TAN-1057 A~D, NEW ANTIBIOTICS WITH POTENT ANTIBACTERIAL ACTIVITY AGAINST METHICILLIN-RESISTANT *Staphylococcus aureus*

TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

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Two Gram-negative bacteria were found to produce the new antibacterial antibiotics TAN-1057 A, B, C and D. The producing bacteria were characterized and designated as *Flexibacter* sp. PK-74 and PK-176. These antibiotics were active against Gram-negative and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. TAN-1057 A inhibited protein biosynthesis in *Escherichia coli* and *S. aureus*. It showed excellent protective effects against an experimental methicillin-resistant *S. aureus* infection in mice.

The nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) have become a serious problem[1], since MRSA is often resistant to not only β-lactam antibiotics but also many other antibiotics[1,2]. Although many antibiotics with anti-staphylococcal activity have been developed, very few antibiotics are available for the therapy of MRSA infection. Therefore, it is highly desirable to develop new anti-MRSA antibiotics.

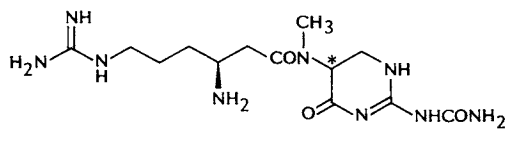
In the course of screening for new anti-staphylococcal antibiotics, we discovered TAN-1057 A, B, C and D in the culture filtrates of *Flexibacter* sp. PK-74 and PK-176[3,4]. TAN-1057 A~D were found to be dipeptide antibiotics (Fig. 1)[5] having potent therapeutic effects against MRSA infection. This paper deals with the taxonomy of the producing organisms and the fermentation and biological activity of TAN-1057.

Materials and Methods

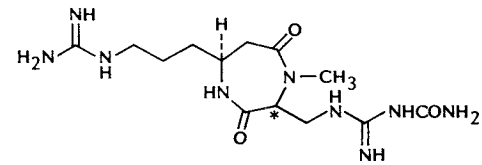
Taxonomy

The utilization of carbohydrates, motility and the degradation of polysaccharides were examined

Fig. 1. Structures of TAN-1057 A, B, C and D.



TAN-1057 A *S*
TAN-1057 B *R*



TAN-1057 C *R*
TAN-1057 D *S*

by the methods of STANIER *et al.*[6], GILARDI[7] and LEWIN[8], respectively. Microcyst formation was tested by the method of DWORKIN *et al.*[9]. Other taxonomical and physiological characteristics were determined by the method of COWAN and STEEL[10]. The identification of the genus was carried out according to the classification standard described in BERGEY's Manual of Systematic Bacteriology[11].

In Vitro and *In Vivo* Activity

MIC values were determined by a two-fold agar dilution method with an inoculum size of 10^6 cfu/ml, unless noted otherwise.

The therapeutic effect was tested as follows. Four-week-old mice were infected intraperitoneally with 0.5 ml of a bacterial suspension. The challenge inoculum of bacteria was: 10^8 cfu/mouse for *S. aureus*, 10^1 cfu/mouse for *Streptococcus pneumoniae* and 10^5 cfu/mouse for *Escherichia coli*. Groups of five mice at each dosage level were given 0.2 ml of antibiotic solution immediately after infection. The 50% effective dose (ED_{50}) was calculated from the survival rate 5 days after infection.

Incorporation of Radiolabeled Precursors

S. aureus FDA 209P was cultivated for 1 day at 37°C in DYAB medium[12] without shaking. Cells were centrifuged and washed with DAVIS' minimal medium[13] and suspended to give an absorbance of 0.5 at 600 nm in DAVIS' minimal medium supplemented with 10 ml/liter of the amino acid solution and 1 ml/liter of the vitamin solution. The amino acid solution contained 20 mg/ml each of lysine, arginine, methionine, cysteine, leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, histidine, threonine, glutamic acid, proline, aspartic acid, alanine, glycine and serine. The vitamin solution contained biotin 10 mg/ml, thiamine hydrochloride 10 mg/ml, pyridoxine hydrochloride 100 mg/ml, riboflavin 500 mg/ml, nicotinamide 100 mg/ml, calcium pantothenate 100 mg/ml, *p*-aminobenzoic acid 100 mg/ml, choline chloride 2,000 mg/ml, inositol 1,000 mg/ml, folate 100 mg/ml and cyanocobalamin 100 mg/ml. For the protein synthesis assay, leucine was omitted from the medium described above. An incubation mixture consisting of 0.45 ml of the above-mentioned cell suspension, 0.025 ml of [methyl-^{3}H]thymidine solution (1.48 TBq/mmol, 1.11 MBq/ml), [2-^{14}C]uridine solution (2.2 GBq/mmol, 0.925 MBq/ml) or L-[U-^{14}C]leucine solution (12.7 GBq/mmol, 1.85 MBq/ml), and 0.025 ml of antibiotic solution was incubated for 45 minutes at 37°C without shaking. After 0.1 ml of 35% TCA was added, the mixture was allowed to stand for 1 hour in an ice bath. In the experiment looking at the incorporation of leucine, the mixture was further heated at 90°C for 10 minutes. The mixture was centrifuged, and the acid-insoluble precipitate was washed with 0.5 ml of 5% TCA and suspended in 0.1 ml of 0.1 M ammonium acetate. Half of this suspension was transferred to a toluene-based scintillator, and the radioactivity was counted with a scintillation counter. In the experiment using *E. coli* LD-2, a lysine and diaminopimelic acid auxotroph, as the test organism, M9 medium[14] supplemented with 20 μg/ml of L-threonine, L-leucine, L-lysine and diaminopimelic acid and 2 μg/ml of thiamine was used instead of DAVIS' minimal medium.

Cell Free Protein Synthesis

According to the modification of the method of NIERENBERG[15] described below, ribosome and S-100 fractions were prepared from *E. coli* LD-2 grown in DYAB medium. The exponentially growing cells were centrifuged and washed twice with medium A (10 mM Tris-acetate buffer pH 7.8, 10 mM magnesium acetate, 60 mM NH_4Cl and 1 mM dithiothreitol) and stored at -80°C. Cells were disrupted by grinding with alumina (twice the weight of the washed cells) at 5°C for 5 minutes. To the disrupted cells, medium A (twice the volume of the washed cells) and DNase-1 (final concentration 0.2 μg/ml) were added. To remove the alumina and cell debris, the suspension was centrifuged twice at $3,000 \times g$ for 60 minutes. The supernatant was incubated for 15 minutes at 37°C. The incubation mixture consisted of 50 mM Tris-acetate (pH 7.8), 1 mM ATP K salt, 5 mM phosphoenolpyruvate (PEP), 20 U/ml pyruvate kinase and 1 mM of each of 20 common amino acids. After incubation, the reaction mixture was dialyzed against medium A for 1 day at 4°C. The dialyzed mixture was centrifuged at $3,000 \times g$ for 30 minutes. The supernatant was again centrifuged at $105,000 \times g$ for 120 minutes. The upper 2/3 portion of the supernatant was used as the S-100 fraction. The precipitate was washed by resuspension in medium A and centrifuged at $105,000 \times g$ for 120 minutes. The precipitate was suspended in medium A, and this suspension was used as the ribosome fraction.

Cell-free protein synthesis was measured according to the modification of the method of WILHELM *et al.*[16] as follows. The reaction mixture for cell-free protein synthesis contained the following components in 250 μl: 50 mM Tris-acetate pH 7.8, 60 mM NH_4Cl, 10 mM magnesium acetate, 10 mM PEP, 1 mM dithiothreitol, S-100 fraction 1.5 mg-protein/ml, ribosome fraction 1.7 mg-protein/ml, poly-U or poly-A 400 μg/ml and 1-^{14}C-Phe (2.06 GBq/mmol, 37 kBq/ml) or ^{14}C-U-Lys (12.0 GBq/mmol, 37 KBq/ml), respectively. After incubation for 1 hour at 37°C, 50 μl of 35% TCA was added to the reaction mixture followed by centrifugation. The precipitate was washed twice with 250 μl of 5% TCA and suspended in 100 μl of 0.1 N ammonium acetate. In experiments using poly-A and ^{14}C-Lys, sodium tungstate (0.25%) was added to the 5% TCA solution. Half of this suspension was transferred to a toluene-based scintillator, and the radioactivity was counted with a scintillation counter.

Results

Producing Organism

Strains PK-74 and PK-176 were isolated from soil samples collected in the Higashimurou district of Wakayama prefecture, Japan and the Kamiagata district of Nagasaki prefecture, Japan, respectively. Colonies of strain PK-74 were semitransparent, orange, lusterless and flat on nutrient agar. On the other hand, colonies of PK-176 were creamy and lustrous. The morphological and physiological characteristics of strains PK-74 and PK-176 are summerized in Table 1. Both strains are Gram-negative, aerobic, slender rods or sometimes filaments, motile by gliding, do not require any growth factors, and do not form spores or microcysts. They showed very weak decomposing activity on starch and colloidal chitin. The mol% G+C of the DNA of PK-74 and PK-176 was determined to be 33.4 and 36.9, respectively. Based upon these characteristics, strains PK-74 and PK-176 were regarded as belonging to the genus *Flexibacter*, and have been designated as *Flexibacter* sp. PK-74 and PK-176. They have been deposited in the Institute for Fermentation, Osaka under the accession number IFO 14731 and IFO 14825, respectively, and in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan under the accession number FERM BP-1831 and FERM BP-2291, respectively.

Table 1. Morphological and physiological characteristics of strains PK-74 and PK-176.

	PK-74	PK-176		PK-74	PK-176
Cell shape	Rod-filament	Rod-filament	Reduction of nitrate	+	−
Size (μm)	0.4~1.0× 0.9~1.2[a]	0.4~1.0× 0.9~1.3[b]	Denitrification	−	−
			Liquefaction of gelatin	+	+
Flagella	None	None	Utilization of		
Gram stain	Negative	Negative	citrate	+	+
Motility	Gliding	Gliding	potassium nitrate	−	−
Resting cell	None	None	ammonium sulfate	+	−
Range of growth (°C)	13~30.2	13~31	Degradation of Tween 80	−	−
optimum	14~25.2	14~25.2	Hydrolysis of		
Oxygen demand	Aerobic	Aerobic	starch	±[c]	±[c]
O-F test	Not reactive	Not reactive	carboxymethyl cellulose	−	−
Oxidase	+	+	colloidal chitin	±[c]	±[c]
Catalase	−	−	Production of		
Urease	−	−	hydrogen sulfate	−	−
GC content of DNA (%)	33.4±1.0	36.9±1.0			

[a] Filamentous cells of 20~40 μm length were often observed.
[b] Filamentous cells of 10~40 μm length were often observed.
[c] Trace.

Fermentation

Seed medium (500 ml) consisting of glucose 2%, soluble starch 3%, soy bean flour 1%, corn-steep liquor 0.3%, Polypepton (Nihon Pharmaceutical Co.) 0.5%, NaCl 0.3% and $CaCO_3$ 0.5% (pH 7.0) in a 2-liter Sakaguchi flask was inoculated with a loopful of cells of strain PK-74 grown on a nutrient agar slant. After incubation at 24°C for 48 hours on a reciprocal shaker, the seed culture was transferred to a 50-liter fermentor containing 30 liters of the same seed medium supplemented with 0.05% Actcol (Takeda Chemical Ind.) as an antifoaming agent. Cultivation was carried out at 24°C for 48 hours with aeration (30 liters/minute) and agitation (200 rpm). Six liters of this culture broth was transferred to a 200-liter fermentor containing 120 liters of the following fermentation medium: dextrin 3%, soy bean flour 1.5%, corn-gluten meal 1.5%, Polypepton 0.2%, sodium thiosulfate 0.1%, $CaCO_3$ 0.5% and Actcol 0.05% (pH not adjusted). Fermentation was carried out at 17°C for 66 hours with aeration (120 liters/minute) and agitation (170 rpm). Under these conditions, the titer of TAN-1057 calculated as TAN-1057 A was about 60 μg/ml.

Strain PK-176 was cultivated in the same manner as that described for strain PK-74, except that the fermentation medium consisted of soy bean flour 1.5%, corn-gluten meal 1.5%, Polypepton 0.2%, $CaCO_3$ 0.5% and Actcol 0.5% (pH 6.0), and agitation (120 rpm). The titer of TAN-1057 calculated as TAN-1057 A was about 100 μg/ml under these conditions. Although we isolated several strains of TAN-1057 producer, TAN-1057 C and D were detected only in the culture broth of strain PK-176.

Biological Activity

TAN-1057 showed stronger antibacterial activity against Gram-positive bacteria than against Gram-negative bacteria (Table 2). TAN-1057 A and D which have the *S* configuration were more active than TAN-1057 B and C having the *R* configuration (Table 2). There was no cross-resistance between TAN-1057 and methicillin, gentamicin or erythromycin (Table 3).

The antibacterial activity of TAN-1057 was not affected by changing the inoculum size from 10^4 cfu/ml to 10^8 cfu/ml but was affected by a change in the pH of the assay medium. TAN-1057 had

Table 2. Antibacterial activity of TAN-1057 A, B, C and D.

Organism	MIC (μg/ml)			
	A	B	C	D
Staphylococcus aureus FDA 209P	6.25	25	50	6.25
S. epidermidis IFO 3762	0.78	3.13	6.25	0.78
Micrococcus luteus IFO 12708	3.13	12.5	12.5	3.13
Bacillus subtilis NIHJ PCI 219	12.5	50	>100	12.5
B. cereus FDA 5	25	100	>100	25
B. megaterium IFO 12108	12.5	50	>100	12.5
Streptococcus faecalis IFO 3989	12.5	50	50	12.5
S. faecium IFO 3181	25	100	>100	25
Escherichia coli NIHJ JC-2	>100	>100	>100	>100
E. coli LD-2	12.5	25	50	12.5
Serratia marcescens IFO 12648	>100	>100	>100	>100
Proteus vulgaris IFO 3988	>100	>100	>100	>100
Pseudomonas aeruginosa IFO 3080	>100	>100	>100	>100
Alcaligenes faecalis IFO 13111	25	100	>100	25
Acinetobacter calcoaceticus IFO 13006	50	100	>100	25

The assay medium was DYAB medium.

Table 3. Susceptibility of *S. aureus* to TAN-1057 A.

Strain	MIC (μg/ml)				
	TAN-1057A	DMPPC	GM	EM	VCM
FDA 209P	0.39	1.56	<0.20	0.39	1.56
N8	0.78	1.56	<0.20	>100	1.56
N235	1.56	1.56	>100	>100	1.56
C3	0.78	50	100	>100	0.78
N262	0.78	100	50	>100	0.78
N267	<0.20	100	100	>100	1.56
N129	0.39	400	50	0.39	0.78
C10	0.78	800	>100	>100	1.56
N28	0.78	1,600	0.39	>100	1.56
N326	0.78	>1,600	>100	>100	1.56

The assay medium was Mueller-Hinton medium.
DMPPC; methicillin, GM; gentamicin, EM; erythromycin, VCM; vancomycin.

Table 4. Antibacterial activity of TAN-1057 A against *S. aureus* 209P on different media.

Medium	MIC (μg/ml)
AM3	3.13
MH	1.56
HI	3.13
TSB	3.13
NB	0.2
AOAC	0.1
CDSM	0.05
CS	0.05

MIC values were determined by a microdilution method.
AM3: Antibiotic medium No. 3 (Difco).
MH: Mueller-Hinton broth (Difco).
HI: Heart infusion broth (Difco).
TSB: Trypticase soy broth (BBL).
NB: Nutrient broth (Difco).
AOAC: Synthetic medium AOAC (Difco).
CDSM: Complete defined synthetic medium[17].
CS: Calf serum.

Table 5. Effect of nutrients on the antibacterial activity of TAN-1057 A against *S. aureus*.

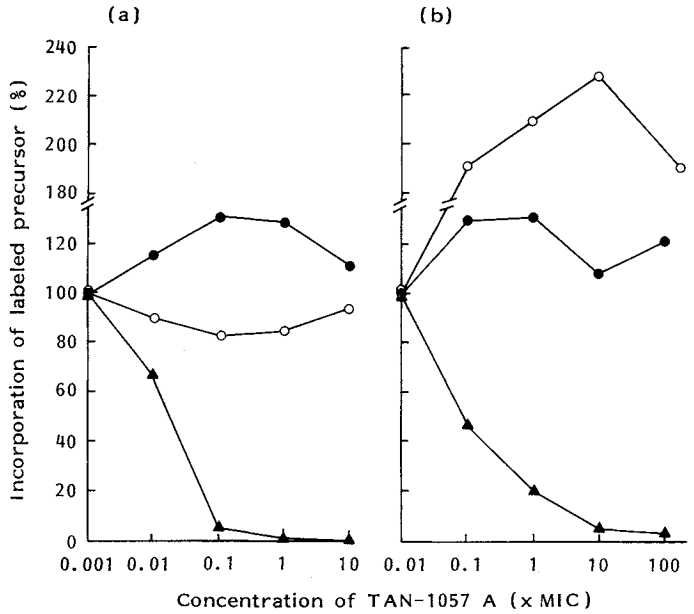
Medium	MIC (μg/ml)
CDSM	0.1
+amino acids[a]	0.1
+beef extract	0.39
+peptone	1.56
+yeast extract	3.13
+casamino acids	1.56
+NaCl	0.1
DYAB	6.25

MIC values were determined by a microdilution method.
[a] Amino acids were added 3-fold of normal CDSM. The other nutrients were added as follows: beef extract; 1.5 g/liter, peptone; 5 g/liter, yeast extract; 6.5 g/liter, casamino acids; 2.5 g/liter, NaCl 3.5 g/liter.

about ten times stronger antibacterial activity at pH 9 than at pH 7 (data not shown). Moreover, the antibacterial activity of TAN-1057 A against *S. aureus* was affected by the assay media. TAN-1057 A showed more than ten times stronger antibacterial activity against *S. aureus* in synthetic media than that measured in the standard assay media (Table 4). The antibacterial activity of TAN-1057 A measured in complete defined synthetic medium (CDSM)[17] was decreased with the addition of peptone, yeast extract or casamino acids (Table 5).

Although the incorporation of radiolabeled thymidine and uridine was not inhibited by TAN-1057 A. TAN-1057 A inhibited the incorporation of leucine into macromolecules in *E. coli* and *S. aureus* at concentrations below the MIC (Fig. 2). Moreover, it inhibited poly-A and poly-U directed peptide synthesis in the *E. coli* cell-free system. The concentration required for 50% inhibition was 10 μg/ml for poly-A directed synthesis and 40 μg/ml for poly-U directed synthesis (Fig. 3).

Fig. 2. Effect of TAN-1057 A on macromolecular synthesis in *S. aureus* FDA 209P (a) and *E. coli* LD-2 (b).
DNA (●), RNA (○) and protein (▲).



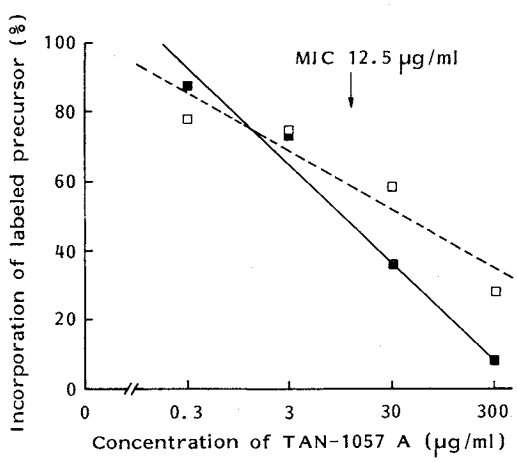
The MIC values of TAN-1057 A against *S. aureus* and *E. coli* were 3.13 μg/ml and 12.5 μg/ml, respectively.

TAN-1057 A showed an extremely strong therapeutic effect against experimental infection with *S. aureus*, including methicillin-resistant strains, in mice (Table 6).

The preliminary acute toxicity (LD_{50}) of TAN-1057 A in mice was *ca.* 100 mg/kg upon intraperitoneal and *ca.* 50 mg/kg upon intravenous injection.

Fig. 3. Effect of TAN-1057 A on protein synthesis in the *E. coli* cell-free system.

Solid line (solid square): poly-A directed protein synthesis. Dashed line (open square): poly-U directed protein synthesis.



Discussion

Although there are some differences in the characteristics of strains PK-74 and PK-176, their similarity suggests that they may belong to the same species.

The mode of action of TAN-1057 is most likely inhibition of protein synthesis, because TAN-1057 A inhibited cell-free peptide synthesis at concentrations near the MIC value (Fig. 3). Since TAN-1057 A did not inhibit aminoacyl-tRNA synthetase (data not shown), the target of TAN-1057 seems to be one of the steps of protein biosynthesis after the formation of aminoacyl-tRNA.

The therapeutic effect of TAN-1057 A against *S. aureus*, especially MRSA, was superior to those of imipenem and vancomycin, although the antibacterial activity of TAN-1057 A measured in the Mueller-Hinton broth was less than that of vancomycin and not so stronger than that of imipenem

Table 6. Therapeutic effect of TAN-1057 A in mice.

Organism	Agent	Route	ED_{50} (mg/kg)	MIC[a] (μg/ml)
Staphylococcus aureus 308A-1	TAN-1057 A	sc	0.027	12.5
		po	1.20	
	IMP/CS[b]	sc	0.1	0.025
	Vancomycin	sc	2.2	0.78
S. aureus N133A[c]	TAN-1057 A	sc	0.026	6.25
		po	0.56	
	IMP/CS	sc	4.20	>25
	Vancomycin	sc	2.3	1.56
S. aureus N295A[c]	TAN-1057 A	sc	0.064	12.5
		po	1.56	
	IMP/CS	sc	15.1	25
	Vancomycin	sc	3.36	0.78
Streptococcus pneumoniae type 1	TAN-1057 A	sc	>25	6.25[d]
Escherichia coli O-111	TAN-1057 A	sc	>12.5	100[d]

[a] MIC values were determined by a broth dilution method using Mueller-Hinton broth.
[b] Imipenem/cilastatin.
[c] MRSA.
[d] MIC values were determined by an agar dilution method using TSA medium. Inoculum size was 10^8 cfu/ml.

(Table 6). TAN-1057 A showed stronger antibacterial activity against *S. aureus* in synthetic media and calf serum than when tested in the standard assay media such as Mueller-Hinton broth (Table 4). The MIC values measured in synthetic media or calf serum correlated with the ED_{50} values of TAN-1057 A. Therefore, the standard assay media may not be suitable for measuring the antibacterial activity of TAN-1057. The addition of peptone, yeast extract or casamino acids, which were the components of the standard assay media, to the synthetic medium decreased the antibacterial activity of TAN-1057. It suggests the presence of factors antagonistic to TAN-1057 activity in these components. Further studies are needed to identify the inhibitory components in complex media, and to identify the mode of action of TAN-1057.

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