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Transvalencin Z, a New Antimicrobial Compound with Salicylic Acid Residue from *Nocardia transvalensis* IFM 10065

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Abstract Transvalencin Z was isolated from a culture broth of *Nocardia transvalensis* IFM 10065, a clinical isolate from a Japanese patient with actinomycotic mycetoma. The transvalencin Z structure was determined using NMR and mass spectrometric analyses. The structure is similar to a partial structure of siderophores such as mycobactins and nocobactins, but the compound has no cytotoxic activity. Transvalencin Z shows a strong antimicrobial activity against Gram-positive bacteria, but shows no activity against Gram-negative bacteria, fungi and tumor cells.

Keywords transvalencin Z, siderophores, *Nocardia transvalensis*, nocobactin

Introduction

Most *Nocardia* species can cause human infections called nocardiosis. The infections that are caused by *Nocardia* are considered rare, but infectious cases have been increasingly reported, especially with the increase of immunocompromised patients [1, 2]. During our screening program of bioactive compounds from pathogenic actinomycetes, we found that most of the culture broth of *Nocardia* clinical isolates produce anti-tumor compounds that are extractable using hydrophobic solvents such as ethyl ether or *n*-hexane [3, 4]. Our studies suggest that most of the active compounds from these pathogenic bacteria belong to siderophore-class antibiotics such as brasilibactin A [4], madurastatin [5] and asterobactin [6]. These siderophores are characterized as having salicylic acid moieties in their structures. We previously reported that *N. transvalensis* IFM 10065 produces a novel zinc-containing antibiotic, transvalencin A [6, 7]. Our continuous screening of new bioactive metabolites from that bacterium has led to isolation of a novel antimicrobial compound with a salicylic acid moiety in its structure, designated as transvalencin Z (Fig. 1, **1**). In this paper, we describe its production, isolation, physicochemical properties, structural characterization and biological activity.

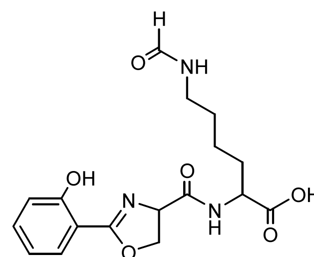

Fig. 1 Structure of transvalencin Z.

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Table 1 Physicochemical properties of transvalencin Z

Appearance	White powder
Molecular weight	363
Molecular formula	$C_{17}H_{21}N_3O_6$
HRFAB-MS (*m*/*z*)	
Found:	364.1503 $(M+H)^+$
Calcd.:	364.1509
$[\alpha]_D^{25.5}$	+15.3° (*c* 1 mg/ml, MeOH)
UV λ_{max}^{MeOH} nm (log ε)	205.5 (3.77), 241.0 (3.28), 301.5 (2.91)

Table 2 Assignments of the main absorption bands (cm^{-1}) of the IR spectrum (KBr) of transvalencin Z

3600~3100	νNH & νOH
2923, 2865	νCH & aldehydic νCH
1653	carboxylic νC=O
1615	νC=O (amide I)
1492	aromatic νC=C
1526	δNH (amide II)
1369	heterocyclic νC=N
1258	νC-O
1234	aromatic δCH
1078	νC-N
965	heterocyclic γCH
754	γCH

ν: stretching vibration, δ: deformation vibration, γ: out-of-plane deformation vibration.

Fermentation and Isolation

N. transvalensis IFM 10065 was cultured as reported previously [6], and the separation of active compounds was monitored by exhibition of antimicrobial activity against *Micrococcus luteus* IFM 2066. The broth was applied on a column (Diaion HP-20; Mitsubishi Chemical Corp.) and washed with 50% CH_3OH. Elution with CH_3OH was undertaken; then it was evaporated to dryness. The residue was dissolved in distilled water, then washed with EtOAc. The active fraction was extracted with BuOH, and the BuOH layer was evaporated to dryness. Then it was purified using silica gel-column chromatography (5×30 cm; Fuji Silysia Chemical Ltd.) with a mixture of $CHCl_3/CH_3OH$ (10 : 0 and 7 : 3) as eluent. The active fraction eluted by $CHCl_3/CH_3OH$ (7 : 3) was applied on preparative TLC plate (Merck and Co., Inc.) using EtOAc/*i*-PrOH/H_2O (4 : 2 : 1, upper phase) as a developing solution. The isolated active fraction was further purified by preparative NH-TLC plate using CH_3OH as mobile phase. Finally, the active compound was applied on LH-20 column chromatography using elution solvent of $CHCl_3/CH_3OH$ (1 : 1). It gave 4.3 mg of pure transvalencin Z (**1**) from a 2.0-liter culture.

Structure Elucidation

Physicochemical properties of transvalencin Z (**1**) are shown in Table 1. The IR absorbencies (FT/IR-300E; Jasco Inc.) at 1369, 1615, and 754 cm^{-1} indicated the presence of heterocyclic and *ortho*-disubstituted benzene rings in its structure (Table 2). The shoulder at 1715 cm^{-1} also suggested the presence of a free carboxyl group. The FAB-MS of **1** gave the $[M+H]^+$ ion at *m*/*z* 364 in the positive mode and HRFAB-MS (JMS-700 and JMS-AX500 instruments; JEOL) displayed the $[M+H]^+$ ion at *m*/*z* 364.1503 (calcd. 364.1509). The molecular formula was established as $C_{17}H_{21}N_3O_6$ based on the HRFAB-MS and NMR (^1H, ^{13}C, DEPT, HSQC, NOESY and HMBC) spectral data (JNM EXP-500; JEOL) (Table 3). The composition therefore indicated the presence of nine double bonds or rings in the molecule. The ^1H-NMR spectrum showed 20 hydrogen atoms, including the presence of two amide NH groups (δ 7.75 and 7.94) and a formyl proton (δ 7.94) in DMSO-d_6. The ^{13}C-NMR spectrum indicated that the compound comprises 17 carbon atoms, which were assigned to five methylenes, six methines, and six quaternary carbons using DEPT and HSQC spectra.

The structure of **1** was determined as follows. A series of aromatic protons of δ 6.50~8.00 in the ^1H-NMR spectrum suggested the presence of an *ortho*-disubstituted benzene ring. In the COSY spectrum (Fig. 2), a spin-spin network from H-4 to H-7 was easily observed. The HMBC spectrum showed a cross peak between H-5 and the carbon signal at δ 159.1 (C-3) that is substituted with an oxygen-functional group and was correlated with H-7. The remaining aromatic carbon (C-2) was assigned to the signal at δ 109.1 by the cross peaks with H-4 and H-6. The chemical shift of C-2 suggested that the C-2 position was substituted with a sp^2 carbon [6]. The sp^2 carbon was assigned to the signal at δ 165.9 (C-1) because of long-range coupling with H-7. These data indicate the presence of the salicylate residue in this structure. The correlation between the signal at δ 4.48 (an H-3 of serine residue) and 4.99 (H-2 of serine residue) was detected in the COSY spectrum. Long-range coupling between the H-3/H-2 and C-1 of salicylate residue (Fig. 2) indicated the presence of a 2-oxazoline ring. Eight sp^3 protons of lysine residue were connected by the COSY spectrum and the correlation between the amido proton at the C-2 position; H-2 of lysine residue was also detected. Long-range coupling between δ 2.98 (H_2-6 of lysine residue) and 160.7 (formic carbon) in the HMBC spectrum indicated that the *N*-terminal of the side chain of lysine residue was formylated. The connection of respective

Table 3 ^{1}H and ^{13}C NMR spectral data of transvalencin Z in DMSO-d_6

Position	^{13}C (ppm)	^{1}H (ppm)	(mult; *J*, Hz)
Salicylic acid			
1	165.9		
2	109.8		
3	159.1		
4	116.5	7.00	(br d; 8)
5	134.0	7.46	(ddd; 1.5, 7.5, 8)
6*	119.1	6.94	(br t; 7.5)
7	128.0	7.63	(dd; 1.5, 7.5)
Serine			
1	168.6		
2	67.4	4.99	(dd; 8, 10)
3	69.7	4.48	(t; 8)
		4.63	(dd; 8, 10)
N-Formyl-lysine			
1	173.1		
1-OH		11.76	(br)
2**	53.8	3.85	(q-like; 6)
2-NH		7.75	(d; 6)
3	31.7	1.54	(m)
		1.68	(m)
4	22.5	1.20	(m)
5	28.8	1.32	(m)
6***	37.1	2.98	(q-like; 6)
6-NH		7.94	(br t; 6)
7	160.7	7.92	(s)

* (in CD_3OD) δ_C 120.0, δ_H 6.86 (dt, J=1 and 7.5 Hz). ** The signal was changed to triplet (J=5.5 Hz) with the addition of CD_3OD. *** The signal was changed to triplet (J=6.5 Hz) with the addition of CD_3OD. The assignment of the OH signal may be changed to 3-OH of salicylic acid moiety[D)].

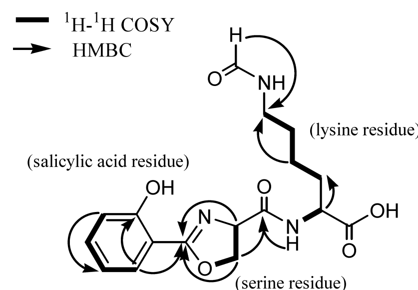

Fig. 2 COSY and HMBC correlations of transvalencin Z.

Table 4 Antimicrobial activity of transvalencin Z (**1**) against bacteria and fungi

Test organisms	MICs (μg/ml)
Nocardia asteroides IFM 0319	1
Nocardia farcinica IFM 0284	1
Nocardia transvalensis IFM 10065	>64
Mycobacterium smegmatis ATCC 607	0.125
Gordonia branchialis IFM 0150	<0.125
Corynebacterium xerosis IFM 2057	0.125
Micrococcus luteus IFM 2066	4
Bacillus subtilis PCI 189	16
Staphylococcus aureus PCI 209P	0.25
Staphylococcus aureus CUH 3666 (MRSA)	1
Escherichia coli NIH JC-2	>64
Candida albicans ATCC 90028*	>64
Aspergillus niger IFM 5368	>64
Cryptococcus neoformans ATCC 90112	>64
Tricophyton mengtagrophytes IFM 10489*	>64

Nutrient Broth, 37°C, 24 hours, *: 37°C, 72 hours. MRSA: Methicillin-resistant *S. aureus*.

moiety was indicated by the correlation between δ 7.75 (2-NH of lysine residue) and 168.6 (C-1 of serine residue) in the HMBC spectrum. These data revealed that this compound comprises salicylate, a 2-oxazoline ring, and *N*-formyl-lysine, suggesting a building block of mycobactin-type siderophores such as mycobactins J, R, S and T [8]. Finally, this compound was determined to be 6-formylamino-2-{[2-(2-hydroxy-phenyl)-4,5-dihydro-oxazole-4-carbonyl]-amino}-hexanoic acid.

Therefore, the transvalencin Z structure was characterized as shown in Fig. 1, except for stereochemistry at the C-2 position of serine moiety and the C-2 position of lysine moiety.

Bioactive Assay

Antimicrobial and antitumor activities of transvalencin Z (**1**) were tested; the results are shown in Table 4. Antibiotic **1** was active against Gram-positive bacteria, especially against acid-fast bacteria such as *Corynebacterium xerosis*, *Gordonia branchialis* and *Mycobacterium smegmatis*, and their MIC values were less than 0.125 μg/ml. The antibiotic was moderately active against Gram-positive bacteria such as *Micrococcus luteus* (4.0 μg/ml) and *Staphylococcus aureus* (0.25 μg/ml), but not active against Gram-negative bacteria, fungi and tumor cells. A similar compound, methyl-2-(2′-hydroxyphenyl)-2-oxazoline-4-carboxylate (salicylic acid and serine moieties of **1**), was isolated from *Actinomadura* species [9]. Antimicrobial activity of **1** against Gram-positive bacteria was stronger than that of the simple compound, but its cytotoxic activity was weaker than that of this compound.

Compound **1** has three binding sites of the six found on

nocobactin-type and mycobactin-type siderophores [10]. For that reason, it is expected that **1** complexes with an iron ion (**1** : Fe=2 : 1 complex). The present study confirmed that **1** has no siderophore activity because its antimicrobial activity is not abolished by addition of Fe^{+3} ion into the medium. Furthermore, the color of the medium was not changed by the addition of the Fe^{+3} ion (Fe-nocobactin and Fe-mycobactin complexes are red) [11]. The inference is also supported by the fact that **1** shows no cytotoxic activity.

Most *Nocardia* strains isolated from clinical specimens were found to produce bioactive secondary metabolites with salicylic acid just as siderophores. For that reason, we were interested in the biosynthesis gene of salicylic acid in *Nocardia* clinical isolates. Our preliminary PCR amplification studies using salicylic acid biosynthesis genes [12] indicated that most *Nocardia* species possess gene(s) for production of salicylic acid containing compounds. Although most such compounds are considered to belong to salicylic acid containing siderophores, there remains the strong possibility of finding novel bioactive secondary metabolites containing salicylic acid, similar to the transvalencin Z antibiotic studied here.

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