167-A, A NEW ANTIBIOTIC PRODUCED BY A MUTANT OF AN INACTIVE WILD STRAIN OF *Amycolata autotrophica*

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Two related antibiotics, 167-A and 167-B, were isolated from the fermentation broth of a mutant of an inactive wild strain of *Amycolata autotrophica*. Antibiotic 167-B was found to be cervinomycin A_2; antibiotic 167-A is a new representative of the same group and has the structure of 18-*O*-demethyl cervinomycin A_2.

Treatment of inactive wild strains discarded in our screening program with intercalating agents ethidium bromide or daunorubicin induce with very high frequency progeny which exhibit antibacterial activity[1]. Antibacterial antibiotics 167-A and 167-B were isolated from the fermentation broth of the mutant EB5 derived from an inactive wild strain of *Amycolata autotrophica* 167 by treatment with ethidium bromide. Antibiotic 167-B was identified with cervinomycin A_2[2,3], component 167-A being a new representative of the cervinomycin group (Fig. 1).

The present paper deals with the taxonomy of wild and mutant strains, production, isolation, biological properties and structure of antibiotics 167-A and 167-B.

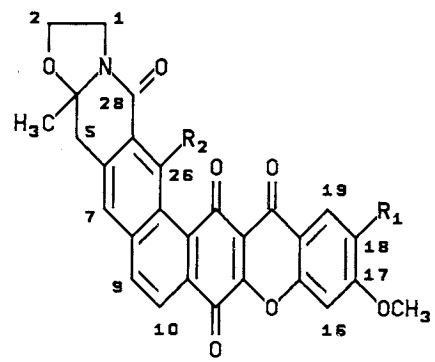
Results

Taxonomy

Microscopic examination of the parent and mutant strains revealed fragmentation of substrate and aerial mycelia of both strains.

The cultural characteristics of the cultures grown on various media at 28°C for 14 days are described in Table 1. The cell-wall analysis showed that the strains contain *meso*-diaminopimelic acid and diagnostic sugars galactose and arabinose, thus falling into cell-wall pattern type IV. The carbon and nitrogen sources utilization patterns and physiological properties of the parent strain 167, mutant strain 167 EB5 and *Amycolata autotrophica* are summarized in Tables 2 and 3. Comparison of the reactions of the parent strain 167 and *Amycolata autotrophica* revealed differences only in glutamic acid utilization and in decomposition of urea. The

Fig. 1. Structures of antibiotics 167-A, 167-B and the diacetyl derivative of 167-A.



	R_1	R_2
167-A	OH	OH
167-B	OCH_3	OH
Diacetyl derivative of 167-A	OAc	OAc

Table 1. Cultural characteristics of strains 167, 167 EB5, *Amycolata autotrophica* IMET 7646, *Amycolatopsis mediterranea* ATCC 13685 and *Amycolatopsis orientalis* ATCC 19795.

Medium		167	167 EB5	*A. autotrophica*	*A. mediterranea*	*A. orientalis*
Inorganic salts-starch[4] (Gause No. 1)	G:	Scant	Scant	Scant	Absent	Moderate
	A:	White	White	White		Absent
	S:	Colorless	Colorless	Colorless		Cream to yellow
	D:	Absent	Absent	Absent		Absent
CZAPEK's agar[5]	G:	Scant	Moderate	Moderate	Absent	Absent
	A:	White	Cream	Cream		
	S:	Colorless	Colorless	Colorless		
	D:	Absent	Light gray	Absent		
Glucose-asparagine agar[5]	G:	Scant	Moderate	Good	Scant	Moderate
	A:	Absent or scant, white to cream	Cream	Cream	Absent	Absent
	S:	Colorless or cream to yellow	Colorless	Cream yellow	Colorless	Yellowish
	D:	Absent	Light gray	Absent	Absent	Absent
Glycerol-nitrate agar[5] (LINDENBEIN's)	G:	Scant	Good	Good	Absent	Good
	A:	Scant, white	Cream	Cream		Absent
	S:	Colorless	Colorless	Beige to yellowish cream		Cream to yellow
	D:	Absent	Light gray	Absent		Absent
Oatmeal agar[6]	G:	Moderate	Moderate	Scant to moderate	Moderate	Good
	A:	White	White	White	Absent	Absent
	S:	Colorless	Colorless	Colorless	Colorless to greenish gray	Cream to yellow
	D:	Absent	Absent	Absent	Absent	Absent
Tryptone-peptone-glucose agar[4] (Gause No. 2)	G:	Abundant with wrinkled surface	Good	Abundant with wrinkled surface	Moderate	Good
	A:	White to cream	Cream to gray	White to cream	Absent	Absent
	S:	Beige	Dark brown	Beige to light brown	Colorless to beige	Cream yellow
	D:	Absent	Dark brown to black	Absent	Absent	Absent

G: growth; A: aerial mycelium; S: substrate mycelium; D: diffusible pigment.

data in Tables 1, 2 and 3 support the assignment of strain 167 to the species *Amycolata autotrophica*.

Morphological observations and cultural characteristics of the mutant strain 167 EB5 were almost identical with those of the parental strain 167. Differences in the properties of the mutant and the parent strains were observed in carbon utilization and in production by the mutant strain of a diffusible pigment in several media (Table 1).

Mutagenesis

Only one out of 395 colonies grown after treatment with different concentrations of ethidium bromide (EB) produced antibiotic. Culture 167 EB5 exhibited antibacterial activity against Gram-positive organisms.

Physico-chemical Properties and Structure

Physico-chemical properties of antibiotics 167-A and 167-B are summarized in Table 4. The antibiotics were slightly soluble in chloroform, poorly soluble in pyridine, dimethylsulfoxide, ethyl acetate and

Table 2. Physiological properties of strain 167, 167 EB5 and *Amycolata autotrophica* IMET 7646.

Test	167	167 EB5	*A. autotrophica*
Hydrolysis of starch	Negative	Negative	Negative
Liquefaction of gelatin	Negative	Negative	Negative
Peptonization of milk	Negative	Negative	Negative
Formation of melanoid pigment on peptone iron agar	Negative	Negative	Negative
Decomposition of urea	Negative	Negative	Positive

Table 3. Utilization of carbon and nitrogen sources by strains 167, 167 EB5 and *Amycolata autotrophica* IMET 7646.

	167	167 EB5	*A. autotrophica*
Glucose	+	−	+
Sucrose	+	−	+
Xylose	+	−	+
Fructose	+	−	+
Arabinose	+	−	+
Inositol	+	−	+
Mannitol	+	−	+
Cellulose	+	−	+
Galactose	+	+	+
Asparagine	+	+	+
Cysteine	+	+	+
Adenine	+	+	+
Glutamic acid	+	−	−
Arginine	+	+	+
Ornithine	+	+	+

+, Growth; −, no growth.

Table 4. Physico-chemical properties of antibiotics 167-A and 167-B.

	167-A	167-B
Appearance	Orange powder	Reddish-orange powder
MP (°C dec)	>250°C (dec)	>290°C (dec)
Optical rotation	$[\alpha]_D^{23}$ −129° (*c* 0.017, $CHCl_3$)	$[\alpha]_D^{23}$ −214° (*c* 0.25, $CHCl_3$)
Molecular formula	$C_{28}H_{19}NO_9$	$C_{29}H_{21}NO_9$
Elemental analysis (%)		
Calcd:	C 65.50, H 3.73, N 2.73	C 66.03, H 4.01, N 2.66
Found:	C 65.7, H 3.9, N 2.70	C 65.45, H 3.92, N 2.59
FAB-MS (*m/z*)	516 $(M+3H)^+$	530 $(M+3H)^+$
UV $\lambda_{max}^{CHCl_3}$ nm ($E_{1cm}^{1\%}$)	268.5 (642), 325 (528), 375 sh (143), 420 (119)	261.0 (717), 324 (546), 375.5 sh (149), 420 (120)
IR ν_{max}^{KBr} cm^{-1}	3550, 2980, 1680, 1630, 1500, 1450, 1420, 1380, 1280, 1230	3450, 2980, 1680, 1620, 1495, 1460, 1425, 1380, 1270, 1230
Rf values (silica gel TLC) $CHCl_3$-MeOH (40:1)	0.16	0.33

methanol and insoluble in ethanol, water and *n*-hexane.

^1H and ^{13}C NMR data for 167-A and 167-B are given in Table 5. Signal assignments have been made by means of homonuclear COSY[7], heteronuclear ^{13}C-^1H correlation *via* direct (HETCOR)[8] and long-range (FLOCK)[9] ^{13}C-^1H coupling constants experiments. Due to low solubility of 167-A only the ^1H NMR spectrum of this antibiotic was recorded. The methyl derivative of 167-A, which possess appropriate solubility, was obtained by methylation with CH_3I in the presence of K_2CO_3 and its NMR spectra were studied.

The ^1H NMR spectrum of 167-A appears to be similar to 167-B with the exception of an additional methoxy group at 4.01 ppm in the latter antibiotic. ^1H and ^{13}C NMR data for 167-B coincide with the methyl derivative of 167-A and the recently described antibiotic cervinomycin A_2[3]. Consequently, antibiotic 167-B and the methyl derivative of 167-A have the same structure as cervinomycin A_2, but antibiotic 167-A has the structure of 17- or 18-*O*-demethyl cervinomycin A_2.

Table 5. ^1H and ^{13}C NMR data for antibiotics 167-A, 167-B and their derivatives (in $CDCl_3$, ppm).

Carbon No.	167-A	167-B		167-A diacetate
	^1H	^1H	^{13}C	^1H
1	3.72 (1H, m), 4.02 (1H, m)	3.72 (m), 4.03 (m)	42.1	3.66 (1H, m), 4.02 (1H, m)
2	4.25 (2H, t)	4.25 (t)	64.3	4.19 (2H, t)
4			92.1	
5	3.22 ABq 3.27	3.22 ABq 3.27	41.1	3.27 ABq 3.32
6			136.6	
7	7.14 (1H, s)	7.15 (s)	117.3	7.66 (1H, s)
8			40.7	
9	7.90 (1H, d)	7.90 (d)	132.2	8.04 (1H, d)
10	8.20 (1H, d)	8.20 (d)	123.8	8.24 (1H, d)
11			129.6	
12			178.6	
13			153.8	
15			151.1	
16	7.14 (1H, s)	7.13 (s)	100.5	7.19 (1H, s)
17			155.6	
18			148.8	
19	7.77 (1H, s)	7.65 (s)	105.2	7.91 (1H, s)
20			119.8	
21			172.5	
22			121.0	
23			181.7	
24			138.2	
25			119.9	
26-OH	13.80 (1H, s)	13.8 (s)	161.5	
27			107.5	
28			164.6	
30	1.40 (3H, s)	1.40 (s)	22.9	1.42 (3H, s)
17-OCH_3	4.05 (3H, s)	4.02 (s)	56.7	3.96 (3H, s)
18-OCH_3	—	4.01 (s)	56.5	
18-$OCOCH_3$				2.34 (3H, s)
26-$OCOCH_3$				2.61 (3H, s)

As hydroxyl and methoxy substitution effects on the H-16 and H-19 chemical shifts are insignificant, the diacetyl derivative of 167-A was synthesized to determine unambiguously the substituent positions in 167-A. The low field shift of H-19 up to 7.91 ppm in diacetate derivative in comparison to 167-A and 167-B clearly indicates that one of the acetoxy groups is at the 18-position (Table 5). Consequently, antibiotic 167-A has the structure of 18-*O*-demethyl cervinomycin A_2.

Biological Activity

Antibiotics 167-A and 167-B have activity

Table 6. Antimicrobial activity of 167-A and 167-B.

Test organisms	MIC (μg/ml)	
	167-A	167-B
Staphylococcus aureus 209 P	0.08	0.08
S. aureus UV-2	0.06	0.08
Micrococcus luteus ATCC 9341	0.12	0.17
Bacillus mycoides R-537	0.04	0.11
Bacillus subtilis ATCC 6633	0.04	0.06
E. coli ATCC 25922	>100	>100
Pseudomonas aeruginosa ATCC 27853	>100	>100
Saccharomyces cerevisiae FL 200	100	100
Aspergillus niger	>100	>100

against Gram-positive bacteria and weak activity against *Saccharomyces cerevisiae* (Table 6).

Discussion

Treatment of inactive wild strains of actinomycetes with intercalating agents[1], as well as protoplast fusion, can activate silent genes responsible for the biosynthesis of antibiotics[10~12].

Experimental

General

Melting points were determined with Buchi SMP-20 apparatus.

IR spectra were run on a Pye-Unicam SP-110 spectrophotometer. UV spectra were recorded with Beckman UV-5260 spectrophotometer. Optical rotation was determined on a Perkin-Elmer 241 B polarimeter. FAB-MS spectra were obtained on a Kratos-50 spectrometer using a glycerol matrix. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR-400 spectrometer operating at 400 and 100.6 MHz for ^{1}H and ^{13}C nuclei, respectively. Spectra were measured at 28°C in $CDCl_3$ using the solvent peak as internal reference.

The *O*-Methyl Derivative of 167-A

Excess CH_3I (1 ml) was added to a suspension of the antibiotic 167-A (10 mg) in DMSO (2 ml) in the presence of K_2CO_3 (5 mg). The reaction mixture was stirred at room temperature for 30 minutes and then filtered. The filtrate was extracted with $CHCl_3$. The extract was washed with water and concentrated to dryness. The reaction product was purified by TLC using Silica gel 60 HF_{254} (Merck) and a solvent system of $CHCl_3$-MeOH (40:1) to afford the *O*-methyl derivative (7 mg) as an orange-reddish powder: MP>290°C (dec); $[\alpha]_D^{23}$ −214° (*c* 0.25, $CHCl_3$); FAB-MS *m/z* 530 $(M+3H)^+$; UV $\lambda_{max}^{CHCl_3}$ nm ($E_{1\,cm}^{1\%}$) 261 (717), 324 (546), 375.5 sh (149), 420 (120); IR ν_{max}^{KBr} cm^{-1} 3450, 2980, 1680, 1620, 1495, 1460, 1425, 1380, 1270, 1230; TLC (silica gel) Rf 0.33 ($CHCl_3$-MeOH, 40:1).

The Diacetyl Derivative of 167-A

A suspension of antibiotic 167-A (15 mg) in pyridine (2 ml) and $(CH_3CO)_2O$ (1 ml) was kept for 4 hours at room temperature. The reaction mixture was poured on an ice-water mixture and extracted with $CHCl_3$. The extract was washed with 1% $NaHCO_3$ and then evaporated to dryness. The product was purified on Silica gel 60 HF_{254} (Merck) by TLC, using a solvent system of $CHCl_3$-MeOH (40:1). The diacetate of 167-A is a yellow powder: MP 275°C; $[\alpha]_D^{23}$ −308° (*c* 0.1, $CHCl_3$); FAB-MS *m/z* 600 $(M+3H)^+$; UV $\lambda_{max}^{CHCl_3}$ nm ($E_{1\,cm}^{1\%}$) 245 (689), 270 sh (417), 309 (498), 369 (153); IR ν_{max}^{KBr} cm^{-1} 3000, 2950, 1780, 1760, 1690, 1660, 1630, 1510, 1450, 1440, 1410, 1380, 1280, 1200, 1170; TLC (silica gel) Rf 0.28 ($CHCl_3$-MeOH, 40:1).

Taxonomy

Strain 167 was isolated from a soil sample collected in Bulgaria. Observations of the cultures were made after incubation at 28°C for 2 weeks. Color names were assigned to the mycelium and diffusible pigments according to method of PRAUSER[13]. Whole-cell sugars were identified by the procedure of LECHEVALIER[14]. Diaminopimelic acid was determined by the method of HASEGAWA[15].

Mutagenic Treatment

The germinating spores of parent inactive strain 167 were grown on Gause No 2[4] medium composed of Hottinguer tryptone broth (content of amino nitrogen 700 mg%) 30 ml, peptone 0.5%, glucose 1%, NaCl 0.5% and $CaCO_3$ 0.3% overnight at 37°C in the presence of various concentrations of EB (5~40 μg/ml). The EB-treated spores were placed on agar plates (Gause No. 2 with 2% agar) and incubated at 28°C for 14 days. Plates were overlaid with soft agar (Gause No. 2, 1% agar) containing *Staphylococcus aureus* 209P as a test organism and incubated overnight at 28°C. The active colonies were transferred to a slant of Gause No. 1 agar[7] (KNO_3 0.1%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%, NaCl 0.05%, $FeSO_4$ 1%,

starch 2% and agar 2%). Activity of the cultures was examined against *Staphylococcus aureus* 209P, its mutant *S. aureus* UV-2, *Micrococcus luteus* ATCC 9341, *Escherichia coli* ATCC 25922, *Saccharomyces cerevisiae* FL 200, *Bacillus subtilis* ATCC 6633, *Bacillus mycoides* R-537 and *Pseudomonas aeruginosa* ATCC 27853.

Fermentation

Fermentation was carried out in 45-liter fermentor charged to a volume of 27 liters. Production medium comprised soy bean meal 1%, sucrose 2%, KNO_3 0.2%, NaCl 0.3% and $CaCO_3$ 0.3%. Incubation was at 28°C for 144 hours with agitation (220 rpm) and aeration (27 liters/minute).

Isolation of Antibiotics

The culture broth (27 liters) was acidified to pH 3.5 with 18% HCl and filtered. The mycelial cake was extracted with acetone. The acetone solution was extracted with chloroform three times. The chloroform extracts were combined and washed with water and dried over anhydrous Na_2SO_4. The solvent extract was evaporated under reduced pressure to an oily residue. The oily residue was subjected to chromatography on a column (3 × 30 cm) packed with Silica gel 60, particle size 0.040 ~ 0.063 mm (Merck). The column was developed with chloroform and then chloroform - methanol. The active chloroform and chloroform - methanol (50 : 0.75) fractions containing antibiotic 167-B were combined. Fractions containing antibiotic 167-A were eluted with chloroform - methanol (50 : 1). Antibiotic 167-B was crystallized from mixture of chloroform - methanol (1 : 5), giving 20 mg of reddish-orange powder. Antibiotic 167-A was crystallized from a mixture of chloroform - methanol (1 : 2), giving 10 mg of orange powder.

Antimicrobial Activity

Antimicrobial activity of the antibiotics was determined by the serial-dilution method using Gause No. 2 agar medium. The MIC is expressed in μg/ml after overnight incubation at 37°C for bacteria and after 48 hours incubation at 28°C for fungi and yeasts.

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