TMC-1 A, B, C and D, New Antibiotics of the Manumycin Group Produced by *Streptomyces* sp.

Taxonomy, Production, Isolation, Physico-chemical Properties, Structure Elucidation and Biological Properties

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Four new antitumor antibiotics, TMC-1 A, B, C and D were isolated from a fermentation broth of *Streptomyces* sp. A-230. Spectroscopic studies have shown that TMC-1 A to D were new members of the manumycin class of antibiotics. These antibiotics showed cytotoxic activities against various tumor cell lines *in vitro*.

In the course of our screening for antitumor agents, seven structurally related active compounds, designated as TMC-1 A (**1**) to G (**7**), have been isolated from an actinomycete strain A-230. Of them, TMC-1 A (**1**), B (**2**), C (**3**) and D (**4**) were found to be new members of manumycin class of antibiotics, represented by manumycins A to G[1~5], asukamycin[6], U-62162[7], U-56,407[8], colabomycin[9], alisamycin[10] and nisamycin[11]. The rest of TMC-1 compounds, TMC-1 E (**5**), F (**6**) and G (**7**), were identified as manumycins D, A and C, respectively, on the basis of their spectral data[3,4]. This paper describes taxonomy of the producing strain, production, isolation, physico-chemical properties, structure elucidation and biological properties of these four new antibiotics.

Results

Taxonomy

The producing strain A-230 was isolated from a soil sample collected in Kochi-shi, Kochi Prefecture, Japan. Taxonomic studies were carried out according to the method of the International Streptomyces Project (ISP)[12]. The strain A-230 has branched substrate mycelium and aerial hyphae. Spiral spore chains of 10 to 30 or more spores were observed at the tips of aerial hyphae on the various ISP agar media. Fragmentation of substrate mycelium, sclerotium and sporangia-like vesicles was not observed. The cultural characteristics of strain A-230 on various agar media are summarized in Table 1. The physiological properties and the utilization of carbohydrates are shown in Table 2.

Whole-cell hydrolysate of the strain contained LL-diaminopimeric acid, which placed the strain in the Type I cell wall group. Microscopic studies and cell wall analysis of strain A-230 indicated that the strain belonged to the genus *Streptomyces*. This strain was deposited as *Streptomyces* sp. A-230 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession number of FERM P-14460.

Production

The producing strain A-230 was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of the medium consisting of glucose 1.0%, dextrin 2.0%, bactosoytone (Difco Co.) 1.5%, yeast extract (Asahi Brewery Co.) 0.1% and $CaCO_3$ 0.3% (pH 7.0, before autoclaving) and cultivated for 5 days at 27°C on a rotary shaker (220 rpm). The entire volume of the seed culture thus obtained was transferred to a 50-liter jar fermenter containing 18 liters of a producing medium having the same composition as the vegetative medium. The fermentation was carried out for 5 days at 27°C at an agitation rate of 250 rpm and an aeration rate of 5.4 liters/minute. The production of the antibiotics was monitored by cytotoxicity against HCT-116 cells.

Isolation

The isolation and purification procedures for each TMC-1 component are summarized in Fig. 1. The fermentation broth was extracted with 1-butanol and the extract was purified by solvent partition to give a crude

Table 1. Cultural characteristics of strain A-230.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract-malt extract agar (ISP No.2)	Good	Abundant, light gray (4.6 PB 7.2/2.7)-grayish white (5.0 PB 8.0/2.6)	Brown (6.2 YR 4.2/4.2)-light brown (6.9 YR 5.5/5.8)	None
Oatmeal agar (ISP No.3)	Good	Abundant, gray (5.4 PB 5.4/3.1)-grayish white (5.0 PB 8.0/2.6)	Dark brown (6.1 YR 3.6/2.3)-light brown (6.7 YR 5.5/4.1)	None
Inorganic salts-starch agar (ISP No.4)	Good	Abundant, grayish white (5.0 PB 8.0/2.6)	Dull red (6.3 R 5.0/6.4)	None
Glycerol-asparagine agar (ISP No.5)	Good	Abundant, light gray (4.6 PB 7.2/2.7)-grayish white (5.0 PB 8.0/2.6)	Brown (7.2 YR 3.6/2.8)-light brown (7.4 YR 4.9/3.6)	None
Peptone-yeast extract iron agar (ISP No.6)	Moderate	None	Pale yellowish brown (2.9 Y 6.7/4.2)	None
Tyrosine agar (ISP No.7)	Good	Abundant, light brownish gray (2.7 Y 6.3/1.6)	Pale yellowish brown (2.9 Y 6.7/4.2)	None
Glucose-asparagine agar	Moderate	Poor, grayish white (5.0 PB 8.0/2.6)	Pale yellow (8.2 Y 8.7/3.8)	None
Nutrient agar	Moderate	None	Light brown (6.7 YR 5.5/4.1)	None
Sucrose-nitrate agar	Poor	Moderate, grayish white (5.0 PB 8.0/2.6)	Light gray (7.9 PB 6.1/3.8)	None

Color names from Guide to Color Standard, Japan Color Research Institute.

Table 2. Physiological properties of strain A-230.

Conditions	Characteristics
Temperature range for growth	10~37 °C
Optimum temperature	27~30 °C
Formation of melanoid pigment	Negative
Liquefaction of gelatin	Positive
Coagulation of milk	Positive
Peptonization of milk	Positive
Hydrolysis of starch	Positive
Decomposition of cellulose	Negative
Reduction of nitrate	Positive
NaCl torelance (%)	8%
Utilization of carbon source *	
L-Arabinose	Positive
D-Fructose	Positive
D-Glucose	Positive
Inositol	Positive
D-Mannitol	Positive
Raffinose	Doubtful
L-Rhamnose	Positive
D-Xylose	Positive
D-Galactose	Positive
Glycerin	Positive
Mannose	Positive
Sucrose	Negative
Starch	Positive

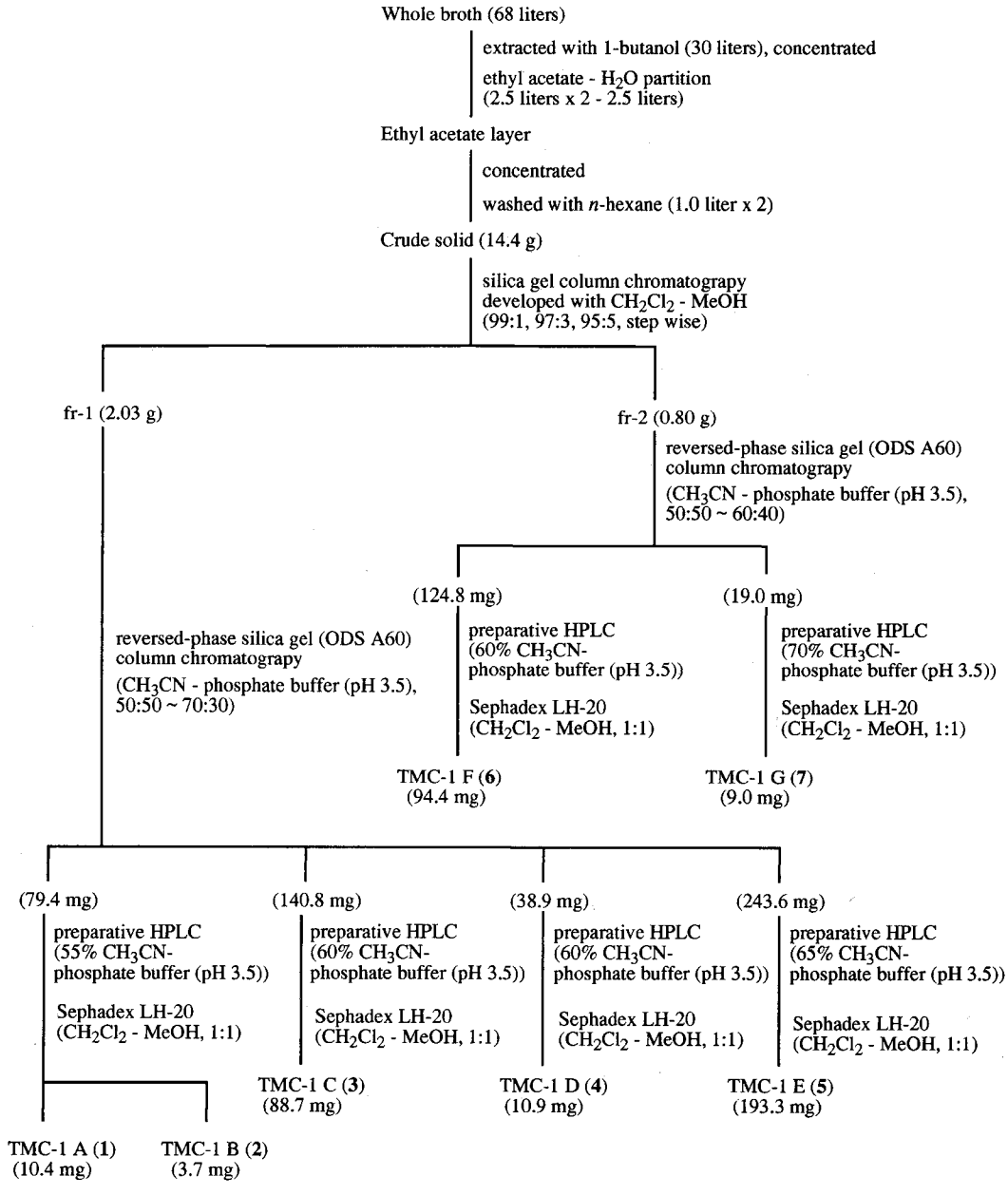
* Positive: positive utilization, Negative: no utilization.

solid (14.4 g). This solid was applied onto a silica gel column chromatography to afford two bioactive fractions. Each fraction was further purified by successive column chromatography on reversed-phase silica gel (ODS) and preparative HPLC followed by Sephadex LH-20 to give pure seven components, **1** (10.4 mg), **2** (3.7 mg), **3** (88.7 mg), **4** (10.9 mg), **5** (193.3 mg), **6** (94.4 mg) and 7 (9.0 mg).

Physico-chemical Properties

Compounds **1** to **4** were isolated as yellow amorphous powders, which were soluble in methanol, acetone, ethyl acetate, chloroform and dimethyl sulfoxide, but practically insoluble in water and *n*-hexane. These compounds gave positive color reaction to iodine vapor and ammonium molybdate-sulfic acid reagent, but negative to ninhydrin. Additional physico-chemical data are summarized in Table 3. All showed similar UV absorption maxima at 262~265 and 304~306 nm, suggesting the presence of the same unsaturated system. Their IR spectra indicated the presence of carbonyl (1660~1670 cm^{-1}), amide (1620 and 1515 cm^{-1}) and triene (1005 cm^{-1}) groups. The molecular formulas of **1** to **4** were determined as $C_{28}H_{36}N_2O_7$, $C_{28}H_{36}N_2O_7$, $C_{30}H_{38}N_2O_7$ and $C_{30}H_{40}N_2O_7$, respectively, on the basis of their HR-FAB-MS, ^1H and ^{13}C NMR spectral data.

Fig. 1. Isolation and purification procedures of TMC-1 A (**1**) to G (**7**).



Structure of TMC-1 C (3)

The ^1H and ^{13}C NMR data of **3** obtained from ^1H, ^{13}C, DEPT, and HMQC spectra are shown in Tables 4 and 5, respectively. The ^{13}C NMR spectrum displayed 30 signals composed of CH_3–C × 3, –CH_2– × 6, >CH– × 2, >C< × 1, –CH= × 10, >C= × 4 and carbonyl C × 4. The ^1H NMR spectrum showed 38 proton signals including two NH protons (δ 7.94 and 7.84), two aliphatic-OH protons (δ 4.05 and 3.36) and one hydrogen-bonding-OH proton (δ 13.64).

The ^1H-^1H COSY spectrum showed the partial structures; a triene chain (C-7 to C-12), =CH–CH(CH_3)–(CH_2)$_3$–CH_3 and –CH(OH)–CH_2– units, and the HMBC experiment established the presence and connectivities of the following three units: 1) The long range couplings from H-3 (δ 7.58) to C-1 (δ 191.8), C-2 (δ 132.1) and C-5 (δ 71.9), from H-6 (δ 2.90 and 2.75) to C-1 and C-4 (δ 73.6), and from NH-0′ (δ 7.94) to C-1 and C-3 showed the presence of an amino-cyclohexenone unit (C-1 to C-6). Furthermore, this unit was revealed to attach to the triene-carbonyl chain by the observation of the couplings from H-7 (δ 6.05) to C-4, from H-12 (δ 6.09) to C-13 (δ 165.7). 2) The long range couplings from H-3′ (δ 7.26) to C-1′ (δ 165.6), C-5′ (δ 149.4) and C-11′ (δ 12.5), and from H-11′ (δ 1.79) to C-4′ (δ 131.0) and C-5′ demonstrated the presence of the acyl chain unit

Table 3. Physico-chemical properties of TMC-1 A (**1**), B (**2**), C (**3**) and D (**4**).

	1	**2**	**3**	**4**
Appearance	Yellow powder	Yellow powder	Yellow powder	Yellow powder
MP	> 95 °C (dec)	> 75 °C (dec)	> 106 °C (dec)	> 89 °C (dec)
$[\alpha]_D^{24}$ (*c* 0.1, $CHCl_3$)	-55°±3°	~0°	+116°±1°	+16°±2°
FAB-MS (m/z)	513 $(M+H)^+$	513 $(M+H)^+$	539 $(M+H)^+$	541 $(M+H)^+$
HRFAB-MS (m/z)				
Found	513.2597	513.2599	539.2743	541.2906
Calcd	513.2601	513.2601	539.2758	541.2914
	for $C_{28}H_{37}N_2O_7$	for $C_{28}H_{37}N_2O_7$	for $C_{30}H_{39}N_2O_7$	for $C_{30}H_{41}N_2O_7$
Molecular formula	$C_{28}H_{36}N_2O_7$	$C_{28}H_{36}N_2O_7$	$C_{30}H_{38}N_2O_7$	$C_{30}H_{40}N_2O_7$
UV λ_{max} (MeOH) nm (ε)	304 (43,400)	304 (38,500)	306 (sh 47,800)	304 (42,700)
	262 (38,800)	280 (34,400)	285 (52,700)	278 (40,200)
		262 (35,600)	265 (sh 49,500)	263 (40,700)
IR ν_{max} (KBr) cm^{-1}	3395, 1670	3385, 1670	3380, 1660	3375, 1670
	1620, 1515,	1620, 1515,	1615, 1515,	1620, 1515,
	1370, 1005	1380, 1005	1350, 1005	1370, 1005

(C-1′ to C-12′). 3) The long range couplings from H-5″ (δ 2.59) to C-1″ (δ 197.5), C-2″ (δ 115.1), C-3″ (δ 174.4) and C-4″ (δ 32.2), and from NH-14 (δ 7.84) to C-3″ suggested the presence of an amino-hydroxy cyclopentenone unit (C-1″ to C-5″). In addition, the cyclohexenone-triene unit was revealed to connect to the acyl chain unit and the cyclopentenone unit through the amide linkage from the observation of the cross peaks from NH-0′ to C-1′ and from NH-14 to C-13. The configuration of the triene chain and the two double bonds was assigned as *E* on the basis of the following results: $^3J_{7,8}=14.7$ Hz, $^3J_{9,10}=14.3$ Hz, $^3J_{11,12}=14.7$ Hz, $^3J_{2',3'}=15.2$ Hz and the observation of NOE between H-3′ and H-5′. These results gave us the planar structure of **3**, as illustrated in Fig. 2.

Structure of TMC-1 D (4)

The molecular formula of **4** ($C_{30}H_{40}N_2O_7$) suggested that **4** is the dihydrogenated compound of **3**. The ^{13}C NMR spectrum of **4** (Table 5) was almost identical to that of **3** except for the signals of the acyl side chain (C-1′~C-12′). The methine carbon at C-4′ (δ 34.2) and the methylene carbon at C-5′ (δ 43.9) for **4** were observed in place of the corresponding olefinic carbon (δ 131.0 and 149.4) for **3**. Moreover, the methyl carbon at C-11′ (δ 20.4) for **4** was shifted lower field relative to that for **3** (δ 12.5). Thus, **4** is concluded to be C-4′, 5′-dihydro analog of **3**.

Structures of TMC-1 A (1), B (2), E (5), F (6) and G (7)

The molecular formulas of **1** and **2** differs from that of **3** by C_2H_2 unit. The ^1H and ^{13}C NMR data of **1** and **2** corresponded to those of **3** except for the signals of the acyl side chain as shown in Tables 4 and 5. Thus, the structures of the acyl chain of **1** and **2** were analyzed by ^1H-^1H COSY. The configuration of the double bond was determined to be *E* on the basis of the small coupling constant between C-1′ and H-3′ as shown in Fig. 3.

The physico-chemical and ^1H and ^{13}C NMR data of **5**, **6** and **7** were possibly identical to those of the reported data for manumycins D, A and C, respectively[3,4]. The identity of these compounds was confirmed by analyzing the 2D NMR data of **5**, **6** and **7**.

Stereochemistry

The relative stereochemistry of the cyclohexenone moiety of **1** to **4**, which has two chiral centers at C-4 and C-5 positions, was elucidated basing on the ^1H-^1H decoupling and the NOE experiments. The small vicinal coupling constants, $^3J_{H5,H6ax}=3.0\sim3.4$ Hz and $^3J_{H5,H6eq}=5.7\sim6.0$ Hz, indicated that H-5 proton was located at quasiequatorial position, which was supported by the observation of the long range "*W*" coupling between H-3 and H-5 ($^4J_{H3,H5}=1.0\sim1.5$ Hz). The observation of NOE between H-6ax and H-7 of the triene chain indicated that the triene chain was attached at

Table 4. ^{1}H NMR data of TMC-1 A (**1**), B (**2**), C (**3**) and D (**4**)[a].

Position	1	2	3	4
3	7.56 (1H, d, 1)[b]	7.55 (1H, d, 1)	7.58 (1H, d, 1.5)	7.58 (1H, bs)
5	4.11 (1H, m)	4.11 (1H, m)	4.11 (1H, m)	4.10 (1H, m)
6eq	2.90 (1H, dd, 6.0, 17.0)	2.89 (1H, dd, 6.0, 17.0)	2.90 (1H, dd, 6.0, 17.0)	2.89 (1H, dd, 5.7, 17)
6ax	2.75 (1H, dd, 3.3, 17.0)	2.75 (1H, dd, 3.4, 17.0)	2.75 (1H, dd, 3.4, 17.0)	2.74 (1H, dd, 3, 17)
7	6.05 (1H, d, 14.7)	6.05 (1H, d, 14.5)	6.05 (1H, d, 14.7)	6.04 (1H, d, 14.7)
8	6.52 ~ 6.61 (1H, m)	6.53 ~ 6.61 (1H, m)	6.52 ~ 6.62 (1H, m)	6.50 ~ 6.62 (1H, m)
9	6.52 ~ 6.61 (1H, m)	6.53 ~ 6.61 (1H, m)	6.52 ~ 6.62 (1H, m)	6.50 ~ 6.62 (1H, m)
10	6.38 (1H, dd, 11.4, 15)	6.39 (1H, dd, 11.4, 15)	6.36 (1H, dd, 11.4, 14.3)	6.37 (1H, dd, 11.4, 14.5)
11	7.32 (1H, dd, 11.4, 14.7)	7.32 (1H, dd, 11.4, 14.7)	7.30 (1H, dd, 11.4, 14.7)	7.31 (1H, dd, 11.4, 14.7)
12	6.05 (1H, d, 14.7)	6.04 (1H, d, 14.7)	6.09 (1H, d, 14.7)	6.08 (1H, d, 14.7)
2'			5.85 (1H, d, 15.2)	5.86 (1H, d, 15.2)
3'	6.23 (1H, dd, 1, 10)	6.44 (1H, dt, 1, 7.3)	7.26 (1H, d, 15.4)	6.77 (1H, dd, 8.3, 15.2)
4'	2.50 (1H, m)	2.19 (2H, m)		2.44 (1H, m)
5'	1.2 ~ 1.4 (2H, m)	1.1 ~ 1.5 (2H, m)	5.70 (1H, bd, 9.7)	1.2 ~ 1.4 (2H, m)
6'	1.2 ~ 1.4 (2H, m)	1.1 ~ 1.5 (1H, m)	2.52 (1H, m)	1.2 ~ 1.4 (1H, m)
7'	1.2 ~ 1.4 (2H, m)	1.1 ~ 1.5 (2H, m)	1.2 ~ 1.4 (2H, m)	1.2 ~ 1.4 (2H, m)
8'	0.88 (3H, t, 7.1)	0.88 (3H, t)	1.2 ~ 1.4 (2H, m)	1.2 ~ 1.4 (2H, m)
9'	1.91 (3H, d, 1)	1.90 (3H, bs)	1.2 ~ 1.4 (2H, m)	1.2 ~ 1.4 (2H, m)
10'	1.02 (3H, d, 6.8)	0.89 (3H, d)	0.87 (3H, t, 7.0)	0.88 (3H, t, 6.9)
11'			1.79 (3H, d, 1.1)	1.04 (3H, d, 6.6)
12'			0.99 (3H, d, 6.6)	0.85 (3H, d, 6.4)
4"	2.53 (2H, m)	2.53 (2H, m)	2.53 (2H, m)	2.53 (2H, m)
5"	2.60 (2H, m)	2.60 (2H, m)	2.59 (2H, m)	2.59 (2H, m)
14-NH	8.27 (1H, s)	8.26 (1H, s)	7.84 (1H, s)	7.89 (1H, s)
0'-NH	7.58 (1H, bs)	7.54 (1H, bs)	7.94 (1H, bs)	7.78 (1H, bs)
OH	3.34 (1H, bs)	3.20 (1H, bs)	4.05 (1H, s)	3.92 (1H, bs)
OH	2.88 (1H, bs)	2.82 (1H, bs)	3.36 (1H, bs)	3.24 (1H, bs)
3"-OH	13.52 (1H, bs)	13.51 (1H, bs)	13.64 (1H, bs)	13.63 (1H, bs)

[a] 400 MHz in $CDCl_3$ at 30°C.

[b] Proton number, multiplicity and coupling constants in Hz.

quasiaxial position. From these results, **1**~**4** were suggested to have two possible half-chair conformations (^{5}H or H_5) and the relative stereochemistry of C-4 and C-5 was assigned to be *cis* leading to (4*R*,5*S*) or (4*S*,5*R*) as shown in Fig. 4.

The absolute stereochemistry at C-4 and C-5 was elucidated by MOSHER and TROST's method[13,14]. Hydroxyl group at C-5 position of **3** was converted to (*R*)-(+)-and (*S*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) esters to give compounds **8** and **9**, respectively. The ^{1}H NMR chemical shift differences of H-6ax and H-6eq of **8** and **9** ($\Delta\delta=\delta(R)-\delta(S)$) were −0.10 and −0.06 ppm, respectively. These results clearly indicated 4*S*,5*R* configuration for **3**. Using the same procedure, manumycin D (**5**) was converted to (*R*)-(+)- and (*S*)-(−) MTPA esters, **10** and **11**. The stereochemistry of manumycin D (**5**) which had been reported as 4*S*, 5*R* or 4*R*, 5*S*[4], was determined 4*S*, 5*R*.

From these structural studies, the structures of TMC-1 A (**1**) to D (**4**) were determined as shown in Fig. 5.

Biological Activity

The cytotoxic activity of TMC-1 A (**1**) to D (**4**) and manumycin D (**5**), A (**6**) and C (**7**) against several tumor

Table 5. ^{13}C NMR data of TMC-1 A (1), B (2), C (3) and D (4)[a].

Position	1	2	3	4
1	191.8 s[b]	191.8 s	191.8 s	191.7 s
2	132.2 s	132.3 s	132.1 s	132.0 s
3	126.0 d	125.9 d	126.8 d	126.8 d
4	73.6 s	73.6 s	73.6 s	73.5 s
5	71.9 d	71.8 d	71.9 d	71.8 d
6	40.6 t	40.6 t	40.7 t	40.6 t
7	138.6 d	138.6 d	138.7 d	138.6 d
8	131.5 [c] d	131.5 [d] d	131.4 d	131.4 [e] d
9	139.7 d	139.7 d	139.8 d	139.7 d
10	131.4 [c] d	131.4 [d] d	131.3 d	131.3 [e] d
11	143.6 d	143.6 d	143.5 d	143.5 d
12	121.4 d	121.4 d	121.5 d	121.5 d
13	165.4 s	165.4 s	165.7 s	165.5 s
1'	168.1 s	168.0 s	165.6 s	165.0 s
2'	129.3 s	130.7 s	117.5 d	121.7 d
3'	144.7 d	139.0 d	148.6 d	153.2 d
4'	33.3 d	29.3 t	131.0 s	34.2 d
5'	36.6 t	35.4 t	149.4 d	43.9 t
6'	29.7 t	34.1 d	33.3 d	30.4 d
7'	22.8 t	26.3 t	36.9 t	37.1 t
8'	14.0 q	12.4 q	29.7 t	29.1 t
9'	12.6 q	11.3 q	22.8 t	23.0 t
10'	20.2 q	19.0 q	14.1 q	14.2 q
11'			12.5 q	20.4 q
12'			20.5 q	19.4 q
1"	197.3 s	-	197.5 s	-
2"	115.0 s	114.9 s	115.1 s	115.0 s
3"	-	173.8 s	174.4 s	174.3 s
4"	32.1 t	32.1 t	32.2 t	32.2 t
5"	25.6 t	25.6 t	25.7 t	25.7 s

[a] 100MHz in $CDCl_3$ at 30°C.

[b] Multiplicity.

[c,d,e] May be exchangeable.

- Not detectable.

cell lines is summarized in Table 6. All compounds showed moderate cytotoxicity to various tumor cell lines.

Manumycin A (**6**) exhibited antibacterial activity against Gram-positive bacteria; *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis* (MIC: 3.1, 12.5 μg/ml) and no activity against Gram-negative bacteria; *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and fungi; *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatas* at 100 μg/ml. TMC-1 A (**1**) to D (**4**) and manumycin D (**5**), on the other hand, showed no antimicrobial activity against above described strains at 100 μg/ml.

Discussion

In this study, we isolated new antibiotics, TMC-1 A (**1**) to D (**4**) from *Streptomyces* sp. A-230, and our structural study demonstrated that TMC-1 A to D have 4,5-dihydroxy cyclohexenone moiety in their structures of manumycin class antibiotics. Manumycin D was first reported as a manumycin group antibiotic without oxirane unit although its stereochemistry at C-4 and C-5 and biological activity had not described[4]. Here, we determined 4*S*, 5*R* configuration for TMC-1 C as well as manumycin D for the first time. TMC-1 A to D and manumycin D showed cytotoxic activities as well as manumycins A and C (Table 6). On the other hand, TMC-1 A to D and manumycin D did not show antibacterial activities observed for manumycin A. These results suggested that the oxirane unit was essential for antibacterial activities, but was not for cytotoxicities. Since manumycin class antibiotics were reported to have inhibitory activity against ras farnesyltransferase,[5,15] further biological studies of TMC-1 A to D will give us new information on structure-activity-relationship of manumycins.

Experimental

General

^1H and ^{13}C NMR spectra were obtained on a JEOL GSX-400 NMR spectrometer; chemical shifts are given in ppm (δ) relative to TMS as an internal standard. Mass spectra were obtained on a JEOL JMS HX-100 spectrometer. UV spectra were measured on a Shimadzu model UV-2200A spectrophotometer. IR spectra were measured on a JASCO model 100 infrared spectrophotometer. Melting point was determined using a Yanagimoto MP-2S micro melting point apparatus and is uncorrected. Optical rotation was determined with a Horiba model SEPA-200 high sensitive polarimeter. Wako gel C-300 and YMC ODS A60 were used for column chromatography. Preparative HPLC was carried out using Gilson HPLC system and YMC D-ODS-5; column size: 20 × 250 mm; flow rate: 12 ml/minute.

R-(+)-and *S*-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl Esters of TMC-1 C (**3**), **8** and **9**

1.4 mg of 4-dimethylaminopyridine (DMAP) was added to a solution of 20.3 mg of **3**, 50.5 mg of *R*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (*R*-(+)-MTPA) and 38.9 mg of dicyclohexylcarbodiimide (DCC) in 2.0 ml of dry methylene chloride. After 1 hour, dicyclohexylurea was removed by filtration, and the

Table 6. Cytotoxicity of TMC-1 A (**1**) to D (**4**), and manumycin D (**5**), A (**6**) and C (**7**) against tumor cells *in vitro*.

Cell line	IC_{50} (μg/ml)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
HCT-116 human colon carcinoma	28.0	16.9	3.4	6.8	6.1	3.2	2.4
SW480 human colon adenocarcinoma	47.7	17.1	9.0	11.0	11.0	10.0	6.5
Saos-2 human osteogenic sarcoma	37.6	23.1	4.6	5.9	9.3	7.2	3.1
WiDr human colon adenocarcinoma	28.6	13.1	10.0	9.3	8.3	6.1	3.3
OVCAR-3 human ovarian adenocarcinoma	28.6	13.5	5.8	6.0	7.6	6.0	2.7
HL-60 human promyelocytic leukemia	29.8	13.1	6.3	12.0	9.4	1.8	1.0
HeLa S3 human epitheloid carcinoma	24.2	10.5	6.7	8.3	7.9	6.9	3.8
P388D1 murine lymphoid neoplasm	15.5	8.0	3.3	3.2	3.4	0.4	0.2

Fig. 2. 2D NMR experiments of TMC-1 C (**3**).

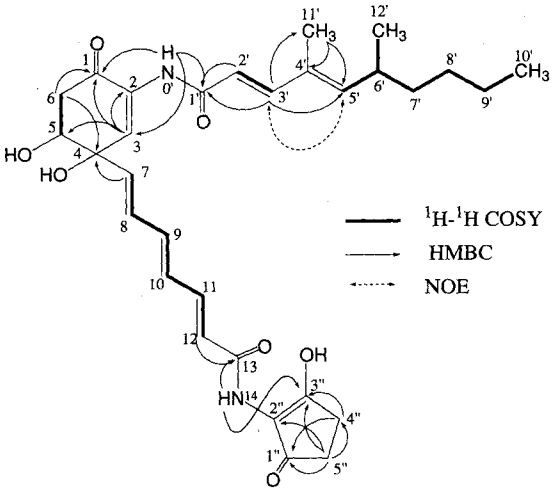


Fig. 3. NMR experiments of TMC-1 A (**1**) and B (**2**).

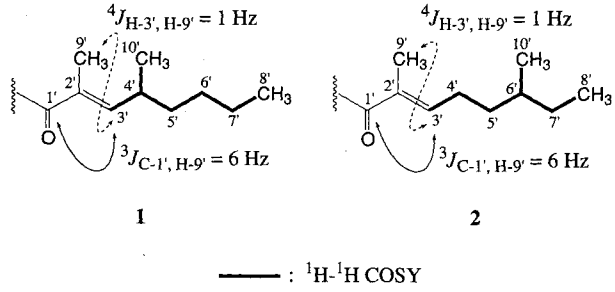


Fig. 4. Relative stereochemistry of the cyclohexenone moiety of TMC-1 A (**1**) to D (**4**).

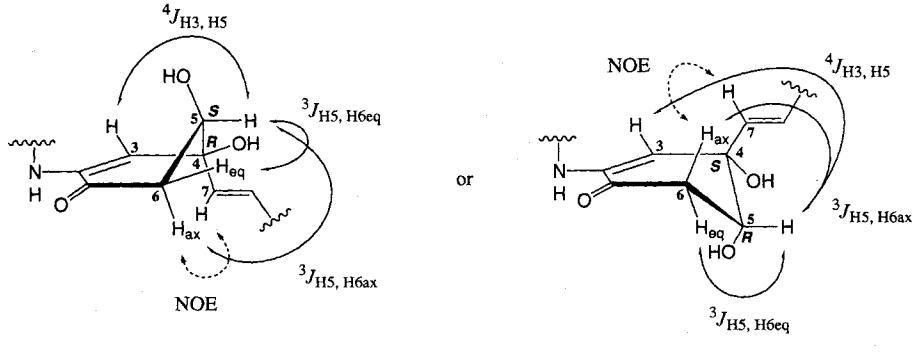
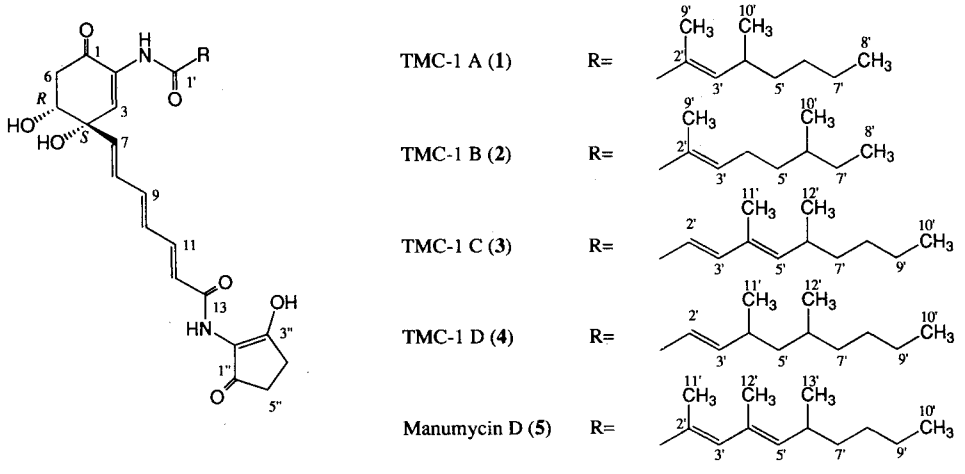


Fig. 5. Structures of TMC-1 A (**1**), B (**2**), C (**3**), D (**4**) and manumycin D (**5**).



filtrate was washed with 2 × 2.0 ml of water. The organic phase was then dried over anhydrous Na_2SO_4 and filtered, and the resulting filtrate was concentrated to give a yellow syrup. This syrup was purified by preparative TLC (CH_2Cl_2-MeOH (95:5)) to afford 15.8 mg of **8** as yellow amorphous powder.

Compound **9** was prepared as similar procedure.

8: FAB-MS *m/z* 755 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ 13.54 (1H, br s, 3″-OH), 7.80 (1H, s, 14-NH), 7.67 (1H, br s, NH), 7.61 (1H, d, J=0.9 Hz, 3-H), 7.48 (2H, m, Ph (*o*-)), 7.42~7.36 (3H, m, Ph (*m*-, *p*-)), 7.34 (1H, dd, J=14.8, 11.4 Hz, 11-H), 7.27 (1H, d, J=15.0 Hz, 3′-H), 6.64~6.54 (2H, m, 8, 9-H) 6.40 (1H, m, 10-H), 6.08 (1H, d, J=14.8 Hz, 12-H), 6.02 (1H, d, J=14.1 Hz, 7-H), 5.81 (1H, d, J=15.2 Hz, 2′-H), 5.71 (1H, d, J=9.7 Hz, 5′-H), 5.44 (1H, ddd, 5-H), 3.48 (3H, bs, OCH3), 3.00 (1H, dd, J=17.2, 6.8 Hz, 6ax-H), 2.88 (1H, dd, J=17.2, 3.7 Hz, 6eq-H), 2.55 (4H, m, 4″, 5″-H), 2.52 (1H, m, 6′-H), 1.79 (3H, d, J=0.9 Hz, 11′-CH_3), 1.4~1.2 (6H, m, 7′, 8′, 9′-H), 0.99 (3H, d, J=6.6 Hz, 12′-CH_3), 0.87 (3H, t, J=7.0 Hz, 10′-CH_3).

9: FAB-MS *m/z* 755 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ 13.55 (1H, br s, 3″-OH), 7.83 (1H, s, 14-NH), 7.67 (1H, br s, NH), 7.61 (1H, d, J=1.1 Hz, 3-H), 7.47 (2H, m, Ph (*o*-)), 7.42~7.36 (3H, m, Ph (*m*-, *p*-)), 7.33 (1H, dd, J=14.8, 11.4 Hz, 11-H), 7.28 (1H, d, J=14.5 Hz, 3′-H), 6.57~6.48 (2H, m, 8, 9-H), 6.37 (1H, m, 10-H), 6.08 (1H, d, J=14.7 Hz, 12-H), 5.94 (1H, d, J=14.3 Hz, 7-H), 5.83 (1H, d, J=15.0 Hz, 2′-H), 5.71 (1H, d, J=9.7 Hz, 5′-H), 5.44 (1H, ddd, 5-H), 3.48 (3H, d, J=1.0 Hz, OCH_3), 3.10 (1H, dd, J=17.0, 7.0 Hz, 6ax-H), 2.94 (1H, dd, J=17.0, 3.7 Hz, 6eq-H), 2.55 (4H, m, 4″, 5″-H), 2.52 (1H, m, 6′-H), 1.79 (3H, d, J=1.1 Hz, 11′-CH_3), 1.4~1.2 (6H, m, 7′, 8′, 9′-H), 0.99 (3H, d, J=6.6 Hz, 12′-CH_3), 0.87 (3H, t, J=7.0 Hz, 10′-CH_3).

R-(+)- and *S*-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl Esters of Manumycin D (**5**), **10** and **11**

Compounds **10** and **11** were prepared using a procedure similar to the one described above.

10: FAB-MS *m/z* 791 $(M+Na)^+$; ^1H NMR ($CDCl_3$) δ 13.56 (1H, br s, 3″-OH), 8.18 (1H, s, 14-NH), 7.71 (1H, s, NH), 7.58 (1H, d, J=1.1 Hz, 3-H), 7.48 (2H, m, Ph (*o*-)), 7.42~7.36 (3H, m, Ph (*m*-, *p*-)), 7.34 (1H, dd, J=14.7, 11.3 Hz, 11-H), 6.78 (1H, br s, 3′-H), 6.64~6.54 (2H, m, 8, 9-H) 6.40 (1H, m, 10-H), 6.10 (1H, d, J=14.7 Hz, 12-H), 6.02 (1H, d, J=14.1 Hz, 7-H), 5.34 (1H, br d, J=9.7 Hz, 5′-H), 5.44 (1H, ddd, 5-H), 5.34 (1H, br d, J=9.7 Hz, 5′-H), 3.49 (3H, d, J=0.9 Hz, OCH_3), 3.01 (1H, dd, J=17.2, 6.8 Hz, 6ax-H), 2.89 (1H, dd, J=17.2, 3.7 Hz, 6eq-H), 2.57 (4H, m, 4″,5″-H), 2.45 (1H, m, 6′-H), 2.04 (3H, d, J=1.3 Hz, 11′-CH_3), 1.82 (3H, d, J=1.3 Hz, 12′-CH_3), 1.4~1.2 (6H, m, 7′,8′,9′-H), 0.99 (3H, d, J=6.6 Hz, 13′-CH_3), 0.89 (3H, t, J=7.0 Hz, 10′-CH_3).

11: FAB-MS *m/z* 791 $(M+Na)^+$; ^1H NMR ($CDCl_3$) δ 13.56 (1H, br s, 3″-OH), 8.22 (1H, s, 14-NH), 7.68 (1H, s, NH), 7.58 (1H, d, J=1.1 Hz, 3-H), 7.47 (2H, m, Ph (*o*-)), 7.42~7.36 (3H, m, Ph (*m*-, *p*-)), 7.33 (1H, dd, J=14.7, 11.2 Hz, 11-H), 6.79 (1H, br s, 3′-H), 6.57~6.48 (2H, m, 8, 9-H) 6.40 (1H, m, 10-H), 6.09 (1H, d, J=14.7 Hz, 12-H), 5.94 (1H, d, J=14.1 Hz, 7-H), 5.34 (1H, br d, J=9.9 Hz, 5′-H), 5.44 (1H, ddd, 5-H), 5.34 (1H, br d, J=9.7 Hz, 5′-H), 3.49 (3H, d, J=0.9 Hz, OCH_3), 3.11 (1H, dd, J=17.2, 7.3 Hz, 6ax-H), 2.95 (1H, dd, J=17.2, 3.7 Hz, 6eq-H), 2.57 (4H, m, 4″, 5″-H), 2.46 (1H, m, 6′-H), 2.06 (3H, d, J=1.3 Hz, 11′-CH_3), 1.83 (3H, d, J=1.3 Hz, 12′-CH_3), 1.4~1.2 (6H, m, 7′,8′,9′-H), 0.99 (3H, d, J=6.6 Hz, 13′-CH_3), 0.89 (3H, t, J=7.0 Hz, 10′-CH_3).

Antimicrobial Activity

Antimicrobial activity was determined by the agar-dilution method in Mueller-Hinton agar (Eiken) for Gram-positive and Gram-negative bacteria and in Sabouraud agar (Difco) for fungi and yeast. The inoculum was adjusted to 1×10^6 cfu/ml for bacteria and 1×10^6 cells or spores/ml for fungi and yeast.

Antimicrobial activity was detected after 20 hours at 37°C for bacteria and 72 hours incubation at 27°C for fungi and yeast.

In Vitro Cytotoxic Activity

The cells used for assay were cultured in following medium; HCT-116 and Saos-2: complete McCoy's 5A supplemented with 10% fetal bovine serum, SW480: complete Leibovitz's L-15 supplemented with 10% fetal bovine serum, WiDr: complete MEM-E supplemented with 1% essential amino acid solution, 10% fetal bovine serum, OVCAR-3: complete RPMI-1640 supplemented with 100 μg/ml insulin, 10% fetal bovine serum, HL-60: complete RPMI-1640 supplemented with 20% fetal bovine serum, HeLa S3: complete MEM-E supplemented with 10% fetal bovine serum, P388D_1: complete RPMI-1640 supplemented with 5% fetal bovine serum.

In vitro cytotoxic activity was tested in 96-well microtiter plates of which well containing 1×10^4 each cell lines in 135 μl medium. The test samples were dissolved in DMSO. The serially diluted DMSO solution (15 μl) was added to each well of plates. After addition, the cells were incubated at 37°C for 72 hours in a humidified 5% CO_2 atmosphere. *In vitro* cytotoxic activity was evaluated by the microculture tetrazolium assay (MTT assay)[17] method for HL-60 and P388 D_1 cells and by the colorimetrical determination method at 540 nm after staining viable cells with neutral red solution for other cells.

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