52B (Fig. 3).

Biological Properties

The enzyme inhibitory activities of TMC-52A, B, C, and D *vs.* several kinds of proteinases are shown in Table 3. TMC-52A, B, C, and D strongly inhibited cathepsin L, B, and papain which are included in the papain superfamily, however, the calpains were inhibited weakly. On the other hand, serine proteinase, metalloproteinase, and aspartic proteinase were not inhibited at a concentration of 100 μM. Compared with inhibitory activities of TMC-52A to D for cathepsin L and B, the IC_{50} value for cathepsin B was approximately 20 to 50 times as large as that for cathepsin L, suggesting that TMC-52s were selective inhibitors of cathepsin L.

Discussion

In this study, we isolated new cysteine proteinase inhibitors, TMC-52A, B, C, and D, and demonstrated that TMC-52A to D are novel epoxysuccinyl peptides. Several compounds structurally related to TMC-52 have been reported from various filamentous fungi. Cathestatin was isolated from *Penicillium citrinum* Thom in 1995[5]. PF1126A and PF1126B were discovered from *Aspergillus terricola* Marchal[6]. *Myceliophthora thermophila* (Apinis) van Oorschot was reported to produce estatin A and B in 1989[7]. The teleomorph of these producers are classified in Ascomycota. *Penicillium* and *Aspergillus* are anamorphs of Eurotiaceae. Whereas, *Myceliophthora* is anamorphic Arthrodermataceae and Ceratostomata-

ceae[8]. All these genera were formally classified in the class Plectomycetes. Our strain F-2665 was, however, related to none of these families; *Gliocladium* is known as an anamorph of Nectriaceae and Hypocreaceae, the class Pyrenomycetes. The taxonomic positions of producer strains together with structural simplicity of these peptidic compounds suggested that TMC-52 and its related derivatives were widely distributed in Ascomycota. These compounds are likely biosynthesized *via* common pathways related to the primary metabolism of peptides.

TMC-52, cathestatins and estatins have similar inhibitory potency and selectivity *vs.* cathepsin L and B. Cathepsin L prefers the bulky and aromatic side chains such as in phenylalanine and tyrosine at the P2 position. But cathepsin B prefers positively charged side chains such as in arginine and bulky aromatic side chains at the same position[9]. TMC-52, cathestatins, and estatins have Phe or Tyr in their molecules and these residues are thought to be involved in enzyme-substrate interaction. This explains why the inhibitory profiles of cathepsin L and cathepsin B are similar.

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