A STREPTOTHRICIN-LIKE ANTIBIOTIC MIXTURE, A-269A (AND A-269A′)

Yutaka Kido, Tarou Furuie, Keitarou Suzuki, Kyoko Sakamoto, Yoshito Yokoyama, Masaru Uyeda, Jun-ei Kinjyo, Shyoji Yahara, Toshihiro Nohara and Motoo Shibata

Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862, Japan

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A streptothricin-like antibiotic, A-269A (referred to as A-269A hereafter) was isolated from the culture broth of *Streptomyces* sp., strain No. A-269. In this paper, the characterization of the producer, and the production, isolation, physico-chemical properties and biological properties of A-269A are reported. The structure of the antibiotic was also examined by ^{1}H NMR, ^{13}C NMR and fast atom bombardment mass spectra studies. From the spectroscopic data, A-269A was assumed to be a mixture of antibiotic LL-BL 136 and an isomeric compound in which the carbamoyl group is substituted on C-12′ hydroxyl instead of C-10 hydroxyl.

In the course of our screening for new antibiotics, antibiotic A-269A was obtained from the fermentation broth of *Streptomyces* sp., strain No. A-269, isolated from a soil sample collected in Kumamoto City.

The antibiotic was basic, water-soluble and active against Gram-negative bacteria, especially *Escherichia coli* and *Proteus vulgaris*. As already pointed out by Mehta *et al.*[1], streptothricin antibiotics are commonly encountered in a conventional screening program, based on detection of activity against Gram-negative microbes. The antibiotic was also found to resemble the streptothricin-like antibiotic, LL-BL 136[2].

In this paper, the production, isolation, physico-chemical and biological activities, and the structure elucidation of A-269A are described together with the taxonomic characteristics of the A-269A-producing strain.

Materials and Methods

Microorganism

The strain No. A-269 was isolated from a soil sample collected in Kumamoto City, Japan.

Morphological Characterization

After incubation on glucose - asparagine agar for 7 days at 28°C, the organism was examined by a light-microscope and an electron-microscope (JEM-50B, Japan Electron Optics Laboratory, Co., Ltd.).

Cultural and Physiological Characterization

For cultural and physiological characterizations, various media recommended by ISP[3] and Waksman[4] were used. Color names were based on the Rayner's description[5]. Utilization of carbohydrates was determined by the method of Pridham and Gottlieb[6].

Fermentation

Shaking culture fermentations were carried out with 50 ml of a medium in 200-ml Erlenmeyer

flasks. Spores from a slant culture were inoculated into a seed culture medium containing glucose 2%, soluble starch 3%, soybean flour 1%, peptone 0.5%, NaCl 0.3% and $CaCO_3$ 0.5% (pH 7.0). The seed culture was incubated for 48 hours at 28°C on a rotary shaker (160 rpm). One liter of the resultant seed culture was inoculated in a 30-liter jar fermentor containing 14 liters of the production medium consisting of glucose 1%, dextrin 3%, soybean flour 1%, peptone 0.5% and NaCl 0.3% (pH 7.0). Fermentations were carried out for 48 hours at 28°C with agitation (350 rpm) and aeration (14 liters/minute).

Antibiotic Assay

The conventional serial agar dilution method and cup or paper-disc method were used in this study. The test organisms used were given in Table 3.

Fast Atom Bombardment Mass Spectrum (FAB-MS)

FAB-MS was recorded using a Jeol JMS-DX 303 ion source (3 kV accelerating potential) and the JMA 3500 data system. A xenon atom beam source (6 kV accelerating potential) was used.

^1H and ^{13}C NMR Spectra

^1H and ^{13}C NMR spectra were taken on Jeol JNM-GX 400 (400 MHz and 100 MHz, respectively) spectrometers in D_2O solution with TMS as an internal standard.

Results and Discussion

Characterization of Strain No. A-269

The strain No. A-269 forms well-developed aerial mycelia with spore chains in the form of compact spirals. Spores are cylindrical with spiny surfaces. In general, a brown colored growth developed well with a spore mass color of smoke gray on yeast - malt agar, oatmeal agar and glycerol - asparagine agar. In carbon source utilization, good or moderate growth was observed with D-glucose, D-galactose, *i*-inositol, D-mannitol, salicin, sucrose and raffinose. L-Arabinose, D-xylose and rhamnose were not utilized by this strain. The growth of the strain occurs between 20°C and 43°C, and its optimum temperature was 37°C. The growth and sporulation occured at pH's ranging from 5.0 to 8.0 and its optimum pH was 6.0. Detailed properties, compared with the description of *Streptomyces acidophilus*[7] will be published in a separate paper. The characteristics of the strain were summarized in Table 1.

Fermentation and Isolation

One liter of the seed culture was inoculated in a 30-liter jar fermentor containing 14 liters of the

Table 1. Characteristics of *Streptomyces* sp. strain No. A-269.

Spore	Compact spiral, cylindrical
Spore surface	Spiny
Color of colony	Aerial mass color is smoke gray or pale mouse gray on glucose - asparagine agar, glycerol - asparagine agar, oatmeal agar, yeast extract - malt extract agar and tyrosine agar.
Color in medium	Melanoid pigments formed in gelatin, milk, nutrient agar, yeast extract agar, glucose or glycerol nutrient agar
Physiological properties	Carbon utilization: (+); D-Glucose, D-galactose, *i*-inositol, D-mannitol, salicin, sucrose, raffinose. (−); L-Arabinose, D-xylose, rhamnose. Optimum temp 37°C Optimum pH 6.0

Fig. 1. Isolation and purification of antibiotic A-269A.

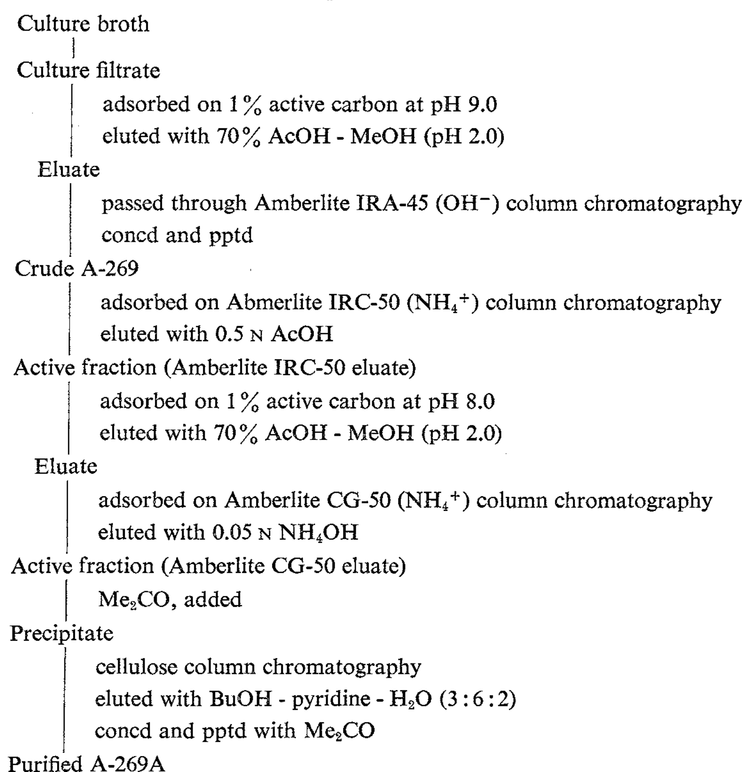


Table 2. Physico-chemical properties of antibiotics A-269A and B.

	A				B	
Nature	Basic, white amorphous powder				Same as in A	
MP (°C, dec)	225				225	
$[\alpha]_D^{25}$ (c 0.2, H_2O)	−115°				−115°	
Elementary analysis (%)	$C_{17}H_{29}N_7O_8 \cdot 1\frac{1}{2}H_2CO_3$		$C_{17}H_{29}N_7O_8 \cdot 2H_2SO_4 \cdot H_2O$		$C_{17}H_{29}N_7O_8 \cdot 2H_2O$	
	Calcd:	Found:	Calcd:	Found:	Calcd:	Found:
C	40.22,	39.13, 39.25,	30.31,	30.91, 30.46,	41.21,	41.57,
H	5.80,	5.71, 5.91,	5.23,	5.54, 5.15,	6.67,	6.77,
N	17.75	17.93, 17.87	14.56,	14.14, 13.99,	19.79	19.09
S			9.52	9.25, 8.95		
Empirical formula	$C_{17}H_{29}N_7O_8$				Same as in A	
MW	459 (FAB-MS)				Same as in A	
Color reaction	(+): Molisch, anthrone, silver nitrate, ninhydrin, Pauly				Same as in A	
	(−): Elson-Morgan, Sakaguchi, maltol				Same as in A	
Solubility	Soluble in H_2O, slightly soluble in MeOH, insoluble in other organic solvents				Same as in A	

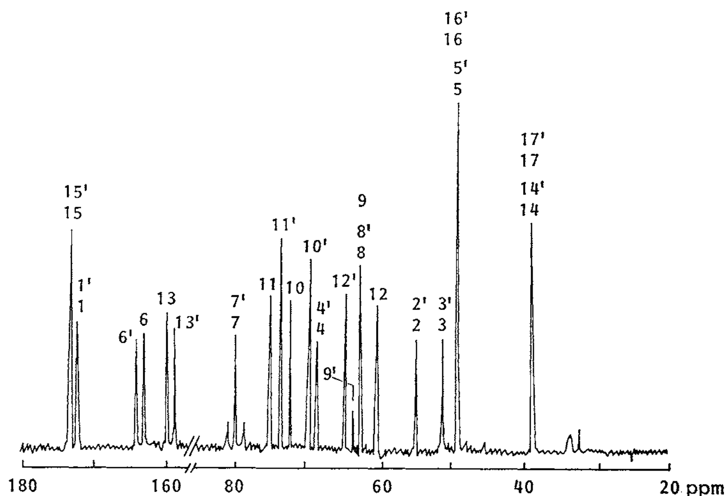
production medium. The fermentation was carried out at 28°C with aeration of 14 liters per minute and agitation of 350 rpm. After filtration of 48 hours fermentation broth, the filtrate was adjusted to pH 9.0 and adsorbed on 1% active carbon. Active fractions were eluted with 70% methanolic acetic acid and concentrated *in vacuo* to give a brownish gray powder of crude A-269. The purified A-269A preparation was obtained as a result of consecutive application of the following procedures;

Table 3. Antimicrobial activity of antibiotics A-269A and B.

Test organism	Medium	MIC (μg/ml)	
		A	B
Escherichia coli IFO 3301	I	1	1
Proteus vulgaris IFO 3167	I	5	5
Pseudomonas aeruginosa IFO 3448	I	>100	>100
Staphylococcus aureus IFO 3060	I	50	50
S. aureus, OM, EM-R[a]	I	50	50
Bacillus subtilis PCI 219	I	10	10
B. cereus IFO 3466	I	>100	>100
Mycobacterium smegmatis IFO 3082	I	20	20
M. smegmatis, NM-R[b]	I	20	20
M. smegmatis, SM-R[c]	I	20	20
Saccharomyces cerevisiae IFO 0305	II	>100	>100
Candida albicans IFO 0583	II	>100	>100
Aspergillus niger IFO 4066	II	>100	>100
Penicillium chrysogenum IFO 4626	II	50	50

Medium I: Bouillon agar, II: glucose bouillon agar.
[a] Resistance to oleandomycin and erythromycin.
[b] Resistance to neomycin.
[c] Resistance to streptomycin.

Fig. 2. 100 MHz ^{13}C NMR spectrum of antibiotic A-269A.



adsorption on an Amberlite IRC-50 (NH_4^+) column, elution with 0.5 N acetic acid, concentration of eluates *in vacuo*, adsorption on an Amberlite CG-50 (NH_4^+) column, elution with 0.05 N NH_4OH, concentration of eluates *in vacuo*, precipitation with acetone and chromatography on a cellulose column using a solvent system of butanol - pyridine - water (3 : 6 : 2). The isolation and purification procedures for purified A-269A is summarized in Fig. 1.

Physico-chemical and Biological Properties

The antibiotic A-269A is a colorless amorphous powder with the physico-chemical properties summarized in Table 2.

The antimicrobial spectrum of A-269A is shown in Table 3. The antibiotic is active mainly

against Gram-negative bacteria, and in particular, *E. coli* or *P. vulgaris* against which the minimum inhibitory concentration were 1 or 5 μg/ml, respectively.

Structure Determination

The IR spectrum of A-269A gave a similar absorption pattern to that of racemomycin A[8]. The hydrolysate (6 N HCl, 105°C, 12 hours) of A-269A was found to contain NH_3 and sarcosine in 1:1 ratio and lesser amounts of an unidentified streptolidine-like compound from the analysis by an amino acid analyzer (Hitachi KLA-3B). Sarcosine was identified as 2,4-dinitrophenylsarcosine.

Anal Calcd for $C_9H_9N_3O_6$: C 42.35, H 3.53, N 16.47.
Found: C 42.59, H 3.59, N 16.22.

A-269A gave a FAB-MS spectrum with a $(M+H)^+$ ion at m/z 460, that corresponded to a molecular weight of 459. From the properties of A-269A mentioned above, it resembles the streptothricin-like antibiotic, LL-BL 136[2], while it differs from SF-701[9] in its optical activity, and in its molecular weight, which is the same as that of LL-BL 136. BORDERS *et al.* have pointed out, that LL-BL 136 was not differentiated from SF-701 reported by TSURUOKA, though different molecular formulae were given for both antibiotics. Moreover, each antibiotic contains sarcosine instead of β-lysine. From the presence of sarcosine in the hydrolysate and the same molecular weight as that of LL-BL 136, A-269A could not be differentiated from LL-BL 136 (*i.e.* from SF-701). The structure elucidation of A-269A was based on the results from ^{13}C and ^1H NMR studies and FAB-MS spectrum studies.

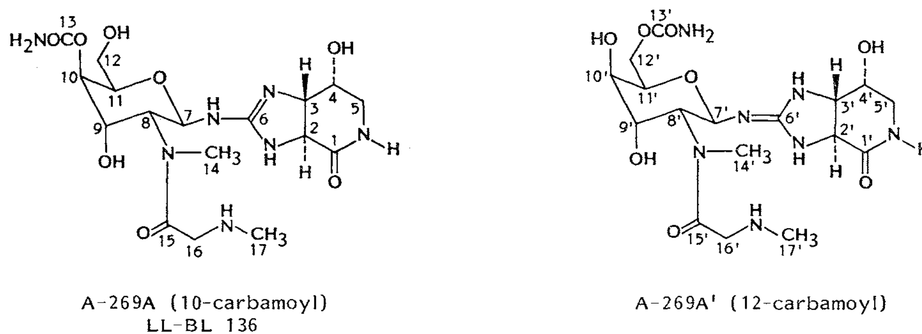
Both ^{13}C NMR[10~12] and ^1H NMR[13] studies have been described for antibiotics of the streptothricin family. Streptothricin F[14] (racemomycin A) is a well-characterized antibiotic; most of its structural features have been known for many years[14,15] and the last detail, the location of the carbamoyl group on the C-10 hydroxyl has recently been defined[16,17]. The ^{13}C NMR spectrum of A-269A is shown in Fig. 2. The assignments of the carbon signals of A-269A, shown in Table 4 were based on the comparison with ^{13}C chemical shifts for streptothricin F[12,18] and LL-AC 541[19]. The proposed structures for A-269A (and A-269A′) was shown in Fig. 3, together with the structures of streptothricin F and LL-AC 541 for references. The respective

Table 4. Comparison of ^{13}C NMR chemical shifts for A-269A and related antibiotics.

Assignment	A-269A (D_2O, ppm)	Streptothricin F (D_2O, ppm)	LL-AC 541 (D_2O, ppm)
15	172.9	173.1	169.0
15′	172.9		
1	172.7	171.1	170.7
1′	172.7		
6′	164.1[a]		
6	163.8[a]	163.7	163.0
13	159.0[b]	158.7	158.5
13′	158.1[b]		
7	79.6	79.9	77.8
7′	79.6		
11	73.9[c]	74.5	74.7
11′	72.7[c]		
10	71.7	71.1	71.5
10′	69.5		
4	69.0	67.4	68.9
4′	69.0		
12′	64.6		
9′	63.4		
8	62.5	62.3	61.7
9	62.5	61.9	61.7
8′	62.5		
12	60.9	61.3	61.1
2	56.3	55.5	55.3
2′	56.3		
3	51.4	50.2	61.7
3′	51.4		
5	49.7	50.0	50.1
5′	49.7		
16	49.7	37.6	44.1
16′	49.7		
14	34.9		33.1
14′	34.9		
17	34.9	30.3	
17′	34.9		

[a~c] May be reversed.

Fig. 3. The proposed structure of A-269A and the structures of streptothricin F and LL-AC 541.



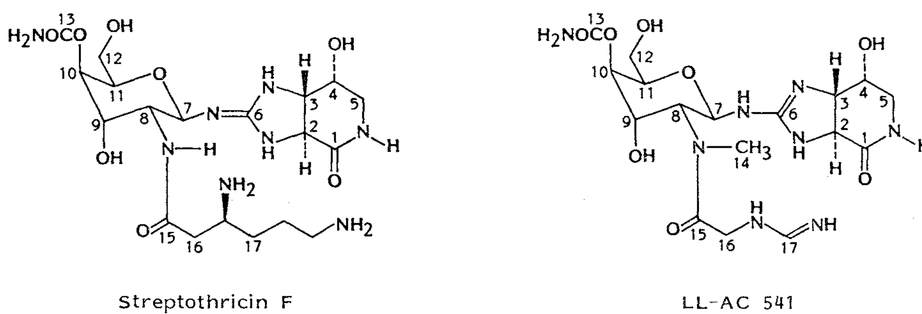
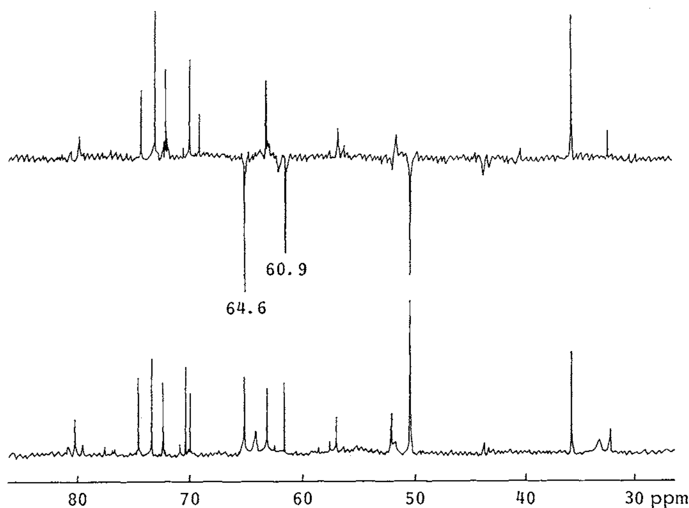


Fig. 4. ^{13}C NMR spectrum (with INEPT) of antibiotic A-269A.



signals ascribable to C-9~13 (9'~13') on the amino sugar moiety along with C-6 (C-6') on the streptolidine part appeared as pairs of singlets in the ^{13}C NMR spectrum of A-269A. Two carbon signals due to the hydroxymethyl group on amino sugar at δ 60.9 and 64.6 could be unambiguously assigned to C-12 and C-12', respectively, by the intensive nuclei enhanced by polarization transfer method (INEPT, Fig. 4). From the facts mentioned above, A-269A was assumed to be a mixture

Fig. 5. 400 MHz ^{1}H NMR spectrum of antibiotic A-269A.

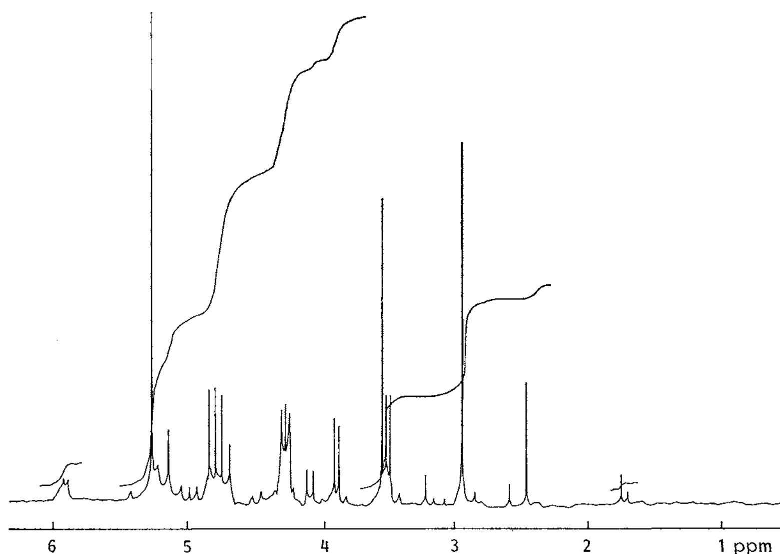
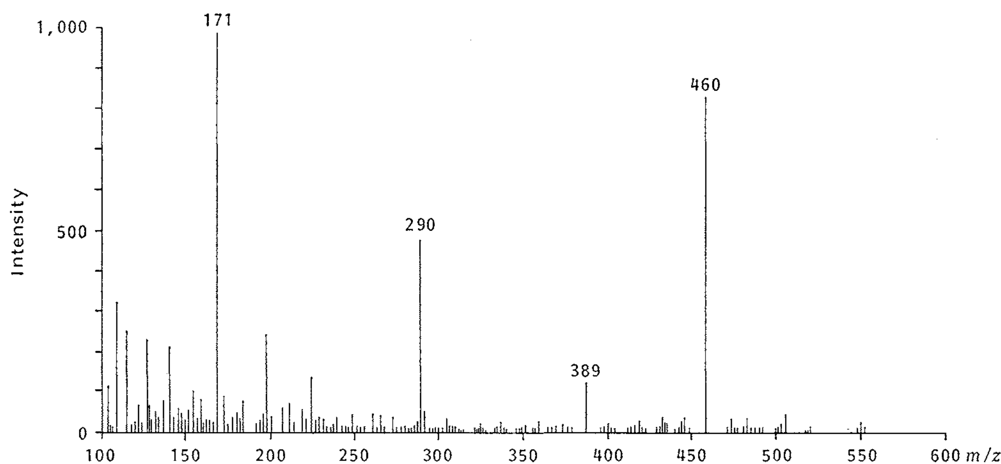


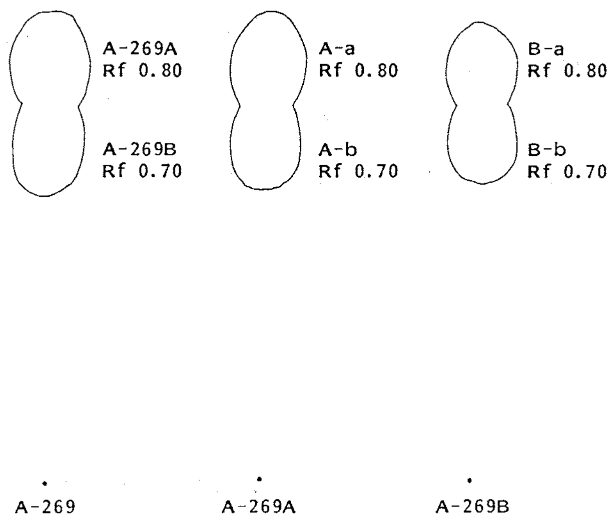
Fig. 6. FAB-MS spectrum of antibiotic A-269A or B.



of 10-carbamoyl compound (LL-BL 136) and 12′-carbamoyl compound (Fig. 3). The ^{1}H NMR and FAB-MS spectra of A-269A were shown in Figs. 5 and 6, respectively. The fragmentation in the FAB-MS spectrum, *m/z* 171 (streptolidine fragment), *m/z* 290 (sarcosine-amino sugar fragment), *m/z* 389 (streptolidine-amino sugar fragment) and *m/z* 460 $(M+H)^+$, also supported the proposed structures for A-269A.

On the other hand, the preparation of A-269A showed a dumbbell-like inhibition zone only on the paper chromatogram using the solvent system of butanol - acetic acid - water (2 : 2 : 3), in contrast to a single inhibition zone on other paper chromatograms. The upper zone of Rf, 0.80 and the lower zone of Rf, 0.70 in a dumbbell-like inhibition zone are termed A-269A and B, respectively (Fig. 7). The preparation of A-269B recovered from the lower zone gave similar physico-chemical and biological properties to A-269A, in addition to the same FAB-MS spectrum (Tables 2, 3 and Fig. 6). It also

Fig. 7. Bioautograms of antibiotic A-269A and B.
Solvent system; BuOH - AcOH - H_2O (2 : 2 : 3), development; ascending, 18 hours.



gave a similar dumbbell-like inhibition zone to that of A-269A on the paper chromatogram mentioned above. This phenomenon was assumed to be due to a tautomerism of the double bond on C-6 (C-6′) in the streptolidine moiety.

Acknowledgment

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References

1) MEHTA, R. J.; Y. K. OH, R. D. SITRIN, E. A. SHULTIS & C. H. NASH, III: Microbial resistances: A predictive test for streptothricin antibiotics. J. Antibiotics 34: 1501~1503, 1981
2) BORDERS, D. B.; J. P. KIRBY, E. R. WETZEI, M. C. DAVIES & W. K. HAUSMANN: Analytical method for streptothricin-like antibiotics: Structure of antibiotic LL-BL 136. Antimicrob. Agents Chemother. 1: 403~407, 1972
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Classification, Identification and Descriptions of Genera and Species. Vol. 2. Williams & Wilkins Co., Baltimore, 1961
5) RAYNER, R. W.: A Mycological Color Chart. Commonwealth Mycological Inst., Kew, Surrey & British Mycological. Soc., Kew, 1970
6) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid of species determination. J. Bacteriol. 56: 107~114, 1948
7) BUCHANAN, R. E. & N. E. GIBBONS (*Ed.*): BERGEY's Manual of Determinative Bacteriology. 8th Ed., p. 827, Williams & Wilkins Co., Baltimore, 1974
8) TANIYAMA, H.; Y. SAWADA & T. KITAGAWA: Characterization of racemomycins. Chem. Pharm. Bull. 19: 1627~1634, 1971
9) TSURUOKA, T.; T. SHOUMURA, N. EZAKI, T. NIWA & T. NIIDA: SF-701, a new streptothricin-like antibiotics. J. Antibiotics 21: 237~238, 1968
10) GOULD, S. J.; K. J. MARTINKUS & C.-H. TANN: Biosynthesis of streptothricin F. 1. Observing the interaction of primary and secondary metabolism with [1,2-$^{13}C_2$] acetate. J. Am. Chem. Soc. 103: 2871~2872, 1981

11) SAWADA, Y.; S. KAWAKAMI, H. TANIYAMA & Y. INAMORI: Incorporation of carboxyl and methyl carbon-13 labeled acetates into racemomycin A by *Streptomyces lavendulae* ISP 5069. J. Antibiotics 30: 630~632, 1977
12) KAWAMURA, T.; T. KIMURA, K. TAGO, T. BEPPU & K. ARIMA: The identity of S15-1-A and B with racemomycins A and C. J. Antibiotics 29: 844~846, 1976
13) BORDERS, D. B.; K. J. SAX, J. E. LANCASTER, W. K. HAUSMANN, L. A. MITSCHER, E. R. WETZEL & E. L. PATTERSON: Structures of LL-AC 541 and LL-AB 664, new streptothricin-type antibiotics. Tetrahedron 26: 3123~3133, 1970
14) VAN TAMELEN, E. E.; J. R. DYER, H. A. WHALEY, H. E. CARTER & G. B. WHITFIELD, Jr.: Constitution of the streptolinstreptothricin group *Streptomyces* antibiotics. J. Am. Chem. Soc. 83: 4295~4296, 1961
15) BYCROFT, B. W. & T. L. KING: Crystal structure of streptolidine, a guanidine-containing amino acid. J. Chem. Soc. Chem. Commun. 1972: 652~653, 1972
16) KUSUMOTO, S.; Y. KAMBAYASHI, S. IMAOKA, K. SHIMA & T. SHIBA: Total chemical structure of streptothricin. J. Antibiotics 35: 925~927, 1982
17) KUSUMOTO, S.; S. IMAOKA, Y. KAMBAYASHI & T. SHIMA: Total synthesis of antibiotic streptothricin F. Tetrahedron Lett. 23: 2961~2964, 1982
18) HUNT, A. H.; R. L. HAMILL, J. R. DEBOER & E. A. PRESTI: A 37812: *N*-Methylstreptothricin F. J. Antibiotics 38: 987~992, 1985
19) KAWAKAMI, Y.; K. YAMASAKI & S. NAKAMURA: The structures of component A_1 (=LL-AB664) and component A_2 (=LL-AC541), streptothricin-like antibiotics. J. Antibiotics 34: 921~922, 1981