A NEW NUCLEOSIDIC ANTIBIOTIC AT-265

EISAKU TAKAHASHI and TERUHIKO BEPPU

Department of Agricultural Chemistry,
Faculty of Agriculture, The University of Tokyo,
Yayoi, Bunkyo-ku, Tokyo 113, Japan

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A novel, chlorine containing sulfamoyl nucleoside antibiotics, AT-265, was found from *Streptomyces rishiriensis* subspecies and its structure and biological activities were determined. The antibiotic is active *in vitro* against some *Enterobacteriaceae* and some Gram-positive bacteria.

During our efforts to search for new antibiotics from soil microorganisms, a *Streptomyces* strain was found to produce a chlorine- and sulfur-containing nucleoside antibiotic which we designated AT-265. This report presents taxonomy of the producing microorganism, purification, characterization of chemical structure and biological properties of the antibiotic AT-265.

Materials and Methods

General

The following instruments were used in this study. NMR spectrometer; JEOL FX-100, UV spectrometer; Hitachi 200-10, IR spectrometer; Shimadzu IR 430, field desorption mass spectrometer; JMS D-300.

Fermentation

Seed culture of the strain No. 265 was carried out in a 500-ml Sakaguchi flask containing 100 ml of a medium composed of 1% peptone, 0.5% yeast extract, 0.5% glucose, pH 7, in distilled water. The inoculated flask was incubated for 2 or 3 days at 26.5°C on a reciprocal shaker at 100 strokes per minute. Two ml (2%, v/v) of the seed culture were transferred to a 500-ml Sakaguchi flask containing 100 ml of a production medium composed of 1% soluble starch, 1% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 0.5% Pharmamedia and 10 ppm $CoCl_2$ in tapped water. The fermentation was carried out for 4~5 days at 26.5°C on a reciprocal shaker.

For jar fermentation, 1.5 liters of the seed culture prepared as described above was inoculated into a 30-liter jar fermentor containing 20 liters of the production medium as described above. Cultivation was carried out with agitation at 300 rpm and aeration of 10 liters per minute at 26.5°C. The fermentation broth was harvested after 3 days of cultivation.

Assay of AT-265 in Fermentation Broth

Assay of AT-265 in large scale fermentation was performed by high performance liquid chromatography (HPLC). Whole broth samples (50 ml) were extracted three times with each 50 ml of ethyl acetate and the combined extracts were dried with anhydrous sodium sulfate, filtered and evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of methanol and assayed by HPLC (Waters Associates) with C_{18} μ Bondapak column and reverse-phase separation was carried out using water - MeOH (1 : 1) as the solvent (flow rate, 0.5 ml/minute). AT-265 was detected with a UV detector at 254 nm.

Extraction and Purification of Antibiotic AT-265

The fermentation broth (120 liters) was filtered using a filter aid (Hyflo Super-cel, Johns-Manville

Products Corp.). The clear filtrate was extracted three times with equal volumes of ethyl acetate. The ethyl acetate extract was concentrated under reduced pressure to an oil and was applied onto a column (45 mm×400 mm) packed with silica gel (Merck, Silica gel 60). After washing with 300 ml of ethyl ether, the column was eluted with a mixture of ethyl ether - methanol (9: 1, v/v). The active fractions were combined and concentrated under reduced pressure. The concentrated material was dissolved in a small amount of methanol and applied on a column (40 mm×600 mm) of Sephadex LH-20 (Pharmacia Fine Chemicals). Elution was carried out with methanol and the eluate was collected in 15.5 ml fractions. The active fractions were pooled (fraction No. 140~No. 170) and concentrated under reduced pressure. The white powder obtained (*ca.* 70 mg) was dissolved in hot methanol and was crystallized at 4°C to obtain colorless crystals of AT-265 in a needle form.

Measurements of Growth

Growth of strain No. 265 was monitored by packed cell volume (PCV) which was determined by centrifugation at 1,000×*g* for 10 minutes.

E. coli were grown aerobically at 37°C in Nutrient broth (Difco). Growth of cells was monitored by absorbance measurement at 550 nm with spectronic 20A spectrophotometer (Shimadzu). Total cell numbers were measured with a Coulter counter (model ZB) equipped with a 30 μm aperture probe.

Determination of Minimal Inhibitory Concentrations

Minimal inhibitory concentrations (MIC) were determined using the agar plate dilution method with heart infusion agar (Difco) or No. 1001 medium (3% glycerol, 0.3% sodium L-glutamate, 0.2% peptone, 0.31% Na_2HPO_4, 0.1% KH_2PO_4, 0.005% ammonium citrate, 0.001% $MgSO_4 \cdot 7H_2O$, 1.5% agar).

Measurement of Bacterial Macromolecular Synthesis

E. coli K12 JE 1011 *thr*, *leu*, *his*, *trp*, *thy*, *thi* was grown aerobically at 37°C to early logarithmic phase in an L-tube consisting 10 ml of supplemented minimal salts medium containing 4 g glucose, 8.8 g $Na_2HPO_4 \cdot 2H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 20 mg $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water. Thiamine (10 μg/ml) and each 20 μg/ml of thymine, L-leucine, L-threonine, L-tryptophan and L-histidine were added. Each 0.5 ml of [^{3}H]thymine (5.0 μCi/ml, 33 Ci/mmole), [^{3}H]leucine (2.5 μCi/ml, 131 Ci/mmole), or [^{3}H]uracil (2.5 μCi/ml, 4.7 Ci/mmole) was added to 10 ml of the culture. AT-265 was added concomitantly with addition of the radioactive compounds. After the addition, 1 ml samples of the culture were taken into 5 ml of 10% ice-cold trichloroacetic acid (TCA) at appropriate intervals. After at least 30 minutes at 0°C, the acid-insoluble materials were collected on glass fiber GF/C filters (2.5 cm-diameter, Whatman), washed twice with 5 ml of 5% cold TCA, twice with 5 ml of cold ethanol, and dried. The filter disks were transferred to glass vials, and were counted with Aloka LSC 670 liquid scintillation counter in 5 ml of scintillation fluid containing 4 g 2,5-diphenyloxazole and 0.1 g 1,4-bis-(4-methyl-5-phenyloxazole-2-yl)benzene per 1 liter of toluene.

Assay of RNA Polymerase

The assay mixture (0.25 ml) were prepared as described by BURGESS,[1] except that the acid-insoluble product was collected on Whatman GF/C filters. Radioactivity of the acid-insoluble product was measured by the method described above.

Chemicals

Methyl-[^{3}H]thymine, 5-[^{3}H]uracil, L-4,5-[^{3}H]leucine, 5′,8-[^{3}H]ATP were purchased from Radiochemical Centre (Amersham, England). 2-Chloroadenosine was purchased from Sigma Chemical Co. RNA polymerase (*E. coli* K12) was purchased from Miles Laboratories Inc. (Indiana, U.S.A.).

Results

Taxonomy of the Producing Organism

Strain No. 265 grows well on many standard media including the ISP media that are recommended by SHIRLING and GOTTLIEB[2,3] for the description of Streptomycete cultures.

The strain produces aerial mycelia forming spiral chains of spores with more than ten spores per

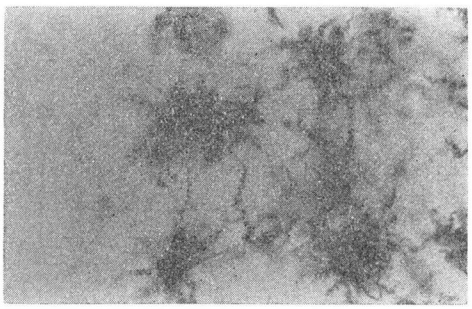
Table 1. Cultural and physiological characteristics of strain No. 265 and *S. rishiriensis* IFO 13407.

	Strain No. 265	*S. rishiriensis*
Growth	Good	Good
Color of aerial mycelium	Gray	Gray
Spore chain morphology	Spirals, long spore chains	Spirals, long spore chains
Spore surface	Smooth	Smooth*
Spore shape	Oval to cylindrical 0.5~0.7×1.0~1.5 μm	—
Reverse side of colony	Yellow to brown	Yellow to brown
Melanoid pigment	+	+
Carbon utilization	Most carbon utilized No growth; mannitol cellulose dulcitol Na-acetate	Most carbon utilized No growth; mannitol cellulose dulcitol Na-acetate
Gelatin liquefaction	+	+
Starch hydrolysis	+	+
Tyrosinase reaction	+	+
Nitrate reduction	−	−
Milk peptonization (26°C)	+	+
(37°C)	+	+
Milk coagulation (26°C)	−	−
(37°C)	+	−

* Data obtained from reference 3. +: Positive reaction. −: Negative reaction.

chain (Plate 1). Spores are cylindrical with smooth surface in size from 0.5~0.7×1.0~1.5 μm.

Plate 1. Photograph of aerial hyphae of strain No. 265 on oat meal agar (ISP 3), 21 days.



Cultural and physiological characteristics of strain No. 265 on various media are summarized in Table 1 in comparison with those of the type strain of *S. rishiriensis* IFO 13407. In general, the vegetative mycelia develop abundantly, and the mass color of the culture is pale yellowish brown to yellowish brown and soluble pigment is produced. Comparison of these properties indicates close resemblance of these two strains. In view of the difference in antibiotic production between the two strains, it may be concluded that strain No. 265 is one of the subspecies of *Streptomyces rishiriensis*.

Fermentation and Purification

Production of AT-265 in shake flasks began at 4 days and reached the maximum of 1.5~1.7 μg/ml at 5 days. Fermentation carried out in a 30-liter jar fermentor gave a time course profile as shown in Fig. 1. Production of AT-265 started just before the stop of cell growth and almost all the antibiotic was secreted into the medium. Maximum amounts of the antibiotic reached about 6 mg/liter after the cultivation of 3 days. It was also found that 2-chloroadenosine, a core compound of AT-265, was also produced during the fermentation concomitantly with the antibiotic production.

AT-265 was extracted from the culture broth with ethyl acetate and purified by silica gel chromatography and Sephadex LH-20 chromatography described in Materials and Methods. About 70 mg pure powder of AT-265 was obtained from 120 liters of the culture broth. Several types of TLC plates and various solvents were used to check the purity of AT-265 (Table 2). The final preparation was homogeneous in HPLC.

Physicochemical Properties of AT-265

AT-265 was isolated as colorless crystals with mp 210~213°C (dec.) and $[\alpha]_D^{22}$ −17.5° (*c* 0.2, MeOH). UV spectra showed absorption maxima at 264 nm (ε 14.0×10^3) in water, 264 nm (13.1×10^3) in 0.05 N HCl and 263.5 nm (14.0×10^3) in 0.05 N NaOH (Fig. 2). These UV spectra strongly suggested the pre-

Table 2. Thin-layer chromatographic mobility of AT-265.

Solvent	Support	Rf
Methanol - ether (1: 9)	Silica gel	0.11
Methanol - ether - 1 N acetic acid (2: 18: 1)	(Merck TLC plates	0.18
Methanol - ether - 1 N ammonia (2: 18: 1)	silica gel 60 F_{254})	0.11
Ethyl acetate - 1 N acetic acid (20: 1)		0.07
Methanol - 1 N acetic acid (20: 1)		0.69
Acetone - water - 1 N acetic acid (5: 5: 1)	Silanized silica gel	0.80
Acetone - water - 1 N ammonia (5: 5: 1)	(Merck TLC plates	0.80
Methanol - acetonitrile - 1 N acetic acid (5: 5: 1)	silica gel 60 F_{254}	0.83
Methanol - acetonitrile - 1 N ammonia (5: 5: 1)	silanized)	0.79

Fig. 1. Time course of AT-265 production in a 30-liter jar fermentor.

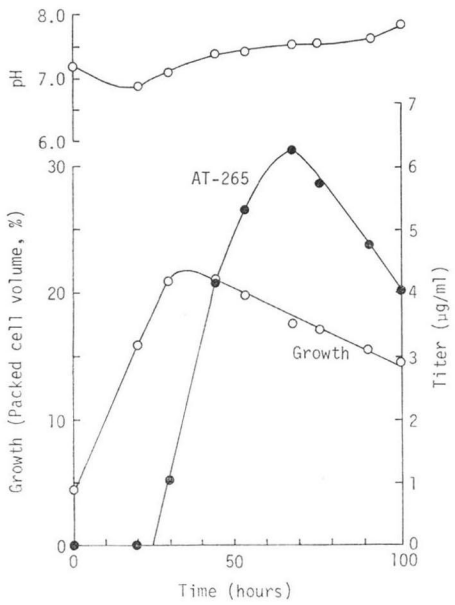


Fig. 2. UV spectra of AT-265.

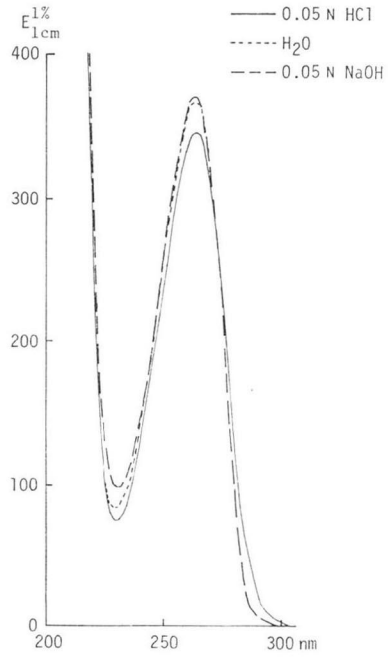
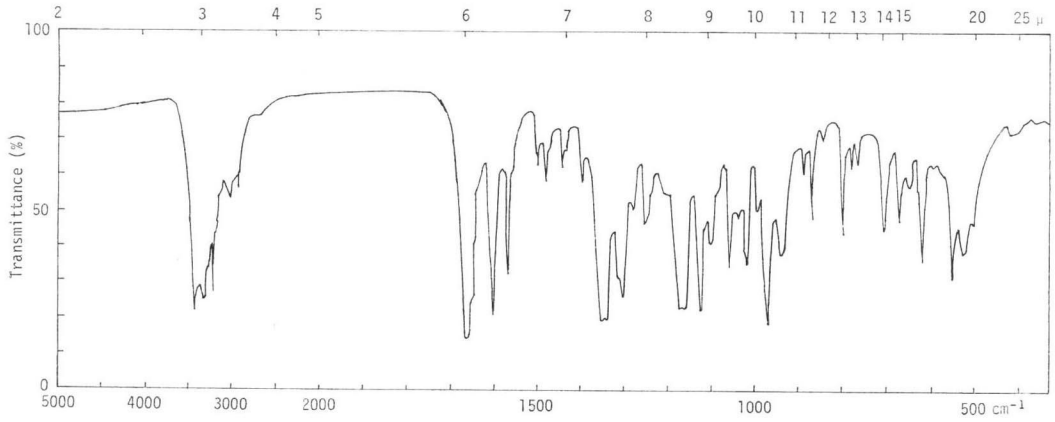


Fig. 3. IR spectrum of AT-265 (KBr).



sence of a 2-chloroadenosine moiety in the molecule of AT-265.

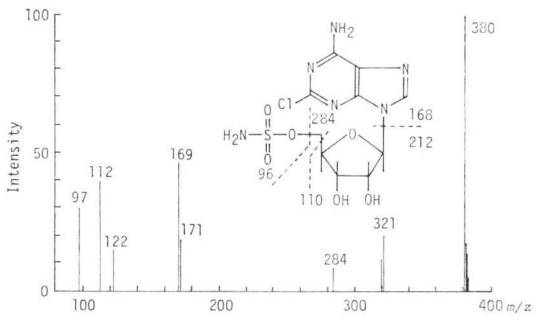
IR spectrum (Fig. 3) showed characteristic absorption at ν_{max}^{KBr} of 1350, 1340, 1176, 1162 cm^{-1} which could be assigned as absorption due to the sulfamoyl moiety.

Elemental analysis indicated a molecular formula $C_{10}H_{13}N_6O_6SCl$.

Anal. Found: C 31.71, H 3.42, N 21.92, O 24.51, S 8.29, Cl 9.38.
Calcd. for $C_{10}H_{13}N_6O_6SCl$: C 31.54, H 3.44, N 22.07, O 25.21, S 8.42, Cl 9.31.

From FD mass spectrometry, the molecular weight of AT-265 was calculated to be 380 (Fig. 4). Strong fragment ion peaks were observed at *m/z* 169 and its isotopic ion peak *m/z* 171. These fragment ion peaks indicated the presence of the protonated base moiety and chlorine in the molecule. Another fragment ion observed at *m/z* 97 gave strong evidence for the presence of the sulfamoyl moiety. The similar fragment ion peask were also observed by electron impact mass spectrum.

Fig. 4. FD mass spectrum of AT-265.



From UV spectra and mass spectrum, chlorine was assumed to bind to the 2-position of the purine nucleus.

Data of ^1H NMR spectra of AT-265 in DMSO-d_6 are shown in Table 3 and Fig. 5. Comparison of the spectral data of 2-chloroadenosine with those of AT-265 indicated that the singlet of δ 8.34 (1H) was assigned to be a proton attached at C-8 of the purine moiety and the pentose fragment was linked to N-9 of the purine moiety. The presence of the anomeric proton as a doublet at δ 5.91 indicated that AT-265 is a carbon-nitrogen linked nucleoside.[4] The signals of δ 5.50 (H) and δ 5.69 (H) disappeared upon the addition of deuterium oxide. It indicated that the doublet of δ 5.50 was the signals of 2′-COH and the doublet of δ 5.69 was the signal of 3′-COH. Spin decoupling studies verified assignment of the C-1′H, C-2′H, C-2′OH, C-3′OH. Irradiation of the C-2′H caused collapse of the C-1′H doublet and C-2′OH doublet to a singlet. Irradiation of the C-1′H caused collapse of the C-2′H multiplet to a pseudo-triplet. Irradiation of the C-2′OH caused collapse of the C-2′H multiplet to a pseudo-triplet, and that of the C-3′H multiplet caused collapse of the C-3′OH doublet to a singlet. The signals at δ

Fig. 5. ^{1}H NMR spectrum of AT-265 (100 MHz, DMSO-d_6).

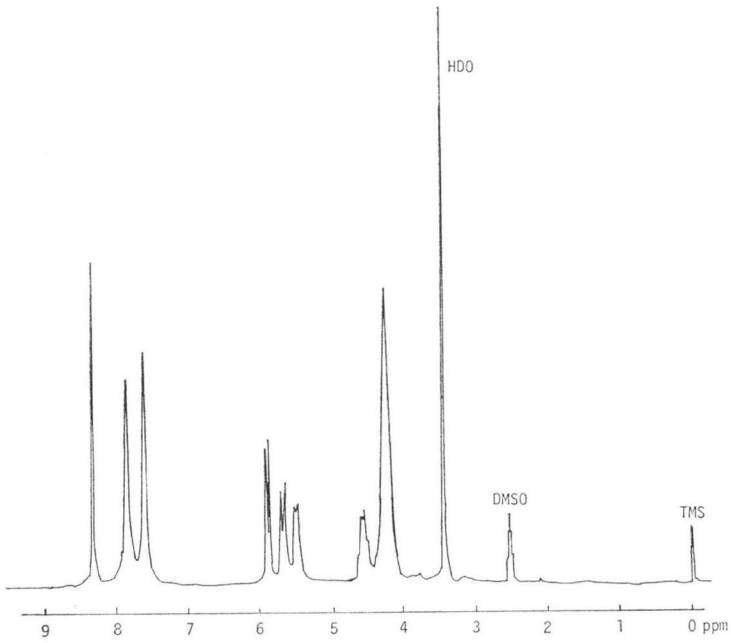


Table 3. Chemical shifts and coupling constants in ^{1}H NMR spectra of AT-265 and 2-chloroadenosine (100 MHz, DMSO-d_6, TMS, ppm, J(Hz)).

Assignment	AT-265				2-Chloroadenosine			
5′-CH_2	4.25	4H	broad		3.62	2H	m	
4′-CH					3.95	H	m	
3′-CH					4.15	H	m	
2′-CH	4.55	H	m		4.52	H	m	
5′-COH	—				5.07*	H	t	J 5.9, 4.4
3′-COH	5.50*	H	d	J 4.9	5.21*	H	d	J 4.9
2′-COH	5.69*	H	d	J 5.9	5.48*	H	d	J 6.7
1′-CH	5.91	H	d	J 5.4	5.82	H	d	J 6.4
5′-OSO_2NH_2	7.63*	2H	s		—			
6-CNH_2	7.87*	2H	s		7.85*	2H	s	
8-CH	8.34	H	s		8.38	H	s	

* These signals disappeared on addition of D_2O.
s=singlet, d=doublet, t=triplet, m=multiplet.

7.63 and δ 7.87 disappeared upon the addition of the deuterium oxide. It was known that the signal of 5′-OSO_2NH_2 (2H) of 5′-*O*-sulfamoyladenosine appears as the singlet at δ 7.73[5] and that of 6-NH_2 (2H) of 2-chloroadenosine at δ 7.85. From these data, the signals were assigned to be those amino groups of AT-265.

The deduced structure of AT-265 was supported by ^{13}C NMR studies (Table 4). Comparison of the spectral data of 2-chloroadenosine with those of AT-265 leads to the assignments of the signals of all carbon atoms of AT-265. From selective proton-decoupling ^{13}C NMR, C-1′, C-2′ and C-3′ could easily be assigned. SUGIYAMA[6] reported that the C-2′ and C-3′ signals of the α-anomer of ribonucleo-

side appeared at the same field strength and the C-2′ signal of the β-anomers of ribonucleoside shifted to downfield of the C-3′ signal by 3~4 ppm. The similar downfield shift of C-2′ signal of AT-265 by 3.0 ppm indicated the β-anomeric configuration. The chemical shifts and multiplicities of C-1′ to C-5′ indicate a ribofuranosyl-structure for the pentose moiety. The signal of C-5′ shifted downfield by 7.1 ppm in the spectrum of the 2-chloroadenosine which was due to the deshielding effect of the sulfamoyl group. This indicated that the sulfamoyl moiety attached to the C-5′ position of the pentose moiety.

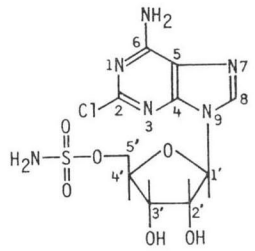
Table 4. ^{13}C NMR of AT-265 and 2-chloroadenosine (25 MHz, DMSO-d_6, TMS, ppm).

Assignment	AT-265	2-Chloroadenosine
6-C	156.5 s	156.6
2-C	153.3 s	153.3
4-C	150.3 s	150.3
8-C	139.6 d	140.4
5-C	117.8 s	118.1
1′-C	87.3 d	87.7
4′-C	81.7 d	85.8
2′-C	73.1 d	73.7
3′-C	70.1 d	70.4
5′-C	68.6 t	61.5

s=singlet, d=doublet, t=triplet.

From all of the results described above, we conclude that the structure of AT-265 is 5′-sulfamoyl-2-chloroadenosine (Fig. 6).

Fig. 6. Structure of AT-265.



Biological Activity of AT-265

AT-265 inhibited growth of many but not all species of Gram-negative and Gram-positive bacteria at relatively low concentration (Table 5). However, this compound was highly toxic to mice. The LD_{50} of AT-265 in mice was 0.2 mg/kg (i.p.) and 3.4 mg/kg (p.o.).

Mode of action of AT-265 was examined with *E. coli* K12 as a test organism. When 5 μg/ml of the antibiotic was added to the exponentially growing culture at 37°C, growth monitored by turbidity stop-

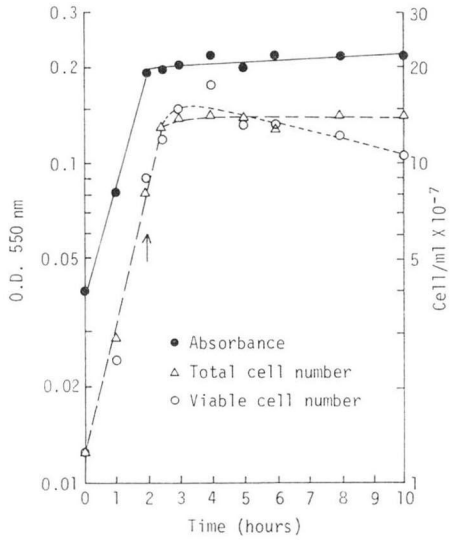
Table 5. Antimicrobial spectrum of AT-265.

Organism	MIC (μg/ml)	Organism	MIC (μg/ml)
Bacillus subtilis IAM 1026	25	*Vibrio metschnikovii* IAM 1039	0.2
B. subtilis PCI 219	25	*Aerobacter aerogenes* ATCC 8724	1.5
Staphylococcus aureus FDA 209P	50	*Enterobacter aerogenes* IAM 12348	100
Corynebacterium fascians IAM 1079	1.5	*Proteus vulgaris* IFO 3167	1.5
C. equi IFO 3730	1.5	*P. mirabilis* IFO 3849	50
Anthrobacter simplex ATCC 6948	1.5	*Klebsiella pneumoniae* IAM 12015	>100
Mycobacterium smegmatis IFO 3082	50	*Serratia marcescens* IAM 1065	50
〃 〃 IFO 3083	25*	*Xanthomonas oryzae* IAM 1657	100
M. phlei IFO 3158	25*	*Erwinia aroideae* IAM 1068	1.5
Escherichia coli ML 3748	3.1	*Salmonella anatum* 1	50
E. coli B	0.8	*Pseudomonas aeruginosa* IFO 3080	>100
E. coli K12	3.1		

* These strains were tested with No. 1001 medium.

Fig. 7. Effect of antibiotic AT-265 on cell growth of *E. coli* K12 in nutrient broth medium.

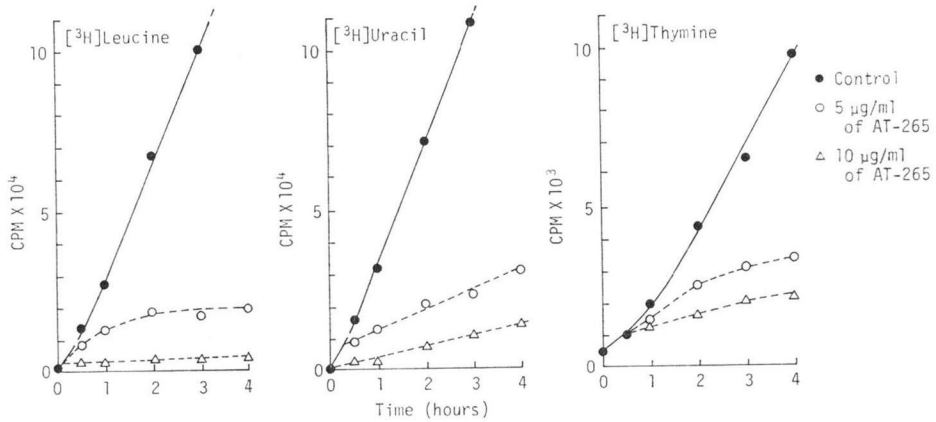
Arrow indicates the time of addition of the drug (5 μg/ml). Total cell numbers were estimated with a Coulter counter. The number of viable cells per milliliter was estimated by counting the number of colonies formed on nutrient agar.



ped immediately while viable and total cell numbers continued to increase for about 1 hour. Then the increase stopped after reaching about twice the number of cells (Fig. 7). As a result, mean cell volume decreased to be halved during the incubation with the drug (data not shown).

Incorporation of radioactive leucine, uracil and thymine into the acid-insoluble materials in the growing *E. coli* cells was severely inhibited but partially continued upon the addition of 5 μg/ml of AT-265. Ten μg per ml of the drug caused complete inhibition of incorporation of [^{3}H]leucine but that of uracil and thymine still continued at a slow rate (Fig. 8). *In vitro* RNA synthesis by *E. coli* RNA polymerase with calf thymus DNA as a primer was not inhibited by 100 μg/ml of AT-265. These results suggest that AT-265 primarily inhibits bacterial protein synthesis *in vivo*.

Fig. 8. Effect of AT-265 on macromolecular synthesis of *E. coli* K12 in minimal salts medium.



Discussion

AT-265 is a new member of the nucleoside group of antibiotics, *i.e.*, an analog of 5′-adenylic acid containing sulfamoyl group and chlorine. A highly toxic antibiotic, nucleocidin, produced by *Streptomyces calvus* has a closely related structure with a sulfamoyl group and fluorine.[7,8] A sulfamoyl adenosine structure seems to play an essential part in their toxicity against animals.

Purine analogues containing 2-chloroadenosine have never been found in nature before. It was found that *Streptomyces rishiriensis* subsp. 265 also produced 2-chloroadenosine. It was isolated from

culture broth, and was identified by ^{1}H NMR, ^{13}C NMR, elemental analysis and UV spectra. Concomitant accumulation of 2-chloroadenosine with AT-265 seems to suggest that the antibiotic might be synthesized from 2-chloroadenosine.

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