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

II. Isolation and Structure Determination

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New dipeptidyl peptidase IV inhibitors, TMC-2A, -2B, and -2C, were isolated from the fermentation broth of *Aspergillus oryzae* A374. On the basis of chemical, spectroscopic and X-ray crystallographic analyses, their structures were established to be peptide-like compounds composed of three moieties, L-tryptophan, mono- or dihydroxy-L-leucine and highly substituted isoquinoline.

In our search for dipeptidyl peptidase IV (DPIV) inhibitors from microbial metabolites, we discovered three novel inhibitors, TMC-2A (**1**), -2B (**2**) and -2C (**3**) (Fig. 1) produced by *Aspergillus oryzae* A374. Our previous paper[1] has described the taxonomy, the fermentation, and the biochemical properties of **1**, **2** and **3**. This paper deals with the isolation, physico-chemical properties and structural determination of these inhibitors.

Results

Isolation

The isolation of TMC-2 compounds from the fermentation broth is outlined in Fig. 2. The fractions containing TMC-2 compounds were identified by assays of DPIV inhibitory activity[2]. Purified **1**, **2** and **3** were isolated as white powder, and **1** was crystallized from aqueous butanol to give fine needles.

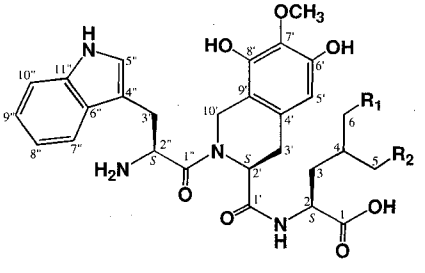
Physico-chemical Properties

The physico-chemical properties of **1**, **2** and **3** are summarized in Table 1. These compounds were soluble in water and methanol but practically insoluble in chloroform, ethyl acetate and acetone. They gave positive color reactions to ninhydrin and ammonium molybdate-sulfonic acid reagents. The molecular formulas of **1**, **2** and **3** were determined to be $C_{28}H_{34}N_4O_9$, $C_{28}H_{34}N_4O_8$ and $C_{28}H_{34}N_4O_8$, respectively, on the basis of their elemental analysis, (HR-)FAB-MS, ^{1}H- and ^{13}C-NMR spectral data. All of the three compounds showed similar UV absorption maxima at 270~290 nm, suggesting the presence of the same unsaturated system. Their IR spectra indicated that they were in the nature of peptides.

Structure of TMC-2A (1)

The ^{13}C NMR spectrum of **1** displayed 28 signals composed of $-CH_2-\times 6$, $>CH-\times 4$, $=CH-\times 6$, $>C<\times 8$, $CH_3-O\times 1$ and $>C=O\times 3$ (Table 2). The ^{1}H NMR data showed 25 protons (Table 3). The ^{1}H-^{1}H COSY spectrum of **1** connected a sequence of 2-H to 6-H, 2′-H to 3′-H, 7″-H to 10″-H (Fig. 3). Further analyses by the ^{1}H-^{13}C long range COSY and the chemical shift led us to interpret the presence of three units, leucine, tetrahydroisoquinoline, and tryptophan, as follows (Fig. 3). (1) A leucine residue with two hydroxy groups attached

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



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

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

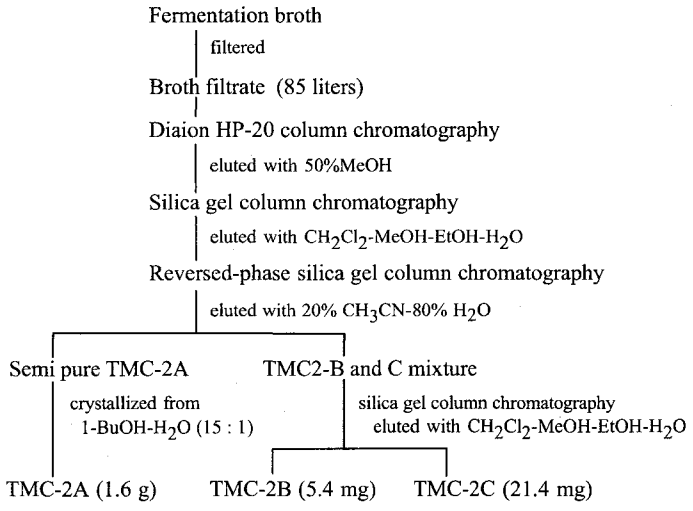


Table 1. Physico-chemical properties of TMC-2 A (**1**), B (**2**) and C (**3**).

	1	**2**	**3**
Appearance	White powder	White powder	White powder
MP	166-168 ℃ (dec)	168-169 ℃ (dec)	175-181 ℃ (dec)
$[\alpha]_D^{24}$	+2.39(*c* 0.2, H_2O)	+11.42(*c* 0.1, H_2O)	-17.5(*c* 0.1, MeOH)
FAB-MS (m/z)	571 $(M+H)^+$	555 $(M+H)^+$	555 $(M+H)^+$
HRFAB-MS (m/z)			
Found	571.2402		
Calcd	571.2403		
	for $C_{28}H_{35}N_4O_9$		
Molecular formula	$C_{28}H_{34}N_4O_9$	$C_{28}H_{34}N_4O_8$	$C_{28}H_{34}N_4O_8$
Elemental analysis			
Found	C 55.55, H 6.33, N 9.09		
Calcd	C 55.45, H 6.27, N 9.24		
	for $C_{28}H_{35}N_4O_9 \cdot 2H_2O$		
UV λ_{max} (MeOH)nm (ε)	212 (9,300)	212 (9,200)	212 (10,400)
	272 (sh 1,550)	272 (sh 1,500)	272 (sh 1,650)
	280 (1,800)	280 (1,650)	280 (1,750)
	288 (1,350)	288 (1,200)	288 (1,300)
IR ν_{max} (KBr) cm^{-1}	3380, 1640, 1520	3380, 1640, 1520	3380, 1640, 1520
Solubility			
Soluble	H_2O, CH_3OH	H_2O, CH_3OH	CH_3OH
Insoluble	$CHCl_3$	$CHCl_3$	$CHCl_3$
Rf. (Silica gel TLC)[*1]	(1) 0.45	(1) 0.51	(1) 0.49
	(2) 0.30	(2) 0.58	(2) 0.52
Rt.(minutes)[*2]	(1) 3.27	(1) 3.82	(1) 3.83
	(2) 12.17	(2) 12.83	(2) 12.90

[*1] Solvent system: (1)CH_2Cl_2 -MeOH - EtOH - H_2O (10 : 4 : 4 : 2)
(2) EtOAc - MeOH -H_2O (10 : 10 : 2)

[*2] Solvent system: (1) CH_3CN20%-H_2O 80% (1.2ml/min.)
(2) Gradient 0-25min; CH_3CN10-35%,H_2O90-65% (1.2ml/min.)
Column YMC-ODS-AM Φ4.6mm × 100mm

Table 2. ^{13}C NMR data of TMC-2 A (**1**), B (**2**) and C (**3**).

Position	1[a]	2[a]	3[b]
1	181.4, s[c]	181.8, s	180.4, s
2	55.5, d	56.1, d	54.4, d
3	32.2, t	33.2, t	32.5, t
4	41.4, d	37.7, d	33.9, d
5	62.7, t	67.6, t	16.3, q
6	65.6, t	19.9, q	69.4, t
1'	174.1, s	174.2, s	172.7, s
2'	59.3, d	59.5, d	59.0, d
3'	33.1, t	34.2, t	37.7, t
4'	132.0, s	132.2, s	130.8, s
5'	109.9, d	110.2, d	108.5, d
6'	151.2, s	151.6, s	151.6, s
7'	137.1, s	137.5, s	136.6, s
8'	148.6, s	148.9, s	148.8, s
9'	113.6, s	114.0, s	112.2, s
10'	41.6, t	41.9, t	40.6, t
1"	173.7, s	174.2, s	172.2, s
2"	54.8, d	55.0, d	54.3, d
3"	30.5, t	30.9, t	30.2, t
5"	128.0, d	128.3, d	126.4, d
4"	109.1, s	109.3, s	109.0, s
6"	129.2, s	129.4, s	128.9, s
7"	120.9, d	121.2, d	120.1, d
8"	122.6, d	122.9, d	121.4, d
9"	125.2, d	125.5, d	124.0, d
10"	114.9, d	115.2, d	113.5, d
11"	139.0, s	139.3, s	138.9, s
OCH_3	63.5, q	63.8, q	61.7, q

[a] 100MHz in D_2O at 30°C.
[b] 100MHz in CD_3OD at 50°C
[c] Multiplicity.

at C-5 and C-6 was deduced from the long range coupling from 2-H (δ 4.11) to carbonyl C-1 (δ 181.4), and the chemical shifts of C-5 (δ 62.7) and C-6 (δ 65.6). (2) The tetrahydroisoquinoline skeleton with a methoxy and two hydroxy groups was inferred from the data of: i) the long range coupling from 3'-H_2 (δ 2.35, 1.14) to C-4' (δ 132.0) and C-9' (δ 113.6), from 5'-H (δ 6.02) to C-6' (δ 151.2), C-7' (δ 137.1) and C-9' (δ 113.6), from 10'-H_2 (δ 4.82 and 3.76) to C-4', C-8' (δ 148.6) and C-9' and from 7'-H_3 (δ 3.73) to C-7', and ii) the chemical shift of 10'-CH_2 (δ_C 41.6, δ_H 4.82 and 3.76) and 2'-CH (δ_C 59.3, δ_H 3.73), suggesting attachment of C-2' and C-10' to N atom. (3) The tryptophan residue was deduced on the basis of the long range couplings from 5"-H (δ 7.34) to C-4" (δ 109.1), C-6" (δ 129.2) and C-11" (δ 139.0), from H-10" (δ 7.46) and H-8" (δ 7.07) to C-6", from H-9" (δ 7.13) to C-7" (δ 120.9) and C-11" (δ 139.0), from H-2" (δ 4.50) to C-1" (δ 173.7), and the chemical shifts of C-2" (δ_C 54.8, δ_H 4.50).

A linkage among the three moieties was established from the data of the long range couplings observed in selective INEPT (optimized at 3.3 and 5 Hz) and NOESY experiments (Fig. 3). Couplings from H-2 to C-1' (δ_C 174.1), from H-2" to C-1" (δ 173.7) and from H-10' to

Table 3. ^1H NMR data of TMC-2 A (**1**), B (**2**), and C (**3**).

Position	1[a]	2[a]	3[b]
2	4.11 (1H, dd, 3.7, 7.8)[c]	4.04 (1H, dd, 4.0, 11.0)	4.18 (1H, t, 7.8)
3a	1.68 (1H, ddd, 3.8, 10.6, 14.5)	1.64 (1H, ddd, 3.9, 9.3, 13.6)	1.45 (2H, ddd, 7.9, 14.3)
3b	1.44 (1H, ddd, 3.8, 10.6, 14.5)	1.22 (1H, m)	↑
4	0.71 (1H, m)	0.63 (1H, m)	0.58 (1H, m)
5a	3.49-3.28 (1H, m)	3.08 (2H, d, 3.8)	0.50 (3H, d, 5.8)
5b	1.14 (1H, dd, 5.0, 15.2)	↑	-
6a	3.49-3.28 (1H, m)	0.63 (3H, m)	3.12 (2H, d, 5.4)
6b	3.19 (1H, dd, 4.9, 11.6)	-	↑
2'	3.73 (1H, m)	3.75-3.70 (1H, m)	3.59 (1H, dd, 2.7, 5.3)
3'a	2.35 (1H, dd, 2.7, 15.2)	2.39 (1H, dd, 2.9, 15.4)	2.37 (1H, dd, 2.7, 14.7)
3'b	1.14 (1H, dd, 5.0, 15.2)	1.22 (1H, m)	2.37 (1H, dd, 5.3, 14.7)
5'	6.02 (1H, s)	6.09 (1H, s)	5.93 (1H, s)
10'a	4.82 (1H, m)	4.80 (1H, d, 16.3)	5.06 (1H, d, 16.3)
10'b	3.76 (1H, m)	3.83 (1H, d, 16.3)	3.69 (1H, d, 16.4)
7'-OCH_3	3.73 (3H, m)	3.75-3.70 (3H, m)	3.74 (3H, s)
2"	4.50 (1H, dd, 4.9, 10.8)	4.50 (1H, dd, 5.0, 9.7)	4.25 (1H, t, 7.8)
3"a	3.49-3.28 (1H, m)	3.51 (1H, dd, 4.7, 13.6)	3.36 (2H, d, 7.8)
3"b	3.49-3.28 (1H, m)	3.41 (1H, dd, 11.2, 13.6)	↑
5"	7.34 (1H, s)	7.38 (1H, s)	7.24 (1H, s)
7"	7.51 (1H, d, 7.8)	7.16 (1H, t, 7.2)	7.53 (1H, d, 7.8)
8"	7.07 (1H, t, 7.3)	7.16 (1H, t, 7.2)	7.06 (1H, ddd, 1.0, 7.5, 8.5)
9"	7.13 (1H, t, 7.4)	7.22 (1H, t, 7.2)	7.14 (1H, ddd, 1.1, 7.5, 8.6)
10"	7.46 (1H, d, 8.1)	7.52 (1H, d, 7.9)	7.40 (1H, d, 7.4)

[a] 400 MHz in D_2O at 30°C
[b] 400MHz in CD_3OD at 50°C
[c] Proton number, multiplicity and coupling constants in Hz.

Fig. 3. Structure of TMC-2A (**1**) from NMR data.

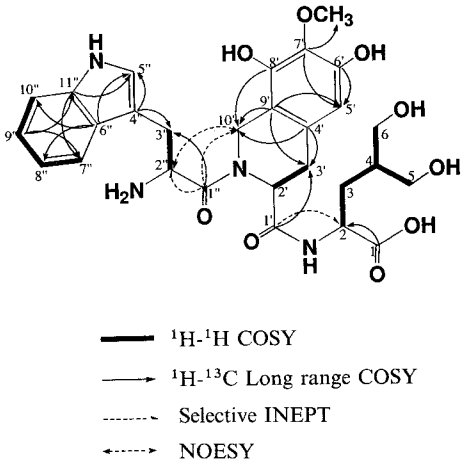
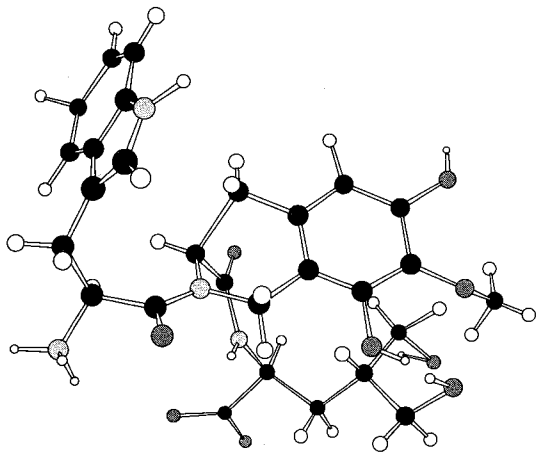


Fig. 4. Perspective view of the molecular structure of TMC-2A (**1**).



C-1″, and NOE between H-10′ and H-2″ were observed. Based on these results, we determined the planar structure of **1** as illustrated in Fig. 3.

Absolute Stereochemistry

The three dimensional structure of **1** was determined by X-ray analysis (Fig. 4). Acid hydrolysate of **1** with 4 N methane sulfonic acid was analyzed by chiral TLC with authentic D- and L-tryptophans. The hydrolysate gave a spot with Rf value identical to that for L-tryptophan. Thus, the absolute structure of **1** was determined to be (2*S*,2′*S*,2″*S*) 2-[2′-[2″-amino-3″-(indol-3‴-yl)-1″-oxopropyl]-1′,2′,3′,4′-tetrahydro-6′,8′-dihydroxy-7′-methoxyisoquinol-3-yl-carbonylamino]-4-hydroxymethyl-5-hydroxypentanoic acid.

Structure of TMC-2B (2) and C (3)

The molecular formulas of **2** and **3** ($C_{28}H_{34}N_4O_8$) suggested that **2** and **3** were the deoxy derivatives of **1**. The ^{13}C NMR data of **2** and **3** were very similar to that of **1** except for the signals of C-4, C-5 and C-6. The methyl carbons at δ 19.9 (**2**) and δ 16.3 (**3**) and the hydroxymethyl carbons at δ 67.6 (**2**) and δ 69.4 (**3**) were observed in place of the corresponding two hydroxymethyl carbons (C-5: δ 62.7 and C-6: δ 65.6) of **1**. In addition, the C-4 methine carbons at δ 41.4 of **1** moved up field to δ 37.7 and 33.9 in **2** and **3**, respectively. Therefore, we concluded that **2** and **3** were C-5 and C-6 deoxy analogs of **1**, respectively (Fig. 1).

Discussion

In this study, we determined the chemical structure of novel DPIV inhibitors, TMC-2A, -2B and -2C, produced by *Aspergillus oryzae* A374. These three compounds were peptide-like and contained L-tryptophan, mono- or dihydroxy-L-leucine and a highly substituted isoquinoline. UMEZAWA *et al.*[2] have reported microbial DPIV inhibitors, diprotins A (Ile-Pro-Ile), and B (Val-Pro-Leu), which have a proline residue at a second position. These compounds are utilized as a substrate of DPIV[3]. On the other hand, TMC-2A is an uncompetitive inhibitor[1] and hence is not degraded by DPIV.

Many compounds containing leucine analog have been reported, *e.g.* leucinostatins, antimicrobial peptide antibiotics, which contain β-hydroxy-L-leucine[4]. δ-Dihydroxy-L-leucine, a constituent of TMC-2A, was discovered for the first time in these natural products, although it had been synthesized as a reduction product of γ-carboxyglutamic acid[5]. The DPIV-inhibiting activity of TMC-2A was more potent than those of TMC-2B and -2C, inferring that the hydroxy group on the leucine residue might be important for the enzyme inhibiting activity. Chemical modification of this leucine moiety might therefore enhance the above activity.

Many higher plants contain polysubstituted tetrahydroisoquinolines, which are derived from L-tyrosine[6,7]. However, 7-methoxy-6,8-dihydroxytetrahydroisoquinoline, a constituent of TMC-2s, has not been reported as a natural product so far. This unique substituted isoquinoline, chemically synthesized, has been described as an intermediate for total synthesis of corpaverine, a plant metabolite[8].

DPIV is widely distributed in cells of animals, plants, fungi and bacteria. Several papers have described that this enzyme might participate in the activation of T cells

and involved in immunological disorders in animals[9]. Some bacteria have been reported to have DPIV, of which physiological functions have not been clarified[10]. TMC-2s are useful as tools for elucidation of the cellular role of DPIV.

Experimental

General

^1H and ^{13}C NMR spectra were obtained with a JEOL GSX-400 NMR spectrometer and chemical shifts are given in ppm (δ) relative to sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal standard. Mass spectra, UV spectra and IR spectra were obtained by using a JEOL JMS HX-100 spectrometer, a Shimadzu model UV-2200A spectrophotometer and a JASCO model 100 infrared spectrophotometer, respectively. Melting points were determined with a Yanaco MP-2S micro melting point apparatus and were uncorrected. Optical rotation was determined with a Horiba model SEPA-200 high sensitive polarimeter.

Isolation of 1, 2 and 3

The fermentation broth was obtained as described in the preceding paper and the mycelium was removed by filtration. The broth filtrate (85 liters) was applied to a column (i.d. 100 × 900 mm) of Diaion HP-20 (Mitsubishi Chemical Industries). The column was washed with water (5 liters) and 20% MeOH (30 liters), followed by the elution of the active principle with 50% MeOH (75 liters). The eluate was concentrated to dryness. The residual solid was applied to silica gel column chromatography (i.d. 60 × 900 mm, Wakogel C-300, Wako Pure Chemical industries), and developed with CH_2Cl_2-MeOH-EtOH-H_2O (10:4:4:1 and 10:4:4:2). The active fraction concentrated was subjected to reversed-phase silica gel chromatography (i.d. 44 × 700 mm, YMC-ODS-A60, YMC) and eluted with 10% CH_3CN. The major active fraction containing **1** was eluted first, and then the mixture of **2** and **3**. Each of the active eluates was evaporated under a reduced pressure. The semi-pure solid of **1** was dissolved in 1-butanol-water (15:1) at 50°C and crystallized at a room temperature, giving fine needles of **1** (1.6 g). The mixture of **2** and **3** was rechromatographed on silica gel, and developed with CH_2Cl_2-MeOH-EtOH-H_2O (10:4:4:1), yielding pure **2** (5.4 mg) and **3** (21.4 mg).

X-Ray Crystallography of 1

A colorless plate crystal of **1** with dimensions 0.50 × 0.40 × 0.10 mm was obtained by recrystallization from an aqueous solution and used for X-ray analysis. The intensity data were collected on a Rigaku AFC5R diffractometer by using graphite-monochromated Cu-Kα (λ=1.5418 Å) radiation by ω-2θ scan technique. Unit cell dimensions were determined by a least squares refinement by using the setting of 25 reflections in the range of $35° < \theta < 45°$. The crystallographic data are summarized as follows: $C_{28}H_{34}N_4O_9 \cdot 3H_2O$, Mr=624.64, plate, $P2_12_12_1$, a=10.688(2) Å, b=29.473(2) Å, c=9.314(1) Å, V=2934.0(7) Å^3, Z=4, D_{calc}=1.414 g/cm^3, μ=0.938 mm^{-1}. The intensities of 2096 reflections with $3.0° < \theta < 60.1°$, $0 < h < 12$, $0 < k < 33$, $0 < l < 10$ were measured. Three standard reflections were monitored every 200 reflection intervals and showed insignificant fluctuations. The data were corrected for Lorentz and polarization effects, but not for absorption.

The structure was solved by a direct method by using SHELXS-86[11] and the subsequent difference Fourier method. The structure refinement on F2 was carried out by a SHELXL-93[12] with anisotropic thermal parameters for all of non-hydrogen atoms. Except for those of water of crystallization, the hydrogen atoms were refined by riding with the atoms to which they were bonded. Six hydrogen atoms of crystal solvent were geometrically located and fixed. The full matrix least squares refinement varied 403 parameters and used all 2096 independent reflections weighted by $\omega = 1/[\sigma^2(Fo^2) + (0.0692P)^2 + 1.6836P]$ where $P = (Fo^2 + 2Fc^2)/3$. Final $R1$=0.0497, $wR2$=0.1167 and *Goodness of Fit*(S)=0.994 for all data; $R1$=0.0489 for 2059 reflections with $I > 2\sigma(I)$. The final difference Fourier map showed maximum and minimum values of 0.275 and 0.243 e$^-$/Å^3, respectively.

Analysis of the Amino Acids

TMC-2A (**1**) was hydrolyzed with 4 N methane sulfonic acid at 115°C for 12 hours in a sealed tube. The hydrolysate was analyzed by chiral TLC (HPTLC plates CHIR, Merck) with a solvent of MeOH-H_2O-CH_3CN (1:1:4), giving a spot of L-tryptophan (Rf 0.88, *cf* Rf 0.81 for D-tryptophan).

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