TMC-2A, -2B and -2C, Novel Dipeptidyl Peptidase IV Inhibitors Produced by *Aspergillus oryzae* A374

I. Taxonomy of Producing Strain, Fermentation, and Biochemical Properties

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TMC-2A (**1**), -2B (**2**) and -2C (**3**), novel dipeptidyl peptidase IV (DPIV) inhibitors, were isolated from the fermentation broth of *Aspergillus oryzae* A374. TMC-2A, -2B and -2C inhibited rat kidney DPIV with IC_{50} value of 8.1 μM, 17 μM, and 20 μM, respectively, as well as human DPIV prepared from mononuclear cells and adenocarcinoma cells. TMC-2 compounds inhibited only DPIV among the proteases tested, indicating their high selectivity for DPIV. The kinetic analyses revealed that TMC-2A was an uncompetitive inhibitor. Taxonomy and fermentation of the producing strain are also described.

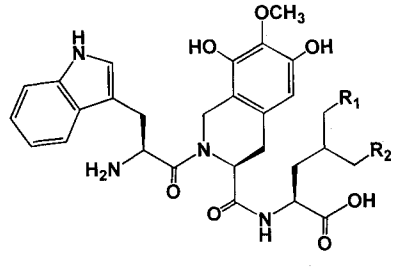
Dipeptidyl peptidase IV (DPIV, EC 3.4.14.5) is a serine protease which cleaves X-proline dipeptides from the NH_2 termini of peptides[1,2]. This enzyme is a membrane-bound glycoprotein and widely distributed on a variety of cells and tissues. The highest activity is found in the kidney and the intestinal brush-border membrane[3,4].

Recently, DPIV has been identified as CD26, a surface differentiation marker in the transduction of mitogenic signals in thymocytes and T lymphocytes[5,6]. DPIV/CD26 not only marks the activated state but is itself involved in the signal transducing process: cross-linking of CD3 and CD26 results in the enhanced T cell activation in the absence of antigen-presenting cells[7]. However, it is unlikely that CD26 directly participates in transducing the activation signal across the T cell membrane, since this molecule has only a very short cytoplasmic region of 6 amino acids[8]. Protein tyrosine phosphatase, CD45RO, has been shown to associate with CD26 and provides a putative mechanism for the costimulation[9]. Other association includes the strong binding of adenosine deaminase (ADA) type I to CD26. This may be of particular importance since ADA activity participates in regulation of the early stages of signal transduction in T lymphocytes[10]. The costimulatory potential possibly depends on the DPIV activity, although a substrate of relevance to T cell activation has not yet been identified[11]. SCHÖN *et al.* have reported that inhibitors and antibodies against DPIV had the activity to suppress lymphocyte proliferation and immunoglobulin synthesis *in vitro*[11]. DPIV inhibitors are expected to be therapeutically useful in the treatment of immunological diseases involving T cell activation such as rheumatoides arthritis.

UMEZAWA *et al.* reported DPIV inhibitors, diprotin A (Ile-Pro-Ile) and B (Val-Pro-Leu), of a microbial origin[12]. These compounds are however utilized as a substrate and degradated by DPIV[13].

We have screened microbial metabolites for DPIV inhibitors and found novel compounds; TMC-2A (**1**), -2B (**2**), and -2C (**3**) (Fig. 1). This report describes the taxonomy and fermentation of the producing strain, and the biological activities of TMC-2 compounds. The isolation, physico-chemical properties, and structural

Fig. 1. Structures of TMC-2A (**1**), -2B (**2**) and -2C (**3**).



	R_1	R_2
TMC-2A (**1**)	OH	OH
TMC-2B (**2**)	OH	H
TMC-2C (**3**)	H	OH

elucidation of TMC-2 compounds are described in an accompanying paper.

Materials and Methods

Chemicals

Glycyl-prolyl-*p*-nitroanilide tosylate (Gly-Pro-*p*NA·Tos) and carbobenzoxy-alanyl-alanyl-leucyl-*p*-nitroanilide (*Z*-Ala-Ala-Leu-*p*NA) were obtained from Peptide Institite Inc. Prolyl endopeptidase and carbobenzoxy-glycyl-prolyl-*p*-nitroanilide (*Z*-Gly-Pro-*p*NA) were purchased from Seikagaku Kogyo Co. Trypsin, subtilisin (BPN'), cathepsin C, leucine aminopeptidase (cytosol), proline aminopeptidase, benzoyl-DL-arginine-*p*-nitroanilide (Bz-DL-Arg-*p*NA), glycyl-phenylalanine-*p*-nitroanilide (Gly-Phe-*p*NA), leucine-*p*-nitroanilide (Leu-*p*NA) and proline-*p*-nitroanilide (Pro-*p*NA) were obtained from Sigma Chemical Co.

Producing Organism and Taxonomical Characterization

The producing fungal strain, A374, was isolated from a soil sample collected in Kochi City, Kochi Prefecture, Japan. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession number FERM P-14934.

Taxonomic studies of strain A374 were conducted according to the method of KLICH and PITT[14], and RAPER and FENNELL[15]. The color standard of Munsell was used for determining and standerizing colors. The media, CZAPEK's solution agar (CZA, Difco), Czapek-yeast extract agar (CYA, CZA supplemented with 0.5% yeast extract), malt extract agar (MEA; malt extract 2.0%, peptone 0.1%, dextrose 2.0%, and agar 0.2%), MY20 agar (peptone 0.5%, yeast extract 0.3%, malt extract 0.3%, glucose 20%, and agar 2.0%), and M40Y agar (sucrose 40%, malt extract 2.0%, yeast extract 0.5%, and agar 2.0%), were used for identification of the fungus.

Fermentation

A spore suspension (1.7×10^4) of *Aspergillus oryzae* A374 was inoculated into 500-ml Erlenmeyer flasks containing 70 ml of a sterilized seed medium composed of glucose 0.5%, glycerol 2.0%, soybean meal 2.0%, yeast extract 0.2%, NaCl 0.25%, $CaCO_3$ 0.4%, and CA-115 (Nihon Yushi Co.) 0.01% (pH adjusted to 7.0 before autoclaving). The inoculated flasks were incubated on a rotary shaker (220 rpm) for 5 days at 27°C. The seed culture (300 ml) was transferred into a 30-liter jar fermentor containing 18 liters of a production medium composed of glucose 0.5%, glycerol 2.0%, soybean meal 2.0%, yeast extract 0.2%, NaCl 0.25%, $CaCO_3$ 0.4%, CA-115 0.01% and CC-438 (Nihon Yushi Co.) 0.1% (pH adjusted to 7.0 before autoclaving). Fermentation was carried out at 27°C for 5 days with aeration at 9.0 liters per minute and with agitation controlled to maintain demanded oxygen at 20% of the oxygen saturation. Dry mycelial weights were measured by filtrating 50 ml of broth on a filter paper and drying the materials trapped on the filter at 60°C for 48 hours.

Preperation of DPIV

DPIV was partially purified from rat kidney, referring to the methods of HOPUS-HAVE & SARIMO[16] and SEIDL & SCHAEFER[17] as follows. One hundred and forty kidneys (200 g) of Sprague-Dawley male rats (8 weeks old) were homogenized in 800 ml of 0.25 M sucrose by using a Polytron homogenizer (Dispergier- und Mischtechnik Co.). The homogenate was centrifuged at 2,500 *g* for 10 minutes, and the supernatant was then centrifuged at 100,000 *g* for 60 minutes. The pellet was suspended in 40 ml of 1% Triton X-100 and stirred for 60 minutes. The suspension was centrifuged at 100,000 *g* for 60 minutes. The supernatant thus obtained was applied to a column of Q Sepharose by FPLC system (Pharmacia Biotech.). The column had been equilibrated with 20 mM Tris-HCl pH 7.6, 0.05% Triton X-100 and washed by the same buffer. The column was then eluted by a linear gradient of 0 to 0.5 M NaCl in 20 mM Tris-HCl, 0.05% Triton X-100 (pH 7.6) at a flow rate of 1 ml per minute. The fractions containing DPIV activity were applied to a Con A Sepharose column (Pharmacia) and washed with ×4 PBS. The column was eluted by 0.3 M α-methyl-D-mannoside. The fractions containing DPIV activity were pooled and used for screening. All procedures were carried out at 4°C.

Procedures for preparing DPIV from other sources are described below.

Human mononuclear cell DPIV: Human mononuclear cells were grown in RPMI-1640 medium containing 10% FCS and 2 μg/ml phytohemagglutinin. Triton X-100 was added to the culture flask at 0.1%. After 30 minutes, the cell suspension was centrifuged at 2,500 *g* for 30 minutes. The supernatant was used as an enzyme source.

Human Caco-2 cell DPIV: Caco-2 cells were grown in D'MEM medium containing 10% FCS. Triton X-100 was added to the flask at 1%. After 30 minutes, the cell suspension was centrifuged at 2,500 *g* for 30 minutes.

The supernatant was applied to a Con A Sepharose column and eluted by 0.3 M α-methyl-D-mannoside. The fractions containing DPIV activity were pooled and dialyzed against PBS containing 5 mM EDTA and 0.1% Triton X-100.

Rat spleen DPIV: Spleens of Lewis rats were cut into pieces and filtered with cell strainer (Falcon, 70 μm). NH_4Cl and Tris were added to the filtrate at 5.8 mM and 2.7 mM, respectively , and then Triton X-100 was added at 0.1%. After 30 minutes, the suspension was centrifuged at 2,500 *g* for 30 minutes, giving the supernatant having DPIV activity.

Enzyme Assays

DPIV activity was measured by the method of NAGATSU *et al.*[18] The substrate (50 μl of 3 mM Gly-Pro-*p*NA·Tos) and a sample (10 μl) to be tested were preincubated for 15 minutes at 37°C. To the mixture, 5 μl of DPIV solution (50 mU/ml, from rat kidney), 10 μl of 710 mM glycine buffer (pH 8.7) and 25 μl of distilled water were added. The enzyme reaction was carried out at 37°C and the absorbance at 405 nm was measured at intervals of 2 minutes for 30 minutes. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of *p*NA per minute under the assay conditions.

The following assays were carried out to assess the enzyme selectivity of DPIV inhibitors. All of the assays were done at 37°C for 30 minutes, and the amounts of the product (*p*NA) were determined by measuring the absorbance at 405 nm kinetically (2 minutes intervals).

Trypsin assay[19]: Reaction mixture contained 10 μl of 60 μg/ml trypsin, 30 μl of 100 mM Tris buffer (pH 8.0) containing 10 mM $CaCl_2$ and 10 μl of a sample . The mixture was incubated at 37°C for 15 minutes. The reaction was started by adding 50 μl of 2.4 mM benzoyl-DL-Arg-*p*NA.

Prolyl endopeptidase assay[20]: Reaction mixture containing 10 μl of 300 mU/ml prolyl endopeptidase, 60 μl of 100 mM phosphate buffer (pH 7.0) and 10 μl of a sample was incubated at 37°C for 15 minutes. Then 20 μl of 1.5 mM *Z*-Gly-Pro-*p*NA was added to the mixture.

Proline aminopeptidase assay[21]: Reaction mixture contained 10 μl of 0.5 U/ml proline aminopeptidase, 60 μl of 100 mM Tris buffer (pH 7.5), 10 μl of sample and 20 μl of 1 mM Pro-*p*NA.

Leucine aminopeptidase assay[19]: Reaction mixture contained 20 μl of 100 U/ml leucine aminopeptidase, 60 μl of 50 mM Tris buffer (pH 7.5), 10 μl of sample and 10 μl of 2 mM Leu-*p*NA.

Cathepsin C assay[22]: Reaction solution contained 10 μl of 5 U/ml cathepsin C, 10 μl of a sample, 70 μl of 150 mM phosphate buffer (pH 6.8) supplemented with 1 mM dithiothreithol and 10 μl of 2 mM Gly-Phe-*p*NA.

Subtilisin assay[23]: Reaction solution contained 10 μl of 0.5 U/ml subtilisin, 70 μl of 50 mM Tris buffer (pH 7.5) supplemented with 1 mM $CaCl_2$, 10 μl of a sample and 10 μl of 950 μM *Z*-Ala-Ala-Leu-*p*NA.

Results

Taxonomy

The fungal strain, A374, was found to produce three compounds, TMC-2A (**1**), -2B (**2**), and -2C (**3**) with DPIV inhibitory activity. Cultural characteristics of this strain is summarized in Table 1 (see Fig. 2 also). The maximum temperature for growth was 45°C when tested on solid media.

Conidial heads showed typically barrel form to loosely columnar on MEA, with size of 160~200×50~100 μm, but radiate heads were also present on CYA, with size of 20~50 μm in diameter. Stipes were 23~1,000×6.5~8 μm, thick, erect, mostly roughened, occasionally septate, and with a distinctive foot cell. Vesicles were globose to subglobose, sometimes clavate and colorless, with size of 19~27×17~24 μm. Aspergilla were almost exclusively uniseriate on MEA, rarely biseriate on CYA (Fig. 2). Phialides were bottle-shaped and yellowish olive, with size of 13×2.5~3.5 μm. Conidia were brown, subglobose and distinctly roughened on MEA to finely roughened on CYA, with size of 4.5~8.0×4.5~7.5 μm (average 5.5 μm in diameter; L/W 1.057) on MEA and 4.5~6.5 μm in diameter (average 6.0×5.5 μm; L/W 1.050) on CYA (Fig. 2).

Based on the above characteristics, strain A 374 was identified as *Aspergillus oryzae* (Ahlburg) Cohn.

Fermentation

A typical time course of the production of TMC-2 compounds in a 30-liter jar fermentor is shown in Fig. 3. The fermentation broth was centrifuged and the supernatant was fractionated by Molcut II (Millipore Ltd.; exclusion molecular weight, 10,000). Low molecular weight fractions were used to measure DPIV inhibitory activity. The amount of TMC-2 compounds produced was assessed by the inhibitory activity: one unit of the inhibitory activity corresponded to 0.44 μg of TMC-2A. The apparent DPIV inhibitory activity was detected on day 2, and the production reached a maximum of 160

Table 1. Cultural characteristics of strain A374, producing TMC-2 compounds.

Media*	Diameter of colony (mm)	Color		Texture and characteristics	Pigment or exudate
		Surface	Reverse		
CZA	52~53	Strong yellow (2.5Y7.5/12)	Light reddish yellow to grayish yellow (2.5Y7.5/12)	Veltinous; powdery appearance due to abundant sporulation; rather irregularly lobate margins	None
CYA	60~71	Dull greenish yellow, dark yellow to grayish deep greenish yellow (5Y6/6~10Y5/6); conidial area turned to olive brown (5Y4/6) after 2 weeks	Dull yellow to dull greenish yellow (10YR8/4~5Y8-9/4-6)	Velutinous; conidial area formed on a tough mycelial mat; occasionally floccose due to colorless mycelial overgrowth	None
CYA**	41~51	Dark yellow to deep greenish yellow (5Y5/8~10Y5/8)	ND***	Velutinous	None
MEA	67~71	Dull greenish yellow (10Y8/2)	Pale yellow to grayish yellow (10Y6~7/6)	Velutinous; no teleomorphic structures	None
MY20	74~78	Dull greenish yellow (10Y8/2)	Pale yellow to grayish yellow (10Y6~7/6)	Velutinous with abundant sporulation	None
MY40Y	74~78	Dull greenish yellow (10Y8/2)	Pale yellow to grayish yellow (10Y6~7/6)	Velutinous with abundant sporulation	None

* Strain A 374 was cultured at 25℃ for 7 days.
** The plate was incubated at 37℃ for 7 days.
*** Not determined.

Fig. 2. Cultural and morphological characteristics of *Aspergillus oryzae* A374.

A, colonies on CYA after 7 days at 25°C. B, colonies on MEA after 7 days at 25°C. C, uniseriate aspergillum with rough stipe, bar = 10 μm. D, lobate-reticulate conidia by SEM, bar = 3 μm. E, conidia, bar = 10 μm.

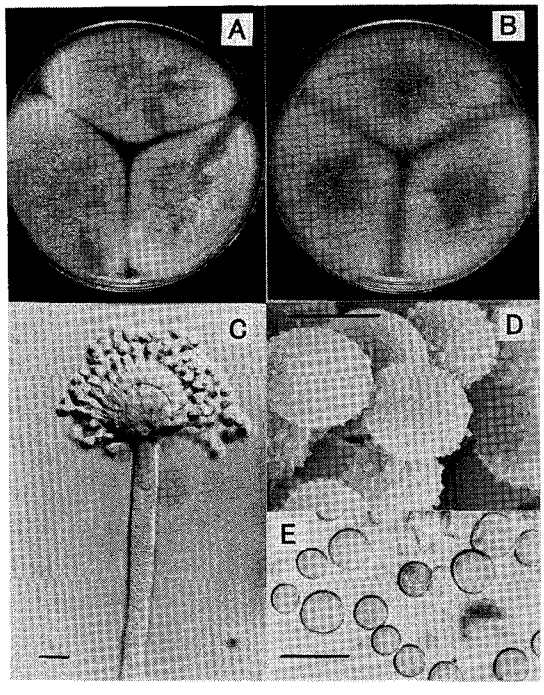
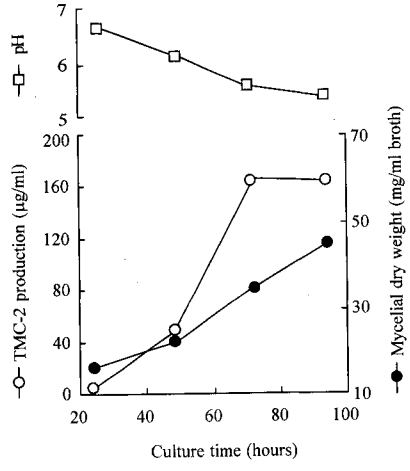


Fig. 3. Time course of TMC-2 production in a 30-liter jar fermentor.



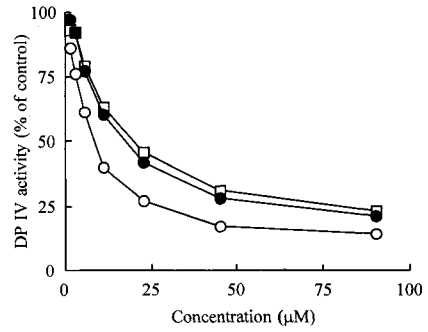
μg/ml on day 3 to 4.

Biological Properties

TMC-2A, -2B, and -2C inhibited the activity of DPIV from rat kidney in a dose-dependent manner (Fig. 4). IC_{50} values of TMC-2A, -2B, and -2C were calculated to be 8.1, 17, and 20 μM, respectively. We tested the inhibitory activity of TMC-2 compounds against DPIV from other sources (human mononuclear cells, human adenocarcinoma Caco-2 cells and rat spleen) (Table 2). TMC-2 compounds were confirmed to inhibit both

Fig. 4. Inhibition of DPIV by TMC-2A (**1**), -2B (**2**), and -2C (**3**).

○ TMC-2A, ● TMC-2B, □ TMC-2C.



The control reaction mixture without the inhibitors liberated 250 pmol of *p*NA per minute, and represents 100%.

Fig. 5. Plots of 1/v versus 1/[S] in the presence of TMC-2A.

○ None, ● 6 μM TMC-2A, □ 12 μM TMC-2A.

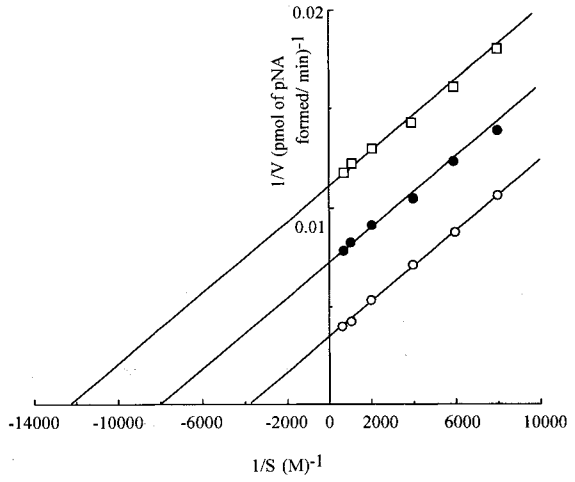


Table 2. Inhibitory activities of TMC-2 compounds against the DPIVs from various sources.

Enzyme source	IC_{50} (μM)		
	TMC-2A	TMC-2B	TMC-2C
Human mononuclear cell	10	16	27
Human Caco-2 cell	4.4	5.8	9.6
Rat spleen	9.1	12	31
Rat kidney	8.1	17	20

Table 3. Effects of TMC-2 compounds on various proteases.

Enzyme	IC_{50} (μM)		
	TMC-2A	TMC-2B	TMC-2C
DPIV	8.1	17	20
Prolyl endopeptidase	>100	>100	>100
Subtilisin	>100	>100	>100
Trypsin	>100	>100	>100
Cathepsin C	>100	ND*	ND*
Proline aminopeptidase	>100	>100	>100
Leucine aminopeptidase	>100	>100	>100

*ND: not determined.

human and rat DPIVs, irrespective of tissues and cells of the enzyme sources.

We performed a detailed kinetic analysis to elucidate the mechanism of inhibition of TMC-2 compounds (Fig. 5). The *Km* value of DPIV from rat kidney was 260 μM in our assay system. A reciplocal plot analysis revealed that TMC-2A behaved as an uncompetitive inhibitor. The *Ki* value of TMC-2A was calculated to be 5.3 μM. In contrast, diprotin A, a known peptide inhibitor of microbial origin, was confirmed to be a competitive inhibitor with a *Ki* value of 9.3 μM (data not shown).

To investigate their enzyme selectivity, we tested the inhibitory activity of TMC-2 compounds towards other serine proteases (proryl endopeptidase, subtilisin, and trypsin), cysteine proteases (cathepsin C and proline aminopeptidase), and a metalloprotease (leucine aminopeptidase). TMC-2 compounds did not inhibit these proteases tested (Table 3).

Discussion

In the cource of screening of microbial products for DPIV inhibitors, we found the novel compounds, TMC-2A (**1**), -2B (**2**) and -2C (**3**), from the culture broth of *Aspergillus oryzae* A374.

Serine proteases had been classified into two subgroups, namely the chymotrypsin and subtilisin families. These two families have unrelated tertiary structure but possess similar catalytic triad residues: His^{57}-Asp^{102}-Ser^{195} in chymotrypsin and Asp^{32}-His^{64}-Ser^{221} in subtilisin[24,25]. Recently, some serine hydrolases, including acyl-amino-acid hydrolase, proryl endopeptidase and DPIV, were found to contain a conserved stretch of *ca.* 200 amino acid residues, in which the catalytic triad residues (Ser^{624}-Asp^{702}-His^{734} in DPIV[5,25]) were organized in a novel sequential order different from those of chymotrypsin and subtilisin. These serine hydrolases were referred to as nonclassical serine hydrolase. They have molecular masses much larger than those of the chymotrypsin and subtilisin families.

TMC-2 compounds inhibited only DPIV among proteases, including the classical (trypsin and subtilisin)

and the nonclassical (prolyl endopeptidase). The high selectivity of TMC-2 compounds to DPIV might result from its uncompetitive inhibitory mechanism. A typical uncompetitive inhibitor binds reversibly to the enzyme-substrate complex yielding an inactive ESI complex. It would be intriguing to know whether the binding site of the TMC-2 compounds represents a site of biological significance.

All of the DPIV inhibitors previously reported, chemically synthesized compounds ((ω-*N*-(*O*-acyl)hydroxyamid) aminodicarboxylic acid pyrrolidides[26], aminoacylpyrrolidine-2-nitriles[27], and Ala-Boro-Pro[28]) and a microbial metabolite (diprotin A[12]), are competitive inhibitors. TMC-2 compounds might provide a new tool for investigating the structure and function of DPIV. Studies on the anti-inflamatory activity of TMC-2 compounds are now in progress.

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