

A-102395, a New Inhibitor of Bacterial Translocase I, Produced by *Amycolatopsis* sp. SANK 60206

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Abstract Bacterial phospho-*N*-acetylmuramyl-pentapeptide translocase (translocase I: EC 2.7.8.13) is a key enzyme in peptidoglycan biosynthesis, and a known target of antibiotics. Here we report a new nucleoside inhibitor for translocase I, A-102395, isolated from the culture broth of the strain *Amycolatopsis* sp. SANK 60206. A-102395 is a new derivative of capuramycin that has the benzene with a uniquely substituted chain instead of an aminocaprolactam. A-102395 is a potent inhibitor of bacterial translocase I with IC_{50} value of 11 nM, but possesses no antimicrobial activity against various strains tested.

Keywords translocase I, peptidoglycan, nucleoside inhibitor, capuramycin, *Amycolatopsis* sp., antimicrobial activity

Introduction

The need for new classes of antibiotics is growing as the emergence of antibiotics resistance continues to spread worldwide. Enzymes responsible for the biosynthesis of the peptidoglycan components of the bacterial cell wall are essential for growth, and therefore are well precedented targets for antibiotics. Among these enzymes, phospho-*N*-

acetylmuramyl-pentapeptide translocase (translocase I) catalyzes the first membrane step of peptidoglycan biosynthesis. It transfers MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to the lipid carrier, undecaprenyl phosphate to form lipid I. There are some compounds reported as translocase I inhibitors [1], such as mureidomycins [2], pacidamycins [3], napsamycins [4], liposidomycins [5], tunicamycin [6], capuramycins [7–13], muraymycins [14] and caprazamycins [15, 16]. They exhibit antimicrobial activity against various strains including multidrug-resistant stains, and show bactericidal activity [15, 17, 18]. Thus translocase I is an established target for the search of novel antibiotics.

In the course of our screening for bacterial translocase I inhibitors, we found inhibitory activity in the culture broth of *Amycolatopsis* sp. SANK 60206. In this paper, we describe the taxonomy and fermentation of the producing microorganism, isolation, physico-chemical properties, structure elucidation and biological activities of A-102395.

Materials and Methods

Materials

Undecaprenyl phosphate was purchased from Larodan Fine

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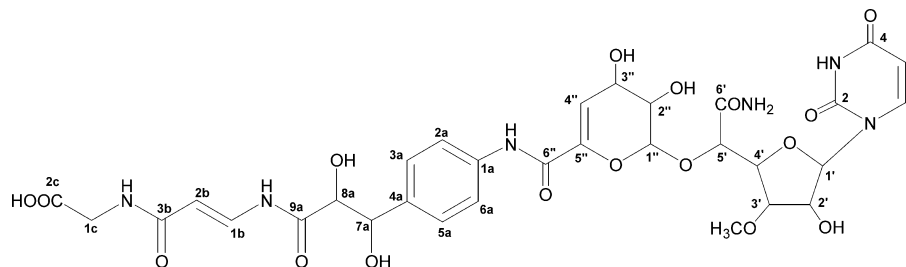


Fig. 1 Structure of A-102395.

Chemicals. Preparation methods for translocase I and a fluorescent substrate (UDP-MurNAc-L-Ala- γ -D-Glu-*m*-DAP-[*N*^ε-dansyl]-D-Ala-D-Ala) were previously reported [10].

Taxonomy of the Producing Organism

The producing strain *Amycolatopsis* sp. SANK 60206 was isolated from a soil sample collected in Hokkaido, Japan. The methods and media described by the International Streptomyces Project (ISP) [19] and Waksman [20] were used to determine the morphological characterizations and the physiological properties of the producing organism. The cell walls and whole-cell hydrolysates were analyzed by the methods of Hasegawa *et al.* [21]. Lipid characteristic of *Nocardia* spp., type A was analyzed by the method of Hecht [22]. The 16S rDNA was amplified by the polymerase chain reaction using genomic DNA of the strain and sequenced. The most related sequences were searched using the BLAST algorithm in the National Center for Biotechnology Information (NCBI).

Measurement of Translocase I Inhibitory Activity

The measurement of translocase I inhibitory activity was carried out in 96-well microtitre polystyrene plate (Corning Coaster, #3694) in 100 μ l containing 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 25 mM MgCl₂, 0.8% Triton X-100, 166 μ M undecaprenyl phosphate and 70 μ M UDP-MurNAc-L-Ala- γ -D-Glu-*m*-DAP-[*N*^ε-dansyl]-D-Ala-D-Ala. The reaction was initiated by the addition of the enzyme (0.625~2.5 μ g protein). The enzyme activity was monitored by measuring the increase in fluorescence at 538 nm (excitation at 355 nm).

Antimicrobial Activities

MICs were determined by the agar dilution method using Mueller Hinton agar (Becton Dickinson and Company).

General Experimental Procedures

The various NMR spectra were obtained on a Bruker AVANCE 500 spectrometer. Methanol-*d*₄ and DMSO-*d*₆

were used as solvent and internal references. FAB-MS spectra were recorded on a JEOL JMS-700QQ mass spectrometer. Optical rotations were recorded on a JASCO DIP-370 spectropolarimeter. UV spectra were obtained on a Shimadzu UV-265FW spectrometer. Fluorescence was measured at room temperature on a fluorescence spectrophotometer, Fluoroskan Ascent (Labsystems).

Results

Taxonomy

The cell walls of the strain contained *meso*-diaminopimelic acid but not glycine. The whole cell sugars had arabinose and galactose in the major amount as the diagnostic component. No nocardomycolic acids were present in cells. The strain showed the highest level of 16S rRNA gene similarity with the type strain of *Amycolatopsis rifamycinica* (AY083603) (1340/1350, 99.3%). Relatively high similarity values were shown with the type strains of *Amycolatopsis lexingtonensis* (AY183358) (1332/1345, 99.0%), *A. vancoresmycina* (AJ508240) (1332/1345, 99.0%). Based on the taxonomic properties, the strain was identified as *Amycolatopsis* sp. SANK 60206.

Fermentation

A loopful of a culture of strain SANK 60206 was inoculated into each of two 500-ml Erlenmeyer flasks containing 80 ml of sterilized seed medium consisting of galactose 2.0%, Bacto-Soytone (Difco) 1.0%, glycerol 1.0%, dextrin 2.0%, corn steep liquor 0.5%, sodium citrate 0.1%, Na₂HPO₄·12H₂O 0.3%, (NH₄)₂SO₄ 0.2%, CaCO₃ 0.2% and CB-442 (NOF Co., Ltd.) 0.05%. The inoculated flasks were incubated on a rotary shaker (210 rpm) at 28°C for 3 days. The cultures were combined and then 2.0 ml aliquots of the culture were transferred into each of sixty-four 500-ml Erlenmeyer flasks containing 80 ml of sterilized production medium consisting of the same seed medium. The inoculated flasks were incubated on a rotary shaker (210 rpm) at 28°C for 6 days.

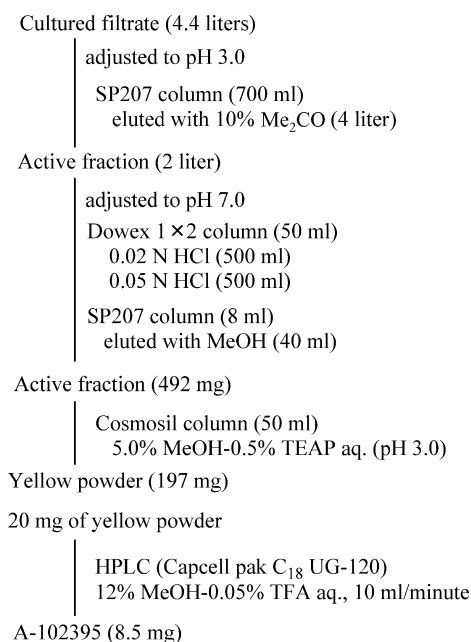


Fig. 2 Isolation procedures of A-102395.

Isolation

The isolation procedure of A-102395 is outlined in Fig. 2. First, 4.4 liters of the culture filtrate were adjusted to pH 3.0 with 1 M HCl and adsorbed onto a DIAION SP207 column (700 ml). The column was washed with water and active substance was eluted with 10% Me₂CO (4 liters) fractionated into 20 ml portions. The combined active fraction was concentrated *in vacuo* to remove Me₂CO (2 liters) and adjusted to pH 7.0 with 1 M NaOH and adsorbed onto a Dowex 1 × 2 column (50 ml). The column was washed with water and the active substance was successively eluted with 0.02 M HCl (500 ml) and 0.05 M HCl (500 ml) and fractionated in 20 ml portions. Active fractions were collected, adjusted to pH 3.0 with 1 M NaOH, subjected to SP207 column (8 ml). After the column was washed with water, active substance was eluted with MeOH and concentrated *in vacuo* to give 492 mg of brown powder. The powder was dissolved in 0.5% triethylamine phosphate (TEAP) aq. (pH 3.0, 2.5 ml) and applied onto a Cosmosil column (50 ml). The column was washed with 0.5% TEAP aq. (pH 3.0, 250 ml) and active substance was eluted with 5.0% MeOH-0.5% TEAP aq. (pH 3.0, 500 ml). The active fractions were collected, concentrated *in vacuo* to remove MeOH and subjected on SP207 column (8 ml). After the column was washed with water, active substance was eluted with MeOH and concentrated *in vacuo* to give 197 mg of yellow powder. Further, 20 mg of this powder was dissolved into 2.0 ml of 20% MeOH aq. containing 0.05% TFA and each

Table 1 Physico-chemical properties of A-102395

Appearance	White powder
$[\alpha]_D^{25}$	+70 (c 0.8, H ₂ O)
UV λ_{\max} nm (ϵ) in H ₂ O	266 (34,900)
Molecular formula	C ₃₁ H ₃₆ N ₆ O ₁₇
FAB-MS (m/z)	765 (M+H) ⁺
HR-FAB-MS (m/z)	
Found:	787.2064 (M+Na) ⁺
Calcd.:	787.2034
IR ν_{\max} (KBr) cm ⁻¹	3336, 3103, 2934, 1670, 1528, 1410, 1251, 1110, 1058, 978

200 μ l portion of the solution was subjected to HPLC column (Capcell pak C₁₈ UG-120, 20 i.d. × 250 mm). The chromatography was performed 10 times with 12% MeOH aq. containing 0.05% TFA at a flow rate of 10.0 ml/minute, under the UV detection at 260 nm. A-102395 was eluted at the retention time of 20.5 minutes. The HPLC fractions were concentrated and freeze-dried, to yield pure A-102395 as a white powder (8.5 mg).

Physico-chemical Properties

Physico-chemical properties of A-102395 are summarized in Table 1. The molecular formula of A-102395 was determined by high resolution FAB-MS to be C₃₁H₃₆N₆O₁₇. The UV spectrum of A-102395 in aqueous solution is characterized by a single maximum absorption at 266 nm (ϵ 34,900).

Structure Elucidation

The ¹H- and ¹³C-NMR assignments of A-102395 in methanol-*d*₄ are listed in Table 2. In the ¹³C-NMR spectrum of A-102395, 29 carbon signals were observed. The multiplicity of carbon signals was determined by DEPT experiments. HSQC [23] experiment established all one-bond ¹H-¹³C connectivities. COSY [24] and HMBC [25] experiments clarified that the partial structure A of A-102395 (Fig. 3) was identical to that of capuramycin and A-500359s [9]. Further analysis of the additional correlations of the signals revealed the partial structure B and C as follows. The ¹H-¹³C long range couplings of ²J and ³J observed in the HMBC experiment gave the following information. The cross peaks from H-2a and H-6a to C-1a and C-4a, and from H-3a and H-5a to C-1a and C-4a as well as the coupling constant (8.5 Hz) observed between H-2a/H-6a and H-3a/H-5a suggested the presence of a 1,4-disubstituted benzene. The cross peaks from H-8a to C-9a elucidated the structure from C-7a to C-9a. The cross peaks from H-7a to C-3a and C-5a, and from H-8a to C-4a

Table 2 ^{13}C - and ^1H -NMR data for A-102395 in methanol- d_4

No.	δ_{C}	δ_{H} ($J=\text{Hz}$)
2	152.3	
4	166.3	
5	102.8	5.73 (1H, d, 8.0)
6	142.0	7.85 (1H, d, 8.0)
1'	91.7	5.75 (1H, d, 3.0)
2'	74.1	4.25 (1H, dd, 3.0, 5.0)
3'	80.1	3.73 (1H, br t, 5.0)
3'-OCH ₃	58.7	3.30 (3H, s)
4'	81.3	4.50 (1H, dd, 2.0, 5.0)
5'	77.4	4.83 (1H, d, 2.0)
6'-CONH ₂	174.0	
1''	101.1	5.28 (1H, d, 4.0)
2''	67.5	4.09 (1H, t, 4.0)
3''	63.7	4.44 (1H, t, 4.0)
4''	110.7	6.07 (1H, d, 1.5)
5''	144.2	
6''	161.7	
1a	138.2	
2a/6a	122.1	7.62 (2H, d, 8.5)
3a/5a	128.3	7.41 (2H, d, 8.5)
4a	139.6	
7a	75.4	5.03 (1H, d, 3.0)
8a	77.6	4.21 (1H, d, 3.0)
9a	174.1	
1b	135.5	7.83 (1H, d, 14.0)
2b	106.3	5.93 (1H, d, 14.0)
3b	169.8	
1c	42.2	3.96 (2H, br s)
2c	173.6	

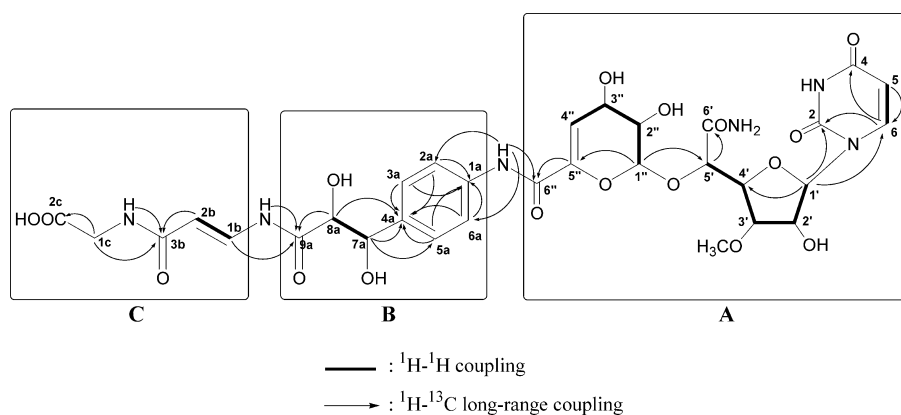
elucidated the connection between C-7a and benzene in the partial structure B. The cross peaks from H-2b to C-3b and from H-1c to C-3b and C-2c revealed the partial structure C from C-1b to C-2c. The geometrical configuration of the double bond at C1b-C2b proved to be *E* based on $J_{\text{H1b-H2b}}$ (14.0 Hz).

To determine the linkages between the three partial structures, a series of NMR experiments were performed again using DMSO- d_6 as a solvent. In the ^1H -NMR using DMSO- d_6 as a solvent, additional three exchangeable proton signals, δ_{H} 10.30, δ_{H} 9.80 and δ_{H} 8.20 were observed. The connection between the partial structure A and B was established by the cross peaks from NH-6'' (δ_{H} 9.80) to C-6'', C-2a and C-6a. The connection between the partial structure B and C was determined by the cross peaks from NH-9a (δ_{H} 10.30) to C-9a as well as from H-8a and H-1b to C-9a in HMBC spectrum. The last exchangeable proton (δ_{H} 8.20) was assigned to NH-3b by HMBC cross peaks from δ_{H} 8.20 to C-3b.

From its molecular formula, two carbonyl residues, C-6' and C-2c, were expected to form amide or carboxylate group. To determine which carbon formed carboxylate group, A-102395 was converted to the methyl ester derivative by TMS-diazomethane. In HMBC experiment of this derivative (using methanol- d_4 as solvent), the long range coupling from δ_{H} 3.72 of methoxy proton to C-2c was observed. It was proved that C-2c formed carboxylate group and C-6' formed amide group. The planer structure of A-102395 was concluded to be as shown in Fig. 1.

Biological Activities of A-102395

A-102395 inhibited translocase I with IC_{50} value of 11 nM, but did not show any antimicrobial activity against Gram-positive and -negative bacteria tested.

**Fig. 3** ^1H - ^1H and ^1H - ^{13}C long-range correlations of A-102395.

Discussion

A new capuramycin derivative, A-102395, was isolated from the fermentation broth of the strain identified as *Amycolatopsis* sp. SANK 60206. A-102395 showed the potent inhibitory activity for bacterial translocase I with IC_{50} values of 11 nM. The inhibitory activity of A-102395 is as potent as that of capuramycin (IC_{50} : 18 nM) [10]. In the previous studies of capuramycin and A-500359s [10, 11], we revealed the core skeleton (2'-methoxyuridine attached an unsaturated uronic acid moiety) was necessary for the inhibition of translocase I. Compared with the structure of A-102395, the core skeleton (partial structure A) is the same as of capuramycin, but A-102395 has the benzene with a uniquely substituted chain instead of an aminocaprolactam. So, it is considered that the potency of the inhibition of translocase I by A-102395 basically comes from the core skeleton, and the other structural features may influence the activity.

A-102395 had potent inhibitory activity for bacterial translocase I, but lacked antimicrobial activity against various strains tested. In the previous studies, it revealed capuramycin and A-500359s showed antimicrobial activities against *S. pneumoniae* and *Mycobacterium*. We think A-102395 has the potency for antimicrobial activity, but the poor permeability of the bacterial membrane may cause the lack of the antimicrobial activity. There are some compounds reported as translocase I inhibitors. Most of them are also natural products containing a nucleoside moiety and have unique spectra of antimicrobial activity. Mureidomycins, pacidamycins and napsamycins are closely related compounds containing a 3'-deoxyuridine attached a peptide chain, and active against *Pseudomonas* strains. Liposidomycins and caprazamycins are uridine compounds containing a diazepine, and an acyl moiety. They have potent antimicrobial activity against *Mycobacterium*. These limited spectra of antimicrobial activity also may be due to a permeability of the bacterial membrane. In the recent reports, some of synthesized analogues of pacidamycins [26], liposidomycins [27], capuramycin [28, 29] and caprazamycins [30, 31] showed broader spectrum and more potent antimicrobial activity. So, there is possibility that some chemical modification of A-102395 at the C-2c or the chain attached to benzene may improve the antimicrobial activity.

Previously, we discovered capuramycin related compounds, A-500359s and A-503083 [32]. Though these producing strains were classified into *Streptomyces* sp., the producing strain of A-102395 is distinct from them taxonomically. It is interesting to note that strains belong to

different families of actinomycetes produce similar compounds. It is possible that both of producing organisms share common biosynthetic genes and synthesize the core skeleton of capuramycin *via* the identical biosynthetic pathway. Therefore, these producing organisms may be useful to study for biosynthesis of capuramycin.

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