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Studies on Novel Bacterial Translocase I Inhibitors, A-500359s

IV. Biosynthesis of A-500359s

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This report describes the isolation of novel A-500359 analogues from the culture broth of *Streptomyces griseus* SANK 60196 and ^{13}C-incorporation studies of A-500359 A to reveal the biosynthetic pathway of A-500359 derivatives. As a result, A-500359 M-3 and J were isolated as novel analogues. The former, isolated from a culture broth fed with unnatural amino acids, was a novel amino acid adduct of A-500359, and the latter was found to be a putative precursor of all A-500359 derivatives, on the basis of the structure. Moreover, ^{13}C-incorporation studies revealed the origin of every carbon atom of A-500359 A.

From these results, it was revealed that the core skeleton of A-500359 was biosynthesized from uridine and phosphoenolpyruvate in the same manner as for polyoxin, a nucleoside antibiotic. Moreover, the uronic acid and aminocaprolactam moiety was derived from hexose and lysine, respectively, and two methyl groups of A-500359 A were derived from methionine.

A-500359s[1~3] were isolated from the culture broth of *Streptomyces griseus* SANK 60196 as potent inhibitors of translocase I, an essential enzyme for the synthesis of peptidoglycan, a major component of the bacterial cell wall. In addition, they have a specific anti-bacterial activity against *Mycobacterium*.[2] We have described their structures and biological activities, and elucidated the absolute configuration of A-500359A (**1**) isolated in previous studies.[1,2]

This class of compounds contains an aminocaprolactam moiety as shown in Fig. 1. In the previous paper, we also described that novel analogues were isolated from the culture broth of SANK 60196 by using a biosynthetic pathway-specific inhibitor.[3] Several de-caprolactam derivatives and unnatural amino acid adducts of the core skeleton of A-500359 were obtained from the culture broth of SANK 60196 fed with *S*-(2-aminuteoethyl)-L-cysteine (AEC), an inhibitor of aspartokinase, an enzyme used for lysine biosynthesis in Corynebacterium species.[3] From these results, it was suggested that novel derivatives could be created using knowledge gathered on the biosynthetic pathway. In order to determine the biosynthetic pathway of A-500359 derivatives, we performed the isolation of novel intermediates and incorporation studies of ^{13}C-labeled precursors to **1**. From these studies, an unnatural amino acid adduct of the A-500359 core skeleton was isolated from the culture broth fed with AEC and unnatural amino acids at the same time.

In this paper, we describe the details of these studies.

Materials and Methods

General Experimental Procedures

The various NMR spectra were obtained on a Bruker AVANCE 500 and AMX 360 spectrometer. HDO served as an internal standard (δ 4.75) for ^{1}H NMR, and 1,4-dioxane was used as an internal standard for ^{13}C NMR (δ 49.2). FAB-MS was recorded on a Micromass Autospec mass spectrometer. Optical rotations were measured with a

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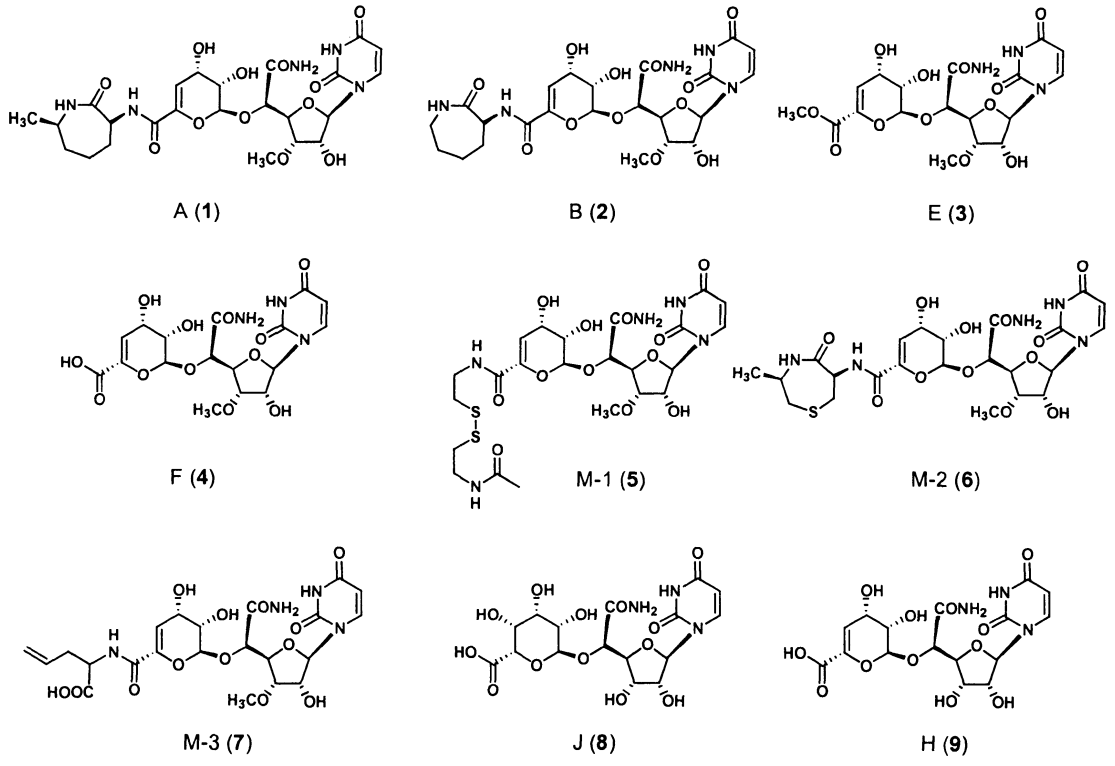
Fig. 1. Structures of A-500359s.



JASCO DIP-370 spectropolarimeter. IR spectra were obtained on a JASCO FT/IR-8900 spectrometer. UV spectra were recorded on a Shimadzu UV-265FW spectrometer. GC/MS analysis was performed by Agilent GC/MSD [EI-MS detector, 5973; GC system, 6890; column, a fused silica capillary column HP-5MS (30 m by 250 μm, Agilent); carrier gas, He].

Labeled Compounds

[*Methyl*-^{13}C]L-methionine (99 atom% ^{13}C), [6-^{13}C]D-glucose (99 atom% ^{13}C), and [1-^{13}C]D-ribose (99 atom% ^{13}C) were purchased from Sigma Co. Sodium [3-^{13}C]pyruvate (99 atom% ^{13}C) was purchased from Aldrich Chemical Co. [1-^{13}C]D-mannose, and [1-^{13}C]L-lysine dihydrochloride were purchased from Isotec Inc.

Bacterial Strain and Culture Medium

Streptomyces griseus SANK 60196, the producing strain of A-500359, was used. The following four media were used. Medium A contains 3% maltose, 0.5% meat extract, 0.5% Polypepton, 0.5% NaCl, 0.3% $CaCO_3$, and 0.05% CB442; Medium B contains 3% maltose, 0.5% yeast extract, 0.5% meat extract, 0.5% Polypepton, 0.5% NaCl, 0.3% $CaCO_3$, and 0.05% CB442; Medium C contains 5% glucose, 0.4% meat extract, 0.3% Polypepton, 1% skim milk, 1% CSL, 0.5% NaCl, and 0.05% CB442; Medium D contains 3% maltose, 0.5% yeast extract, 0.5% meat extract, 0.5% Polypepton, 0.5% NaCl, 0.3% $CaCO_3$, and 0.001% $CoCl_2$. The pH of all media was adjusted to 7.4 with 0.5 N NaOH prior to sterilization.

Production of A-500359 M-3 (7)

A loopful of the spore of a slant culture of the strain SANK 60196 was inoculated in 100 ml of medium A in a 500-ml Erlenmeyer flask and the seed culture was incubated on a rotary shaker at 23°C for 3 days. The seed culture (3%, v/v) was transferred into each 100 ml of medium B in 500-ml Erlenmeyer flasks and after six hours, filter-sterile *S*-(2-aminoethyl)-L-cysteine (AEC) and D,L-allylglycine solution were added to the flasks at a final concentration of 10 mM. Then the flasks were shaken at 23°C for 7 days.

Isolation of 7

The fermentation broth (1 liter) was centrifuged to separate the supernatant and mycelial cake. The supernatant

(1 liter) adjusted to pH 3.0 with 1 N HCl was subjected to column chromatography using a DIAION HP-20 (200 ml, Mitsubishi Chemical Corporation) equilibrated with water containing 0.05% trifluoroacetate. The column was washed with water containing 0.05% trifluoroacetate (500 ml), and then the adsorbed material was eluted with water (500 ml). The eluate was concentrated *in vacuo* and lyophilized to obtain a crude powder. The powder was purified by HPLC [column, Pegasil ODS (i.d. 20×250 mm), Senshu Scientific Co., Ltd.; mobile phase, water containing 0.05% trifluoroacetate; flow rate, 10.0 ml/minute; detection, UV 260 nm; retention time, 28.0 minute]. The eluate was concentrated *in vacuo* and lyophilized to give **7** as a colorless powder (11.1 mg).

Production of A-500359 J (**8**)

A loopful of the spore of a slant culture of strain SANK 60196 was inoculated in 100 ml of medium A in a 500-ml Erlenmeyer flask and the seed culture was incubated on a rotary shaker at 23°C for 3 days. The culture was transferred into 100 ml of medium C in 500-ml Erlenmeyer flasks (3%, v/v) and then the flasks were shaken at 23°C for 11 days.

Isolation of **8**

The fermentation broth (1 liter) was filtered with 5% (w/v) celite 545. The filtrate (1 liter) was applied to a DIAION HP-20 column (200 ml), and the column was washed with water (500 ml). The flow through and wash fractions were combined, adjusted to pH 9.0 by 6 N NaOH, and then it was applied to Dowex SBR-P (OH^- type, Dow Chemical Co., Ltd.) column (300 ml). The column was washed with water (300 ml) and the adsorbed material was eluted with 1 N HCl. The eluate was adjusted to pH 7.0 with 6 N NaOH, and then purified on an activated charcoal column (50 ml). The column was washed with water (300 ml) and the active material was eluted with 60% aqueous acetone (200 ml). The eluate was concentrated *in vacuo* and lyophilized to obtain a crude powder (558 mg). The powder was finally purified by HPLC [column, Pegasil ODS (i.d. 20×250 mm); mobile phase, 0.05% aqueous trifluoroacetate; flow rate, 10.0 ml/minute; detection, UV 260 nm; retention time, 11.1 minutes]. The eluate was concentrated *in vacuo* and lyophilized to give **8** as a colorless powder (16.2 mg).

Determination of the Absolute Configuration of the Uronic Acid Moiety in **8**[4]

A solution of **8** (0.5 mg) in MeOH (0.2 ml) was treated with 2.0 M (trimethylsilyl)diazomethane (0.05 ml) at 25°C for 15 minutes. The reaction mixture was evaporated *in vacuo*, and the residue was re-dissolved in MeOH (0.5 ml). The solution was treated with $NaBH_4$ (5 mg) at 25°C for 10 minutes, and then 1 N HCl was added to stop the reaction. The reaction mixture was then concentrated *in vacuo* to dryness. The residue was heated with 5% HCl-MeOH (0.2 ml) and (−)-2-butanol (0.2 ml) in a sealed ampoule at 80°C for 12 hours. After that, the reaction mixture was concentrated *in vacuo* to dryness and treated with acetic anhydride (0.2 ml) in pyridine (0.2 ml) and at 60°C for 30 minutes. The resultant mixture was subsequently analyzed by GC/MS analysis (initial temperature, 60°C; column oven, 5°C/minute from 170 to 240°C; He gas flow, 2 ml/minute; injector temperature, 300°C).

The retention times of 1-((−)-2-butyl)-2,3,4,6,-tetraacetyl hexopyranoside derived from authentic hexose and **8** were as follows: 1-((−)-2-butyl)-2,3,4,6,-tetraacetyl-D-glucoside, 10.31 minutes; 1-((−)-2-butyl)-2,3,4,6,-tetraacetyl-L-glucoside, 10.07 minutes; 1-((−)-2-butyl)-2,3,4,6,-tetraacetyl-D-mannoside, 9.71 minutes; 1-((−)-2-butyl)-2,3,4,6,-tetraacetyl-L-mannoside, 9.62 minutes; 1-((−)-2-butyl)-2,3,4,6,-tetraacetyl-D-taroside, 10.40 minutes; 1-((−)-2-butyl-2,3,4,6,-tetraacetyl-L-taroside, 10.34 minutes; derivative from **8**, 10.39 minutes; *m/z*: 57, 81, 98, 115, 140, 157, 200, 242, 331).

In Vitro Biological Assays

In vitro translocase I inhibitory activity and antimicrobial activity were measured according to the method reported in the previous paper.[2]

Addition of ^{13}C-Labeled Precursor[5]

A loopful of cells from the slant culture of strain SANK 60196 was inoculated in 20 ml of medium A in a 100-ml Erlenmeyer flask and incubated on a rotary shaker at 23°C for 3 days. The seed culture (3%, v/v) was transferred into 20 ml of medium D in 100-ml Erlenmeyer flasks, and then shaken at 23°C for 3 days. Filter-sterilized ^{13}C-labeled compounds (0.2 mmol) in 2 ml of water were added to the flasks. And fermentation was continued for another 3 days before harvest.

Isolation of ^{13}C-Labeled **1**

Each 60-ml of each culture broth fed with ^{13}C-labeled precursor was centrifuged to obtain the supernatant. The supernatant was purified on an HP-20 column (5 ml). After the column was washed with water, the adsorbed material was eluted with 10% aqueous acetone (10 ml). The eluate was concentrated *in vacuo*. The residue was purified by HPLC [column, Capcell pak C18 UG-120 (i.d. 10×

250 mm), Shiseido Co., Ltd.; mobile phase, 10% aqueous acetonitrile containing 0.05% trifluoroacetate; flow rate, 4.5 ml/minute; detection, UV 260 nm]. The eluate was evaporated *in vacuo* and lyophilized to give ^{13}C-labeled **1**.

Assignment of ^{1}H and ^{13}C NMR Spectra[1)]

The 360 MHz ^{1}H and 90 MHz ^{13}C NMR spectra were unambiguously assigned based on DEPT, COSY, HSQC, and HMBC experiments. The intensity ratios of ^{13}C NMR signals of labeled **1** to those of unlabeled **1** were calculated.

Results

A-500359 M-3 (**7**)

Isolation of A-500359 F (**4**) and M-2 (**6**) from a SANK 60196 culture broth fed with *S*-(2-amino ethyl)-L-cysteine (AEC) has been previously reported.[3)] From these results, it was suggested that the lysine biosynthetic pathway was blocked by AEC to produce **4**, and AEC was incorporated to make **6**. In other words, the origin of the aminocaprolactam moiety in the structure of A-500359 was lysine. Furthermore, according to the results, there is a possibility that an unnatural product was created by the co-addition of AEC and certain amino acids. Therefore, co-addition trials were performed.

Fermentation and Isolation of **7**

According to a method described in the previous report[3)] on the discovery of **6**, supernatants from the culture broth fed with both, various unnatural amino acids and AEC, were analyzed by reversed-phase HPLC with a photodiode array detector. After D,L-allylglycine addition, one peak with a similar UV spectrum as that of A-500359 newly appeared. Therefore, the compound corresponding to the peak was isolated from the supernatant by HP-20 column chromatography followed by reversed-phase HPLC.

Structure Determination of **7**

The physicochemical properties, and the ^{1}H and ^{13}C NMR data of **7** in D_2O are summarized in Tables 1 and 2, respectively. The molecular weight of **7** was determined to be 556 by FAB-MS and the molecular formula was determined to be $C_{22}H_{27}N_4O_{13}$ by high-resolution FAB-MS as well as by NMR studies. Although the ^{1}H NMR spectrum was almost similar to that of **1**,[1)] an exomethylene (δ 5.07, 5.11), a methylene (δ 2.44, 2.55), and an olefinic proton (δ 5.53) signal were newly observed instead of the signal from the aminocaprolactam moiety in **1**.[1)] From a further analysis of the COSY, HMQC, and HMBC spectra, the newly appeared signals were assigned to be an allylglycine moiety and the structure was finally determined as shown in Fig. 2.

Stereoselectivity of Allylglycine Incorporation

In this study, racemic allylglycine was used. However, only one of the single isomer was obtained from the broth. This result suggested that a D- or L-isomer was stereoselectively incorporated into **7** or that after the incorporation of both isomers, one isomer was converted to the other by the producing strain. Therefore, in order to clarify the stereoselectivity, a feeding experiment was carried out with the addition of D- or L-allylglycine, separately. After fermentation, broth supernatants were analyzed by HPLC. **7** was detected in the supernatants from the broth fed with the racemic amino acid or L-isomer, but not detected in the supernatants from the broth fed with

Fig. 2. COSY and HMBC correlations of **7** and **8**.

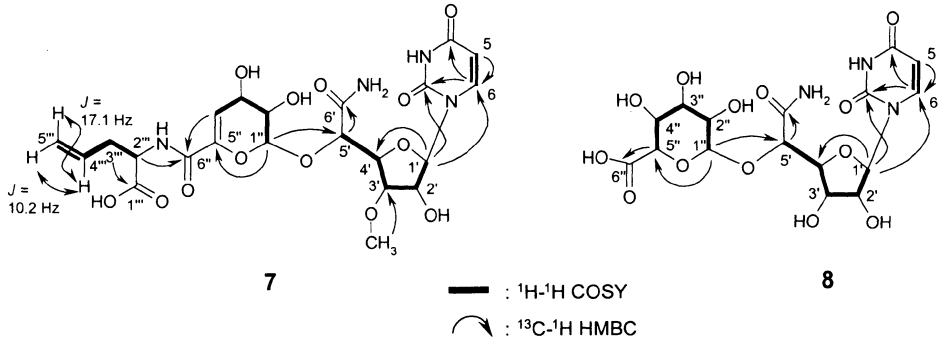


Table 1. Physico-chemical properties of **7** and **8**.

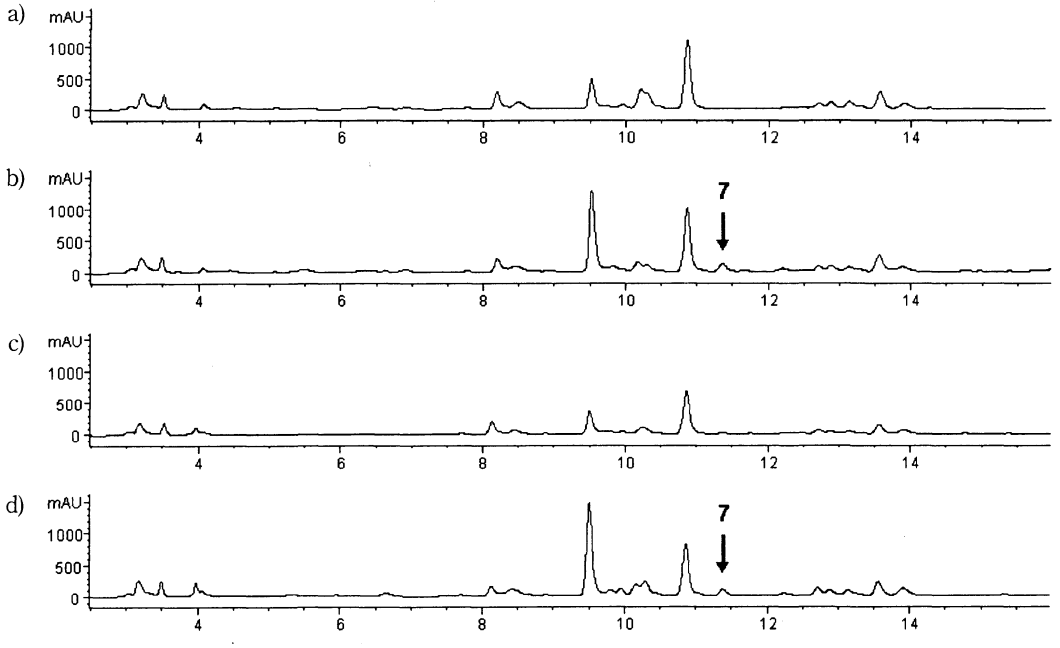
	7	**8**
Appearance	Colorless powder	Colorless powder
$[\alpha]_D^{20}$	+92° (*c* 0.1 , H_2O)	+82° (*c* 0.1 , H_2O)
FAB-MS (*m/ z*)	557 $(M+H)^+$	462 $(M-H)^-$
HRFAB-MS (*m/ z*)	for $C_{22}H_{29}N_4O_{13}$	for $C_{16}H_{20}N_3O_{13}$
	Found: 557.1754	Found: 462.0996
	Calcd.: 557.1731	Calcd.: 462.0996
Molecular formula	$C_{22}H_{28}N_4O_{13}$	$C_{16}H_{21}N_3O_{13}$
UV $\lambda_{max}^{H_2O}$ nm (ε)	263 (10,000)	262 (9900)
IR ν_{max} (KBr) cm^{-1}	3407, 2938, 1684,	3371, 2931, 1684, 1467,
	1524, 1465, 1399,	1407, 1273, 1204, 1107,
	1385, 1335, 1268,	1058
	1205, 1139, 1118,	
	1095, 1063, 1021	

Table 2. ^1H and ^{13}C NMR data for **7** and **8**.

	7		**8**	
Position	δ_C[b] (mult.[a])	δ_H[b] (mult., *J* in Hz)	δ_C[c] (mult.[a])	δ_H[c] (mult., *J* in Hz)
2	152.0 (s)		151.0 (s)	
4	166.8 (s)		165.9 (s)	
5	102.6 (d)	5.58 (1H, d, 8.1)	101.7 (d)	5.70 (1H, d, 8.2)
6	141.5 (d)	7.72 (1H, d, 8.1)	141.6 (d)	7.60 (1H, d, 8.2)
1'	90.6 (d)	5.74 (1H, d, 3.7)	90.7 (d)	5.50 (1H, d, 3.1)
2'	72.6 (d)	4.32 (1H, dd, 3.7, 5.5)	73.0 (d)	4.13 (1H, dd, 3.1, 5.6)
3'	78.9 (d)	3.66 (1H, t, 5.5)	68.8 (d)	4.11 (1H, t, 5.6)
3'-OCH_3	58.6 (q)	3.27 (3H, s)		
4'	82.4 (d)	4.45 (1H, m)	82.8 (d)	4.05 (1H, dd, 5.3, 5.6)
5'	75.7 (d)	4.73 (1H, overlapped with HDO)	75.4 (d)	4.30 (1H, d, 5.3)
6'-$CONH_2$	173.6 (s)		172.6 (s)	
1''	99.8 (d)	5.36 (1H, d, 3.1)	99.2 (d)	4.90 (1H, d,1.9)
2''	65.3 (d)	4.17 (1H, ddd, 1.1, 2.5, 3.1)	68.9 (d)	3.83 (1H, ddd, 1.4, 1.9, 3.9)
3''	62.6 (d)	4.46 (1H, m)	64.4 (d)	3.75 (1H, t, 3.4)
4''	109.9 (d)	5.95 (1H, dd, 1.1, 1.9)	69.7 (d)	4.02 (1H, ddd, 1.4, 1.7, 3.4)
5''	142.2 (s)		71.4 (d)	4.33 (1H, d,1.7)
6''	162.3 (s)		171.9 (s)	
1'''	177.6 (s)			
2'''	55.4 (d)	4.33 (1H, t, 4.3)		
3'''	37.1 (t)	2.44 (1H, ddd, 4.3, 7.3, 13.3)		
		2.52 (1H, ddd, 4.3, 7.5, 13.3)		
4'''	134.0 (d)	5.53 (1H, m)		
5'''	119.0 (t)	5.07 (1H, brd, 10.2)		
		5.11 (1H, brd, 17.1)		

[a] Multiplicity inferred using the DEPT pulse sequence.
[b] ^1H and ^{13}C NMR were measured at 360 MHz and 90 MHz, respectively.
[c] ^1H and ^{13}C NMR were measured at 500 MHz and 125 MHz, respectively.

Fig. 3. Stereoselectivity of incorporation of allylglycine into **7**.



Supernatant of culture broth fed with a) *S*-(2-aminoethyl)-L-cysteine (AEC), b) AEC and D,L-allylglycine, c) AEC and D-allylglycine and d) AEC and L-allylglycine. Column, Pegasil ODS (6 x 150 mm); solvent, A: water containing 0.05% trifluoroacetic acid., B: 90% acetonitrile; B (%), 5-30/ 2-15 minutes; flow rate, 1.0 ml/minute; detection, UV 240 nm

AEC (control) nor D-isomer as shown in Fig. 3. These results indicated that the L-isomer was incorporated into **7**, stereoselectively.

A-5000359 J (**8**)

It has been suggested that H (**9**) was an important intermediate in the biosynthesis of A-500359 on the basis of the structure.[3] However, no intermediate prior to **9** was discovered in the previous experiment.[3] Therefore, a further search to find novel intermediates was done.

Fermentation and Isolation of **8**

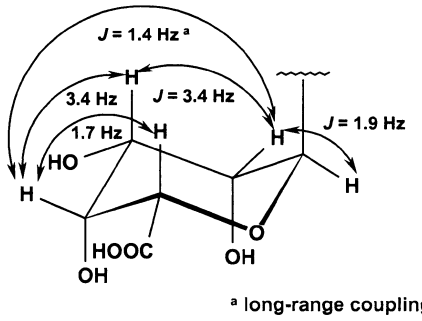
It was considered that the intermediate prior to **9** would have a reduced C-4″ double bond. The only precursor resembling such a moiety would be uridine. Thus, we attempted to discover compounds with a similar UV spectrum to uridine (UV λ_{max}, 260~265) by reversed-phase HPLC and photodiode array detection. When the supernatant from the broth cultured in the high production medium of **3** and **4** (medium C) was analyzed by HPLC, a peak in the UV spectrum was detected. The compound corresponding to the peak was purified on an HP-20, Dowex SBR-P (OH^- type) and activated charcoal columns and by reversed-phase HPLC. The compound was found to be **8**. The physico-chemical properties, and ^1H and ^{13}C NMR data measured in D_2O of **8** are summarized in Tables 1 and 2, respectively.

Structure Determination of **8** and Absolute Configuration of the Uronic Acid Moiety in **8**

The molecular weight of **8** was determined to be 463 by FAB-MS, and the molecular formula was determined to be $C_{16}H_{21}N_3O_{13}$ by high resolution FAB-MS as well as by NMR studies. The olefinic signal of H-4″ in **9** disappeared in the ^1H NMR spectrum and two *O*-methine protons were newly observed. Finally, on the basis of the analysis of various 2D NMR data, the structure of **8** was determined to be as shown in Fig. 2 except for the location of the carbamoyl group. Since C-6′ and C-6″ are possible positions to which an amino group may attach to make a carbamoyl, both were evaluated chemically to determine the correct position.

In general, a methyl ester can be converted to an alcohol by treatment with an excess amount of $NaBH_4$, but a carbamoyl can not. **8** was treated with (trimethylsilyl)-diazomethane in MeOH. The methyl ester was converted to the corresponding alcohol by $NaBH_4$ in MeOH. The alcohol derivative was treated with (−)-2-butanol in 5% HCl/MeOH, and then with acetic anhydride in pyridine.[4] The reaction mixture was analyzed by GC/MS directly. The acetylated-2-butyl derivative was detected as a peak with a retention time of 10.39 minutes, which was identical to that of the authentic D-talose derivative (see materials and methods section). On the basis of the structure, **8** was assumed to be an intermediate of all A-500359 analogues, and was found to be converted to **9** by dehydration at C-4″ and -5″. The carboxyl group in the uronic acid moiety was indeed converted to a corresponding alcohol. Therefore, the carbamoyl carbonyl was assigned to position C-6′ of **8**. This result also indicated that the configuration of the uronic acid moiety in **8** was identical to that of D-talose. The conformation was determined by the application of the Karplus rule to the ^1H-^1H coupling constants of **8** as shown in Fig. 4.

Fig. 4. Relative stereochemistry of the uronic acid moiety in **8**.



Biological Properties of 7 and 8[2]

Table 3 shows the biological properties of **7** and **8**. While **7** exhibited inhibitory activity against translocase I with an IC_{50} value of 600 ng/ml, **8** showed no inhibitory activity at 1000 ng/ml. **7** was active against *Mycobacterium smegmatis*, but **8** showed no antimicrobial activity.

Biosynthesis of 1

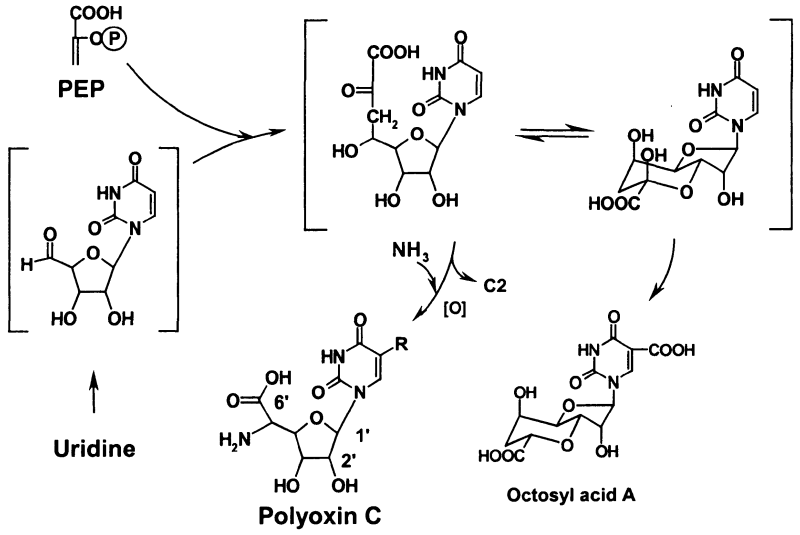
From the viewpoint of biosynthesis, the origin of the carbon skeleton of **1** could be envisioned as an adduct of one uridine, one uronic acid derived from franose and one aminocaprolactam derived from lysine. However, the origin

Table 3. Antimicrobial spectra and translocase I inhibitory activity of **7**, **8** and **1**.

Test organisms	Diameter of inhibition zone (mm)		
	7[a]	**8**[a]	**1**[b]
Staphylococcus aureus FDA 209P	0	0	0
Enterococcus faecalis S-299	0	0	+[d]
Bacillus subtilis PCI 219	(12)[c]	0	12
Mycobacterium smegmatis ATCC 607	(12)[c]	0	(39)[c]
Escherichia coli NIHJ JC-2	(11)[c]	0	15
Klebsiella pneumoniae PCI 602	16	0	19
Proteus vulgaris OX 19	0	0	0
Pseudomonas aeruginosa SANK 73575	0	0	0
Bacteroides fragilis SANK 71176	0	0	0
Mycoplasma mycoides PG-1	0	0	0
Mucor hiemalis SANK 11669	0	0	0
Aspergillus niger SANK 22667	0	0	0
Trichophyton metagrophytes SANK 11868	0	0	0
Candida albicans YU 1200	0	0	0
IC_{50} (μM)	1.8	>2.0	0.017

[a] 40 μg/disk of 10 mg/ml solution, [b] 40 μg/disk of 5 mg/ml solution, [c] partial inhibition zone, d: < 10 mm

Fig. 5. Biosynthetic pathway of polyoxin.[6]



of the C-6′ carbamoyl carbon was unclear. Since in the case of polyoxins[6], a nucleoside antibiotic, it has been reported that the C-6′ carbonyl carbon is derived from phosphoenolpyruvate, it is suggested that the C-6′ amide carbon in the nucleoside moiety of **1** is derived from a pyruvate in the same manner as polyoxins (Fig. 5).[6] Therefore, to clarify the origin of these components and methyl group donors, glucose, mannose, lysine, methionine, ribose, and pyruvic acid were selected as ^{13}C-labeled biosynthetic precursors.[5] These precursors were added individually to the culture at 3 days after inoculation, and the cultivation was continued for an additional 9 days. **1** was produced and purified using an HP-20 column and by reversed-phase HPLC. Each result of the precursor incorporation trials is discussed below and summarized in Table 4 and Fig. 6.

Incorporation of ^{13}C-Labeled Glucose and Mannose

First, feeding experiments using [6-^{13}C]D-glucose and [1-^{13}C]D-mannose were carried out. In the feeding experiment of [6-^{13}C]D-glucose, ^{13}C-incorporation was confirmed at C-6″, and in the case of [1-^{13}C]D-mannose, a high level of ^{13}C-incorporation at C-1″ was observed. These results indicate that the acetal ring moiety of **1** is derived directly from hexose. Moreover, in each case, ^{13}C incorporation into the caprolactam ring, and uracil, and at C-1′ and -5′ was also observed. It has suggested that these hexose rings were degradated by primary metabolism and converted to amino acids. They were incorporated into the caprolactam ring and uracil moiety. The selective incorporation at C-1′ and C-5′ of both precursors also suggests that C-1′ and C-5′ of the carbon skeleton were synthesized from ribose *via* the pentose phosphate cycle.

Incorporation of ^{13}C-Labeled Pyruvate

In the feeding experiment with sodium [3-^{13}C]pyruvate, a 2.1- to 4.2-fold enrichment of carbons at the positions of the C-2, -5, -6, -6′, -5″, -6″, -3″ and -5″ were observed. The 2-, and 6-fold incorporation of ^{13}C into C-6′, compared to the ratio of 1.5 as in the case of [6-^{13}C]D-glucose, indicated that the amide carbonyl is derived not from the direct incorporation of hexose but from a pyruvate in the same manner as in the polyoxin biosynthesis.[6]

Incorporation of ^{13}C-Labeled Ribose

In the feeding experiment with [1-^{13}C]D-ribose, C-1′ was enriched by 17-fold. This result clearly indicates that the C-1′ to -5′ unit is directly derived from ribose, and that C-6′ is formed after the ribose biosynthesis. This result agrees reasonably well with the results described above.

Incorporation of ^{13}C-Labeled Methionine

The feeding experiment with [*Methyl*-^{13}C]L-methionine was carried out to clarify the origin of the 6‴-methyl and 3′-methoxy residues of **1**. Although it is well known that the *O*- or *N*-methyl group is derived from methionine *via* *S*-adenosyl-L-methionine (SAM) in the case of the biosynthesis of natural products,[7,8] the addition of a *C*-

Table 4. Enrichment ratios of labeled **1** from feeding experiments with ^{13}C-labeled precursors.

Position	δ_C	Relative enrichment ratio (Area $_{enriched}$/ Area $_{unenriched}$) [a, b]					
		^{13}C-6-D-glucose	^{13}C-1-D-mannose	^{13}C-3-pyruvate	^{13}C-3-ribose	^{13}C-3-L-methionine	^{13}C-1-D, L-lysine
2	151.1	1.3	2.3	2.2	1.5	-[c]	-
4	165.9	1.6	2.6	1.7	2.0	-	-
5	101.6	2.0	4.2	3..0	1.7	-	-
6	140.8	2.0	4.0	2.2	1.7	-	-
1'	89.9	1.2	9.8	1.2	16.8	-	-
2'	71.7	0.9	1.9	1.1	0.9	-	-
3'	77.9	0.9	2.1	1.4	1.2	-	-
3' OCH_3	57.6	1.9	5.9	1.6	2.0	7.6	1.00
4'	81.4	1.1	2.3	1.3	1.1	-	-
5'	75.3	2.9	6.7	1.1	1.4	-	-
6'	172.8	1.5	3.9	2.6	1.1	1.00	3.0
1''	99.1	1.2	11.1	1.6	3.0	-	-
2''	64.5	1.1	1.6	1.0	0.8	-	-
3''	61.6	1.1	2.2	1.6	2.0	-	-
4''	109.1	1.0	1.9	1.7	1.4	-	-
5''	141.3	1.0	1.0	2.1	1.0	-	-
6''	161.2	2.7	3.8	2.2	1.5	-	-
1'''	174.9	1.3	2.6	1.3	1.4	-	16.4
2'''	52.1	1.5	4.0	2.0	1.1	-	-
3'''	29.8	3.0	8.8	4.2	2.6	-	-
4'''	26.6	1.9	5.2	2.9	1.8	-	-
5'''	35.2	2.5	6.2	3.2	1.7	-	-
6'''	48.7	1.6	3.2	1.8	1.5	-	-
6''' CH_3	20.7	1.9	6.1	1.3	2.1	7.8	-

[a] the smallest peak is arbitrarily defined as 1 unit. [b] The relative enrichment ratio was obtained by calculating the area under the integral curves of the enriched sample to that of the unenriched sample under identical conditions.
[c] Enrichment not detected.

methyl group to the ε-carbon of lysine is not usually observed. Therefore, the origin is unclear. In this case, the resulting ^{13}C NMR spectrum indicated that both the signal of 6‴-methyl and 3′-methoxy carbons were enriched by 8-fold. Thus, it is revealed that the two methyl groups were derived from methionine.

Incorporation of ^{13}C-Labeled Lysine

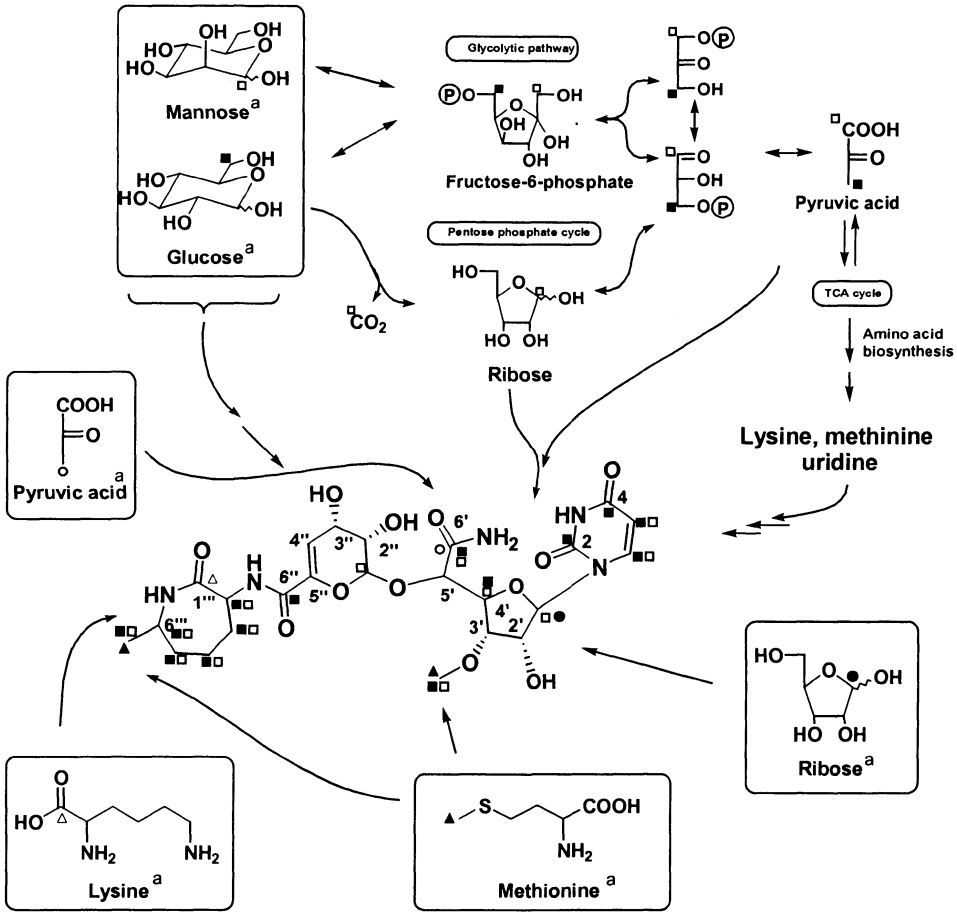
In the feeding experiment with [1-^{13}C]L-lysine dihydrochloride, a 16-fold enrichment of C-1‴ was observed. In contrast, no enrichment was observed at C-6‴. This result indicated that the aminocaprolactam moiety of **1** was directly derived from lysine and the C-6‴-methyl was added after lysine biosynthesis. This result as well as the production of **6** which possesses a methylated AEC in the structure also confirmed that lysine is not converted back to 2,6-diaminopimeric acid in the 2-amino-6-methyl caprolactam formation. This is consistent with the results of the methionine feeding experiment.

Discussion

From the results shown above, we may illustrate the biosynthetic pathway of **1** and the derivatives as summarized in Fig. 7. Furthermore, the origin of all carbon atoms of **1** has been clarified.

The ^{13}C-labeling studies reported here quite clearly demonstrate that **1** is derived from uracil, ribose, pyruvate, hexose, lysine and two methionines. No minor component

Fig. 6. Biosynthetic origin of the carbon skeleton of **1**.

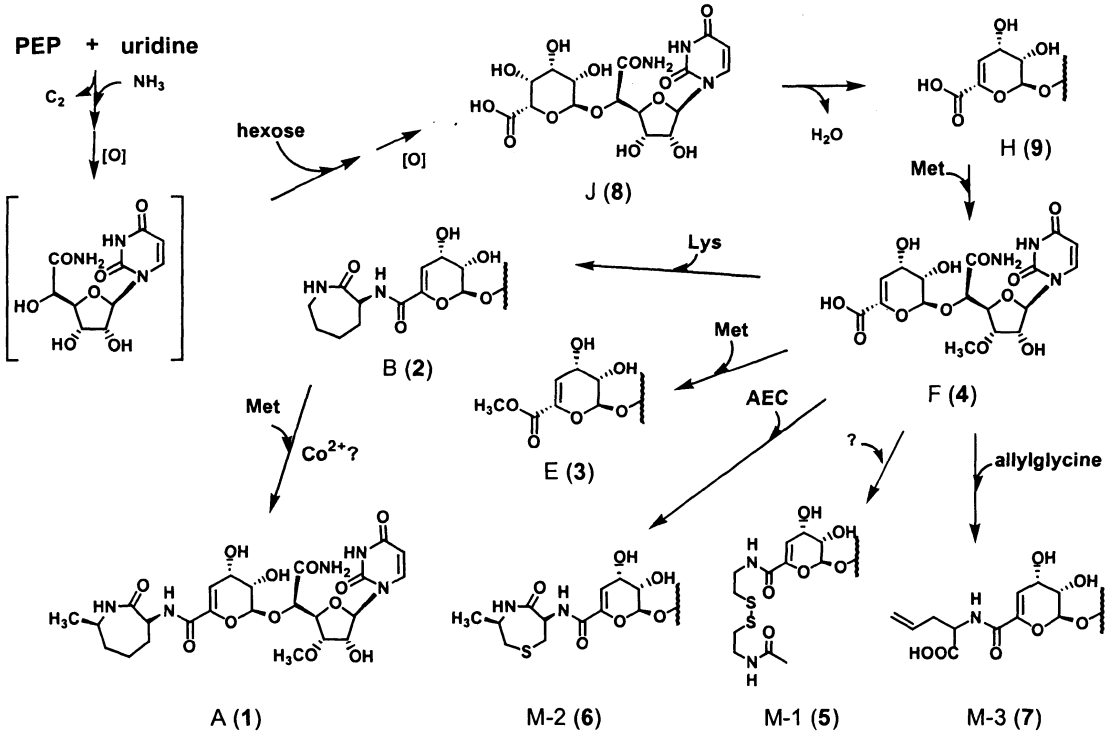


[a] ^{13}C-Labeled precursors used in incorporation studies

which possessed a uridine moiety was identified during our studies and all of them were elongated by C1 at C-5′. It is strongly suggested that the first step of the biosynthesis starting from uridine is the condensation of a uridine and pyruvate in the case of polyoxin biosynthesis.[6] Thereafter, **8** would be formed by the condensation of a uronic acid derived from some hexose such as glucose or mannose and the uridine derivative would then be converted to **9** by dehydration at C-4″ and C-5″. **9** would be converted to **3** or **4** by methylation of the hydroxyl group at C-3′ or the carboxyl group at C-6″. Subsequently, **4** would be converted to **2**, an ε-aminocaprolactam derivative, by condensation with lysine. At the final stage, it is clear that unnatural amino acids like AEC or allylglycine were incorporated into the molecule. The results of ^{13}C-labeled methionine feeding studies revealed that the biosynthesis of **1** was accomplished by the C-6‴ methylation with methionine. In the course of the media improvement study, addition of cobalt(II) chloride enhanced the titer of **1** relative to that of **2** (data not shown). Futhermore, it is commonly known that methylation with methionine occurs *via* *S*-adenosyl-L-methionine and methylcobalamine as the methyl donor.[7~9] It is clear that methylcobalamine is responsible for the methylation at C-6‴ of caprolactam in **1**. Although we attempted to elucidate the timing of the methylation and cyclization reactions of the ε-caprolactam group in **1**, novel intermediates, which help us to identify other possible reactions, have not yet been isolated from the medium of A-500359 production strain, and **2** could not be converted to **1** by methyl transformation using whole-cell and cell-free reactions (data not shown). Therefore, there remains a possibility that C-6‴ methylation occurs directly at the ε-position of lysine before condensation and cyclization.

Allylglycine incorporation was occurred only with the

Fig. 7. Proposed biosynthetic pathway of A-500359s.



co-addition of AEC in the medium. This suggests that the lysine-diaminopimelate pathway is strongly favored in the biosynthesis of **1**. All of the incorporation trials of unnatural amino acids failed except for the allylglycine addition. Furthermore, the incorporation of allylglycine was very specific in that only L-allylglycine was incorporated into the carbon skeleton. It is possible that the permeability of the allylglycine through the membrane of the producer is superior to the other unnatural amino acids and that the stereoselectivity of the enzyme against the substrate was high.

Stereochemical studies of **8** revealed that the configuration of the uronic acid moiety was identical to D-talose. From the standpoint of biosynthesis, **8** might be the most important intermediate of **1** and our result indicates that the uronic acid moiety was derived from glucose or mannose. Based on the configuration of C-4″ and C-5″ of **8**, a reaction mechanism involving the formation of a double bond *via* trans elimination seems reasonable.

Here, we revealed the origin of all carbon atoms of A-500359, but several important questions regarding, the enzymatic mechanisms of C1 elongation at C-5′, condensation of the aminocaprolactam ring and the conversion of hexose to talose still remain. Further investigations will be needed to clarify these issues.

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