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NOTES

TMC-89A and B, New Proteasome Inhibitors from *Streptomyces* sp. TC 1087

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The ubiquitin/proteasome system is a major pathway of selective protein degradation in eukaryotic cells. The system degrades a number of important cellular proteins such as oncogene products, cyclins, and transcriptional factors. The target proteins are conjugated to polypeptide ubiquitin, and then degraded by a proteasome in an ATP dependent manner[1]. The catalytic core of the proteasome is the 20S proteasome. New 20S proteasome inhibitors would contribute to further understanding of the system[2~4]. In the course of our screening program, we have isolated several classes of 20S proteasome inhibitors including (i) the novel cyclic peptides, TMC-95s, and (ii) the new members of α',β'-epoxyketone containing peptides, TMC-86s and TMC-96, from microbial metabolites[5~8]. Our recent search resulted in the isolation of TMC-89A and B, new members of the α',β'-epoxyketone containing peptides, from a microbial metabolite. We describe here the taxonomy of producing strain, production, isolation, structural studies,

Table 1. Cultural characteristics of strain TC 1087.

Medium		TC 1087
Yeast extract-malt extract agar (ISP No.2)	G:	Good
	AM:	Abundant, Olive gray (9-16-1)*
	R:	Dark brownish gray (3-12-1)
	S:	Pale yellowish brown (7-18-2)
Oatmeal agar (ISP No.3)	G:	Good
	AM:	Abundant, Brownish gray (7-15-1)
	R:	Brownish black (3-11-1)
	S:	Grayish brown (5-16-2)
Inorganic salts-starch agar (ISP No.4)	G:	Good
	AM:	Abundant, Grayish yellow green (9-16-2)
	R:	Grayish red brown (3-13-2)
	S:	Pale brown (5-18-2)
Glycerol-asparagine agar (ISP No.5)	G:	Good
	AM:	Abundant, Grayish yellow brown (6-16-2)
	R:	Brownish black (3-11-1)
	S:	Reddish brown (2-12-4)
Peptone-yeast extract iron agar (ISP No.6)	G:	Poor
	AM:	None
	R:	Pale yellowish brown (7-18-3)
	S:	None
Tyrosine agar (ISP No.7)	G:	Good
	AM:	Abundant, Light olive gray (9-17-1)
	R:	Grayish red brown (3-13-2)
	S:	Reddish brown (2-12-4)

* Color codes from the Guide to Color Standard

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

and biological activities of them.

The producing strain TC1087 was isolated from a soil sample collected in Hiroshima City, Japan. The cultural characteristics are summarized in Table 1. The substrate mycelia developed well and were irregularly branched. Each spore chain, which was in the form of curves, hooks, or loops, had 5 to 30 or more spores per chain. The spores were oval to ellipsoidal, with size of 0.7~0.9×1.5~1.8 μm, and the surface was smooth. Fragmentation of substrate mycelia, sporangia, or motile spores 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, the strain TC 1087 was assigned to the genus *Streptomyces*.

The fermentation was carried out at 27°C in a 50-liter jar fermentor with agitation of 200 rpm and aeration at 15 liters per minute by using 30 liters of a medium composed of 1.0% glucose, 4.0% dextrin, 2.5% Bactosoytone, 0.1% yeast extract, 0.3% $CaCO_3$, 0.01% defoaming agent (CC-438; NIPPON OIL & FAT CO., LTD.), adjusted at pH 7.0 before autoclaving. The inhibitory activity of the fermentation broth against 20S proteasome was found after 48 hours of cultivation and reached maximum after 120 hours.

The broth filtrate (27 liters) was applied to a Diaion HP-20 column and eluted with 50% aqueous methanol. The eluate was concentrated and subjected to a medium-pressure reverse-phase silica gel column chromatography (MPLC, YMC ODS A60), followed by elution with 10% aqueous acetonitrile. The active eluate was concentrated and rechromatographed on a silica gel MPLC (Wako gel C300) developed with CH_2Cl_2-MeOH (7:1) to afford semi-pure TMC-89s (560 mg). The semi-pure TMC-89s were purified by preparative HPLC (column: YMC D-ODS-5B (30 mm×250 mm), mobile phase: acetonitrile-water (12:88), flow rate: 25 ml/minute, detection: UV

Table 2. Physiological properties of strain TC 1087.

Characteristic	TC 1087
Temperature range for growth (ISP No. 2)	25~45°C
Optimum temperature for growth (ISP No. 2)	25~37°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)	6%
Utilization of carbon source	
L-Arabinose	+
D-Fructose	+
D-Glucose	+
Inositol	±
D-Mannitol	+
Raffinose	−
L-Rhamnose	+
Sucrose	−
D-Xylose	+

+, Positive; ±, doubtful; −, negative.

Table 3. Physico-chemical properties of TMC-89A and B.

	TMC-89A	TMC-89B
Appearance	White powder	White powder
MP (dec.)	99°C	97°C
$[\alpha]_D^{20}$	− 7.7° (*c* 0.3, H_2O)	− 6.8° (*c* 0.3, H_2O)
Molecular formula	$C_{21}H_{36}N_4O_9$	$C_{21}H_{36}N_4O_9$
FAB-MS (*m/z*)	489 $(M+H)^+$, 511 $(M+Na)^+$	489 $(M+H)^+$, 511 $(M+Na)^+$
HR-FAB-MS (*m/z*)		
Found	511.2350	511.2355
Calcd.	511.2382 for $C_{21}H_{36}N_4O_9Na$	511.2382 for $C_{21}H_{36}N_4O_9Na$
UV λ_{max} (MeOH)	End absorption	End absorption
IR ν_{max} (KBr) cm^{-1}	3300, 3070, 2970, 2900, 1720, 1640, 1530, 1460, 1380, 1260, 1220, 1045	3300, 3070, 2960, 2940, 1720, 1640, 1530, 1460, 1380, 1340, 1220, 1045
Solubility		
soluble in	H_2O, DMSO, MeOH	H_2O, DMSO, MeOH
insoluble in	$CHCl_3$, EtOAc, *n*-hexane	$CHCl_3$, EtOAc, *n*-hexane

Table 4. ^{1}H and ^{13}C NMR data of TMC-89A and B.

Position	TMC-89A		TMC-89B	
	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]
1	47.9 (t)[c]	3.05 (1H, d, 5.4)[d]	47.9 (t)	3.04 (1H, d, 5.4)
		3.07 (1H, d, 5.4)		3.07 (1H, d, 5.4)
2	62.9 (s)		62.9 (s)	
3	206.7 (s)		206.6 (s)	
4	50.4 (d)	4.38 (1H, m)	50.4 (d)	4.37 (1H, m)
5	37.0 (t)	1.25 (1H, m)	37.0 (t)	1.26 (1H, m)
		1.44 (1H, m)		1.44 (1H, m)
6	24.4 (d)	1.67 (1H, m)	24.3 (d)	1.67 (1H, m)
7	23.1 (q)	0.89 (3H, d, 6.6)	23.1 (q)	0.89 (3H, d, 6.6)
8	59.7 (t)	3.31 (1H, dd, 5.2, 12.4)	59.7 (t)	3.33 (1H, dd, 5.4, 12.4)
		4.12 (1H, dd, 6.4, 12.4)		4.11 (1H, dd, 6.5, 12.4)
9	20.8 (q)	0.84 (3H, d, 6.6)	20.8 (q)	0.83 (3H, d, 6.6)
1'	169.8 (s)		169.9 (s)	
2'	57.7 (d)	4.33 (1H, dd, 5.5, 8.6)	57.9 (d)	4.30 (1H, m)
3'	66.6 (d)	3.95 (1H, m)	66.6 (d)	3.93 (1H, m)
4'	18.7 (q)	1.02 (3H, d, 6.9)	18.8 (q)	1.03 (3H, d, 6.5)
1"	169.4 (s)		169.7 (s)	
2"	57.4 (d)	4.35 (1H, m)	57.8 (d)	4.29 (1H, m)
3"	66.7 (d)	3.91 (1H, m)	66.2 (d)	4.04 (1H, m)
4"	18.7 (q)	1.04 (3H, d, 6.7)	19.0 (q)	1.05 (3H, d, 6.5)
1"'	170.1 (s)		170.7 (s)	
2"'	46.2 (d)	3.27 (1H, q, 7.1)	45.9 (d)	3.35 (1H, q, 7.1)
3"'	172.2 (s)		172.9 (s)	
4"'	14.7 (q)	1.17 (3H, d, 7.1)	14.6 (q)	1.20 (3H, d, 7.1)
4-NH		8.07 (1H, d, 7.3)		7.92 (1H, d, 7.1)
8-OH		5.25 (1H, dd, 5.2, 6.4)		5.02 (1H, dd, 5.4, 6.5)
2'-NH		7.73 (1H, d, 8.5)		7.88 (1H, d, 8.5)
3'-OH		4.70 (1H, d, 5.1)		4.70 (1H, d, 5.0)
2"-NH		7.91 (1H, d, 8.2)		8.04 (1H, d, 7.8)
3"-OH		5.07 (1H, d, 4.7)		5.07 (1H, d, 4.6)
3"'-NH		7.03 (1H. br-s)		7.13 (1H, br-s)
3"'-NH		7.36 (1H, br-s)		7.43 (1H, br-s)

[a] 100 MHz in DMSO-d_6
[b] 400 MHz in DMSO-d_6
[c] Multiplicity.
[d] Proton number, multiplicity and coupling constants in Hz.

absorption at 210 nm). TMC-89A was eluted at the retention time of 27 minutes, followed by TMC-89B at 31 minutes. The appropriate eluates were pooled, concentrated and further purified by Sephadex LH-20 column chromatography with methanol, yielding pure TMC-89A (126 mg) and TMC-89B (61 mg).

The physico-chemical properties of TMC-89A and B are summarized in Table 3. The molecular formula of TMC-89A and B was determined as $C_{21}H_{36}N_4O_9$ on the basis of their HR-FAB-MS and ^{1}H and ^{13}C NMR spectral data. Their IR spectra exhibited the presence of hydroxyl (3300 cm^{-1}), ketone or ester (1720 cm^{-1}), and amide (1640 and 1530 cm^{-1}) groups. The NMR spectral data of TMC-89A and B are summarized in Table 4. The structure of TMC-89A was determined by the analyses of the NMR experiments such as ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC, and MS spectrum (Fig. 1), along with the degradation studies. The HMBC correlations from C-1"' (δ 170.1) to 4"'-H (δ 1.17); C-3"' (δ 172.2) to 4"'-H and 3"'-NH_2 (δ 7.03, 7.36), and the NOEs observed between 2"'-H (δ 3.27) and 3"'-NH_2; 2"'-H and 2"-NH (δ 7.91) demonstrated the structure of C-1"' to 3"'-NH_2, which was attached to 2"-NH. The remaining sequence of TMC-89A was confirmed by EI-MS spectrum as shown in Fig. 1. The

Fig. 1. 2D-NMR and EI-MS fragmentation of TMC-89A.

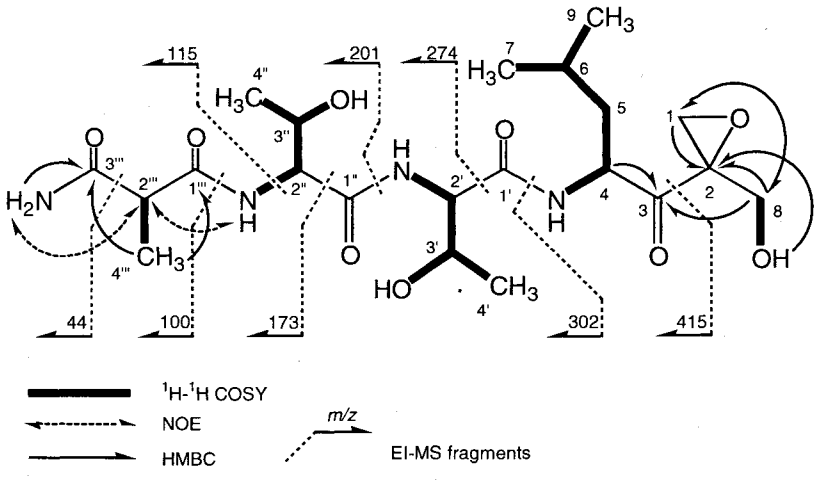
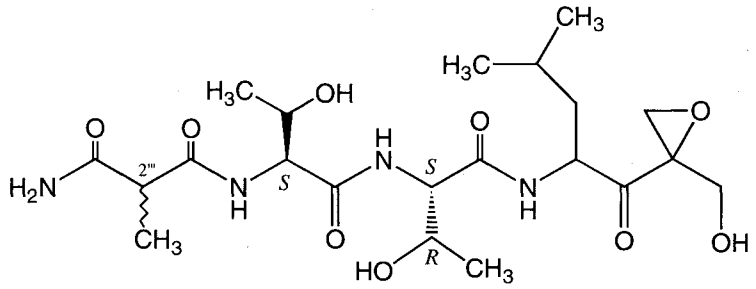


Fig. 2. The structures of TMC-89A and B.



	C-2‴
TMC-89A	*R*
TMC-89B	*S*

NMR data of TMC-89B was almost identical to that of TMC-89A except for the slight shifts of C-1‴~C-3‴ signals (Table 4). No difference, however, was observed in the MS data of them. Therefore, TMC-89B was considered to be a stereoisomer of TMC-89A. Acid hydrolysis of TMC-89A and B with 6 N HCl gave one spot of L-threonine as analyzed by chiral TLC. Thus, the stereochemistries of C-2′, C-3′, C-2″, and C-3″ were assigned to *S*, *R*, *S*, and *R*, respectively (Fig. 2). In order to confirm the stereochemistry of C-2‴ of TMC-89A and B, the carboxamide group (C-3‴-NH_2) was reduced to an amine group by [bis(trifluoroacetoxy)iodo]benzene[9]. Acid hydrolysis of each reduced product of TMC-89A and B gave L-alanine and D-alanine, respectively, along with

Table 5. Inhibitory activities of TMC-89A, B and ALLN against the ChT-L, T-L, and PGPH activities of 20S proteasome.

Compound	IC_{50} (μM)		
	ChT-L	T-L	PGPH
TMC-89A	1.1	0.39	7.2
TMC-89B	1.1	0.51	7.1
ALLN	6.6	6.0	21

Table 6. Cytotoxicities of TMC-89A and B against tumor cells *in vitro*.

Cell line	IC_{50} (μM)	
	TMC-89A	TMC-89B
HCT-116 human colon carcinoma	>20	>20
HeLa S3 human epitheloid carcinoma	>20	>20
SK-BR-3 human breast adenocarcinoma	12	13
WiDr human colon adenocarcinoma	>20	>20
HL-60 human promyelocytic leukemia	13	12
B-16 murine melanoma	8.2	7.6
P388D1 murine lymphoid neoplasm	>20	>20

L-threonine by the chiral TLC analyses. Thus, the configurations of C-2‴ of TMC-89A and B were determined to be *R* and *S*, respectively (Fig. 2).

20S proteasome has three catalytic subunits and shows at least three distinct peptidase activities, chymotrypsine-like (ChT-L), trypsin-like (T-L), and peptidylglutamyl-peptide hydrolyzing (PGPH) activities, which cleave peptide bonds on carboxyl side of hydrophobic, basic, and acidic amino acids, respectively[10]. The inhibitory activities of TMC-89A, B and *N*-Acetyl-Leu-Leu-nLeu-CHO (ALLN) against the ChT-L, T-L, and PGPH activities are shown in Table 5. TMC-89A and B inhibited the T-L activity much stronger than TMC-86s and TMC-96, while all these compounds inhibited the ChT-L and PGPH activities to a similar extent. The same inhibitory activities of TMC-89A as TMC-89B indicated that the stereochemistry of C-2‴ was not vital to their activities. TMC-89A and B did not inhibit m-calpain, cathepsin L, and trypsin at 100 μM, suggesting their high specificity for 20S proteasome. TMC-89A and B did not show remarkable cytotoxicities to various tumor cell lines (Table 6).

Experimental

Enzyme Assays

20S proteasome was isolated from THP.1 monocytic cells[5]. Other enzymes were purchased from commercial sources. The activities of enzymes were measured by using fluorescence substrates according to the method reported previously[5].

Cytotoxic Assays

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[5].

Reduction of the Carboxamide Group

TMC-89A (4.2 mg) was dissolved in 0.5 ml of CH_3CN-H_2O (1 : 1). A solution of [bis(trifluoroacetoxy)iodo]benzene (9 mg in 0.1 ml CH_3CN) was added, and the mixture was stirred for 6 hours under N_2 atmosphere. The solvent (CH_3CN) was evaporated *in vacuo*. The resultant liquid was acidified with 6 N HCl, and washed with ether (1 ml). The water layer was concentrated to dryness and hydrolyzed with 0.2 ml of 6 N HCl in a sealed tube (110°C, 16 hours). The hydrolysate was analyzed by chiral TLC (HPTLC-CHIR, Merck) with a solvent of MeOH-H_2O-CH_3CN (1 : 1 : 4). Rf; L-alanine: 0.48, D-alanine: 0.44, L-threonine: 0.57, D-threonine: 0.52.

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References

1) CIECHANOVER, A. & A. L. SCHWARTZ: The ubiquitin-proteasome pathway: The complexity and myriad functions of proteins death. Proc. Natl. Acad. Sci. USA 95: 2727~2730, 1998
2) PALOMBELLA, V. J.; O. J. RANDO, A. L. GOLDBERG & T. MANIATIS: The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell 78: 773~785, 1994
3) ROCK, K. L.; C. GRAMM, L. ROTHSTEIN, K. CLARK, R. STEIN, L. DICK, D. HWANG & A. L. GOLDBERG: Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78: 761~771, 1994
4) HE, H.; X. M. Qi, J. GROSSMANN & C. W. DISTELHORST: c-Fos degradation by the proteasome. An early, Bcl-2-regulated step in apoptosis. J. Biol. Chem. 273: 25015~25019, 1998
5) KOGUCHI, Y.; J. KOHNO, S. SUZUKI, M. NISHIO, K. TAKAHASHI, T. OHNUKI & S. KOMATSUBARA: TMC-86A, B and TMC-96, new proteasome inhibitors from *Streptomyces* sp. TC 1084 and *Saccharothrix* sp. TC1094. I. Taxonomy, fermentation, isolation, and biological activities. J. Antibiotics 52: 1069~1077, 1999
6) KOGUCHI, Y.; J. KOHNO, S. SUZUKI, M. NISHIO, K. TAKAHASHI, T. OHNUKI & S. KOMATSUBARA: TMC-86A, B and TMC-96, new proteasome inhibitors from *Streptomyces* sp. TC 1084 and *Saccharothrix* sp. TC1094. II. Physico-chemical properties and structure elucidation. J. Antibiotics 53: 63~65, 2000
7) KOGUCHI, Y.; J. KOHNO, S. SUZUKI, M. NISHIO, K. TAKAHASHI, T. OHNUKI & S. KOMATSUBARA: TMC-95A, B, C, and D, novel proteasome inhibitors produced by *Apiospora montagnei* Sacc. TC 1093. Taxonomy, production, isolation, and biological activities. J. Antibiotics 53: 105~109, 2000
8) KOHNO, J.; Y. KOGUCHI, M. NISHIO, K. TAKAHASHI, T. OKUDA, T. OHNUKI & S. KOMATSUBARA: Structures of TMC-95 A, B, C and D: Novel proteasome inhibitors from *Apiospora montagnei* Sacc. TC 1093. J. Org. Chem. 65: 990~995, 2000
9) F.-ZAKUSKI, M. C.; E. L.-SOROCA, J. DEVIN & B. P. ROQUES: ^{1}H NMR configurational correlation for retro-inverso dipiptides: application to the determination of the absolute configuration of "enkephalinase" inhibitors. Relationships between stereochemistry and enzyme recognition. J. Med. Chem. 29: 751~757, 1986
10) ORLOWSKI, M. & C. MICHAUD: Pituitary multicatalytic proteinase complex. Specificity of components and aspects of proteolytic activity. Biochemistry 28: 9270~9278, 1989