ANTIBIOTIC AB-64, A NEW INDICATOR-PIGMENT ANTIBIOTIC FROM *ACTINOMADURA ROSEOVIOLACEA* VAR. *RUBESCENS*

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A new antibiotic designated as antibiotic AB-64 inhibiting gram-positive bacteria was isolated from a rare Actinomycete. The producing strain was classified as *Actinomadura roseoviolacea* var. *rubescens*. Antibiotic AB-64, obtained as red powder, has a molecular formula $C_{26}H_{21}NO_9$(MW 491.46), and seems to be a new heteroaromatic compound based on its physical and chemical properties.

During the course of screening for new antibiotics from rare actinomycetes in our laboratory, a rare actinomycete initially designated as strain A-3416 was found to produce an antibiotic with strong activity against staphylococci resistant to various antibiotics. From its unique physicochemical properties, it was judged to be a new antibiotic and named antibiotic AB-64.

In this paper, taxonomical studies of strain A-3416, fermentation, isolation and characterization of antibiotic AB-64 are described.

Taxonomical Studies on Strain A-3416

Strain A-3416, that produces antibiotic AB-64, was isolated from a soil collected at Hikami, Hyogo Prefecture, Japan. According to the taxonomical studies described below, this organism was identified with a new variant of *Actinomadura roseoviolacea*[1] and designated as *Actinomadura roseoviolacea* var. *rubescens* nov. var. TAMURA *et* KOTANI. It shows the following properties:

1. Morphological Properties

The morphology of the culture on malt-yeast agar was microscopically observed (Plates 1 and 2).

Plate 1. Aerial mycelium of *A. roseoviolacea* var. *rubescens* A-3416 on glycerol-asparagine agar-V after 21 days.

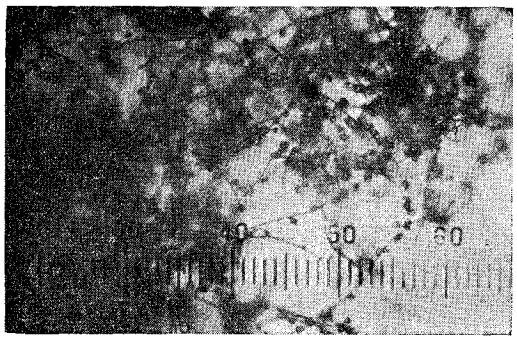


Plate 2. Electron-micrograph of spores of *A. roseoviolacea* var. *rubescens* A-3416 on malt-yeast agar after 30 days.

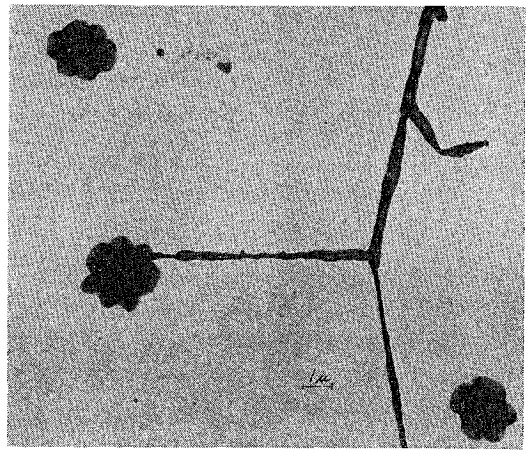


Table 1. Cultural properties of strain A-3416

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Moderate, scant spreading, cream ($1\frac{1}{2}$ ca)**	None	None
Sucrose-nitrate agar-V*	Moderate, scant spreading, Lt. coral rose (6 ga) with small red patches	Moderate, powderly, brite shell pink (6 ea)	None
Glucose-asparagine agar-V	Moderate, scant spreading, rust brown (5 pg)	None	None
Glycerol-asparagine agar	Scant growth, spreading, white	None	None
Glycerol-asparagine agar-V	Moderate, raised, brown mahogani (6 pi)	Moderate, powderly, pale pink (6 ca)	Pale light brown
Starch agar-V	Thin, small colonies, not spreading, persimmon (5 nc)	None	None
Tyrosine agar-V	Moderate, raised, rust brown (5 pg)	Moderate powderly, pale pink (6 ca)	None
Bouillon agar	Moderate, wrinkled, melon yellow (3 ea)	None	None
Malt-yeast agar	Abundant, raised, terra cotta (5 pe)	Abundant, powderly, pale pink (6 ca)	None
Oatmeal agar	Abundant, flat, lacquer red (6 pe)	Scant powderly, white or shell pink	None
Milk-V	Moderate, ring, sunset red (6 la)	None	None
Peptone-yeast-iron agar	Abundant wrinkled, shell pink (5 pa)	None	None
Glycerol-calcium malate agar-V	Moderate, flat, Dk. lacquer red (6 pe)	None	None

Glucose-asparagine agar, starch agar, tyrosine agar and glucose-peptone-gelatin-V: no growth or scant growth.
* -V: added B-vitamins[2].
** Color No. on Color Harmony Manual (1958).

Spore chains, spirals, thightly closed and forming pseudosporangia on long aerial hyphae. Pseudosporangia usually about 3~5 μ diameter. Spores seemed to be enveloped in slimy substance. Spore surface: smooth.

2. Cultural Properties

The cultural properties on different media are listed in Table 1. The experiments to determine the following cultural characteristics were carried out at 30°C for 3~4 weeks. The gelatin stab culture was observed after incubation at room temperature for 60 days.

3. Carbon Utilization

The carbon utilization of strain A-3416 was examined according to the method described by NONOMURA and OHARA[2] with results as shown in Table 2.

4. Physiological Properties

The physiological properties are summarized in Table 3.

5. Cell Wall Components

Amino acids and carbohydrates from cell wall hydrolysates were examined according to the method described by BOONE and PINE[3].

Amino acids: meso-diaminopimelic acid (present), lysine (none).

Sugars: arabinose (none), galactose (none), xylose (none).

The distinctive characters of strain A-3416 are as follows:

(1) Cell wall components: Type III[4] (*Thermoactinomyces*, *Actinobifida*, *Microbispora* and *Actinomadura*).

(2) Melanoid pigments not produced (in the sense of ISP[5]).

(3) Spore chains: tightly closed spirals or pseudosporangia. Spore surface: smooth.

(4) Aerial mass color: pale pink.

(5) Substrate mycelium: red (pH sensitive).

From the taxonomical studies performed and the key to the new species of *Actinomadura*[1], it is evident that strain A-3416 should be classified in the genus *Actinomadura*, and strain A-3416 most closely resembles *Actinomadura roseoviolacea* A-5 (NONOMURA and OHARA, 1971)[1]. However, detailed comparison experiment, which was pointed out by H. NONOMURA (personal communication, Jan. 1972), show some differences between strain A-3416 and *A.*

Table 2. Carbon utilization of strain A-3416 (C-2 medium[2])

Carbon source	Results
No carbon	±
L-Arabinose	±
D-Xylose	+
D-Glucose	++
D-Fructose	++
Sucrose	++
Inositol	++
L-Rhamnose	+
Raffinose	++
D-Mannitol	++
Salicin	+
α-Methyl-glucoside	++
Galactose	++
Lactose	+
Cellulose	±

++ Good growth + Moderate growth
± Little growth

Table 3. Physiological properties of strain A-3416

Determination	Results	Medium used
Melanoid pigment	Negative	Peptone-yeast-iron agar, Tyrosine agar-V
Starch hydrolysis	Positive	Starch agar-V
Nitrite from nitrate	Positive	Nitrate broth
Milk peptonization	Positive	10% Difco skim milk (after 3 weeks)
Gelatin liquefaction	Weakly positive	Glucose-peptone-gelatin-V (after 60 days)
Production of H_2S	Weakly positive	Peptone-yeast-iron agar
Solubility of Ca-malate	Positive	Glycerol-Ca malate agar-V
Relation to temperature	Optimum growth at 32~34°C, no growth at 45°C	Malt-yeast broth
Relation to pH of medium	Optimum growth at pH 6~8	Malt-yeast broth
B-vitamins	Essential for growth	

Table 4. Comparison of strain A-3416 and *A. roseoviolacea* A-5

		Strain A-3416	*A. roseoviolacea* A-5
Color change of colony (reverse side) by addition of 0.05 N NaOH[1] on yeast-malt agar, oatmeal agar and glycerol-asparagine agar-V after 30 days.		from rust brown (or brown red) to deep ruby red	from dark purple (or dull red) to violet
Hydrolysis of	Casein[2]	+	+
	Xanthine[3]	−	−
	Hypoxanthine ⟶ xanthine[3]	+	+
	Adenine ⟶ hypoxanthine[3]	−	+
	Tyrosine[4]	+	+
	Starch[5]	+	+
	Esculine[6]	+	+
	Urea[7]	−	−
Nitrite from nitrate[1]		+	+
Acid production from[8]	Arabinose	+	+
	Erythritol	−	−
	Mannitol	(+)	−
	Melibiose	+	+
	Rhamnose	+	+
	Xylose	+	(+)

1) ISP method[5].
2) Starch-K_2HPO_4-V (soluble starch 10 g, K_2HPO_4 0.5 g, B-vitamins, water liter, pH 7.0). Casein 1 g/liter. Detection: Avicel (cellulose) TLC, EtOH-water (7 : 3), ninhydrin reaction.
3) Trypton-yeast broth (ISP medium 1) and starch-K_2HPO_4-V. Test sample 1 g/liter. Detection: Avicel TLC, water saturated *n*-BuOH - NH_4OH (100:1), UV light (280 nm).
4) Trypton-yeast broth. Tyrosine 1 g/liter. Detection: Avicel TLC, *n*-BuOH - acetic acid - water (4 : 1 : 2), α-nitroso-β-naphtol, ninhydrin, MILLON reaction.
5) Trypton-yeast broth. Soluble starch 1 g/liter. Detection: iodo-starch reaction.
6) Trypton-yeast broth. Esculine 1 g/liter. Detection: Avicel TLC, *n*-BuOH - acetic acid - water (8 : 2 : 2), UV light (280 nm).
7) Trypton-yeast broth. Urea 1 g/liter. Detection: CONWAY's microanalysis of diffusion method[6], NESSLER reaction.
8) L-Asparagine-casamino acid broth. Test sugar 1 g/liter. Detection: pH indicator (BTB).

roseoviolacea A-5 (Table 4). Characteristics which distinguish strain A-3416 from standard strain A-5 of *Actinomadura roseoviolacea* are:

(1) Reverse side of colony: changed to red with 0.05 N NaOH (*A. roseoviolacea* A-5: violet).
(2) Nucleic acid hydrolysis: hypoxanthin from adenine: negative (*A. roseoviolacea* A-5: positive).

From these considerations, strain A-3416 is resonably recognized as a variant of *A. roseoviolacea* and designated as *Actinomadura roseoviolacea* var. *rubescens* nov. var. referring to the "red" substrate mycelium.

Fermentation and Isolation of Antibiotic AB-64

Strain A-3416 was grown on a slant of yeast-malt agar (ISP medium 2) for 2 weeks at 30°C.

Flask fermentations were run using 70 ml of medium in 500-ml SAKAGUCHI flask. All fermentations were carried out at 30°C on a reciprocal shaker. Germination and fermentation medium: glucose 2 g, defatted soybean meal 2 g, soluble starch 1 g, meat extract 0.1 g, yeast extract 0.4 g, NaCl 0.2 g, K_2HPO_4 0.05 g, $CaCO_3$ 0.4 g, soybean oil 0.2 g, tap water to 100 ml, pH 8.8. Maximum yield was obtained by incubation for 10 days in 500-ml SAKAGUCHI flask containing 70 ml of the medium and 3 ml of 4 days old germinators.

The antibiotic produced was determined by paper disk method using *Staphylococcus aureus* P-213 (aminobenzyl penicillin resistant).

The cultured broth (7 liters, pH 8.0) was separated continuously in S-type ultracentrifuge at 10,000 rpm. The supernatant broth was adjusted to pH 4.0 with concentrated HCl, and centrifuged. The precipitate (110 g, wet) was extracted two times with 1 liter of 80% aqueous acetone. The aqueous acetone extracts were concentrated to one fifth of its volume *in vacuo*. The aqueous solution was adjusted to pH 6.0 with NaOH, and extracted four times with the equal volume of *n*-butanol. The butanol extracts were concentrated to dryness *in vacuo*. The residue (crude active substance) was purified two times by chromatography on a column of Sephadex G-25. The residue was dissolved in 10 ml of 0.1 N NH_4OH, and charged on top a column (5 cm×50 cm) to which a suspension of Sephadex G-25 in 0.01 N NH_4OH had previously been applied. The chromatographic development was carried out with 0.01 N NH_4OH, and equal volume (15 ml) fractions were collected. The antibiotic activity of the elutes was monitored by paper chromatography and its bioautogram. Antibiotic AB-64 was obtained after 1,100 ml of the elute. The active fractions (300 ml) were concentrated to dryness *in vacuo*. The residue (red powder) was dissolved in 2 ml of dimethylsulfoxide and precipitated by addition with the equal or double volume of water. The red powder was purified further by reprecipitation from DMSO-water, washed with water and acetone, and filtered off and dried *in vacuo*. From 7 liters of the broth, 100 mg of pure red powder (antibiotic AB-64) was isolated. The following Rf values were obtained by ascending paper strip chromatography: water, 0; 50% aqueous acetone, 0.59; *n*-BuOH saturated with water, 0.54; *n*-BuOH - MeOH - water (4 : 1 : 2), 0.74; *n*-BuOH-pyridine - water (6 : 4 : 3), 0.72.

Fig. 1. Procedure of isolation of antibiotic AB-64

Fermentation broth, pH 8.0
↓ adjusted to pH 4 with conc. HCl, centrifuged
Precipitate
↓ extracted with 80% aqueous acetone
Aqueous acetone extract
↓ concentrated *in vacuo*
Aqueous solution
↓ adjusted to pH 6 with NaOH, extracted with *n*-BuOH
BuOH extract
↓ evaporated *in vacuo*
Residue
↓ gel filtration on Sephadex G-25, eluted with 0.01 N NH_4OH
Active fraction
↓ evaporated *in vacuo*
Red powder (Antibiotic AB-64)

Physicochemical Properties of Antibiotic AB-64

Antibiotic AB-64 is a pigment obtained as red powder having m.p. 275~277°C (dec.). The molecular weight of antibiotic AB-64 was not directly obtained owing to its low solubility in common organic solvents. The molecular formula $C_{26}H_{21}NO_9$ of antibiotic AB-64 was confirmed indirectly by mass spectrometry and by the analysis of derivatives described below.

Antibiotic AB-64 is soluble in aqueous

Fig. 2. Ultraviolet and visible spectra of antibiotic AB-64 and its derivatives.

A. AB-64 in 0.02 N NaOH
B. AB-64 in acidic solution (pH 2~3)
C. AB-64-pentaacetate in EtOH
D. AB-64-methyl ether in EtOH

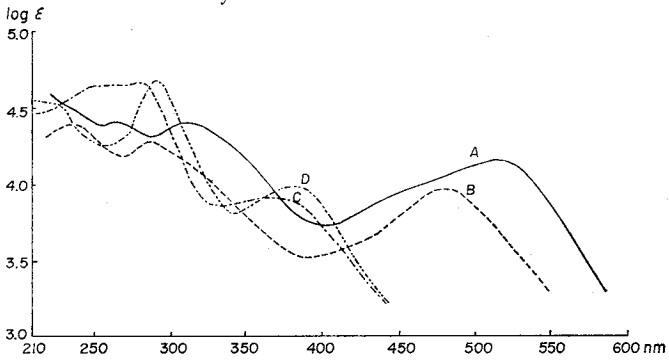
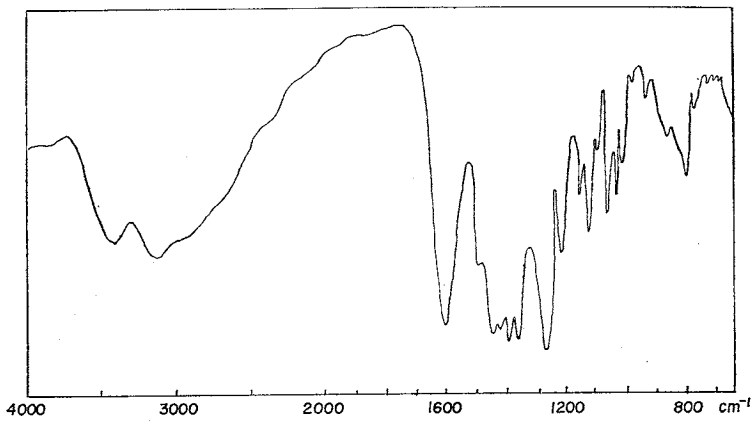


Fig. 3. Infrared absorption spectrum of antibiotic AB-64 in KBr disk.



alkaline solution, *e.g.* ammonia and alkali hydroxides. The change of UV and visible spectra depending upon pH value of the solution as shown in Fig. 2 suggests that antibiotic AB-64 is classified as a phenolic pH-indicator. In the IR spectrum, antibiotic AB-64 showed hydroxyl bands at 3400 and 3100 cm^{-1}, and broad carboxyl bands at 1600~1630 cm^{-1} (Fig. 3). The latter indicated that the carbohydroxyl group of antibiotic AB-64 formed a strong intramolecular hydrogen bond. The NMR spectrum taken in diluted NaOD-D_2O solution showed two aromatic protons at δ 6.67 (1 H, singlet) and 6.94 (1 H, singlet), methoxyl protons at δ 3.50 (3 H, singlet) and aromatic C-methyl protons at δ 2.03 (3 H, singlet).

Antibiotic AB-64 was acetylated with acetic anhydride in aqueous ammonia followed with acetic anhydride-pyridine to give antibiotic AB-64-pentaacetate lactone, pale yellow prisms, m. p. 285~290°C, $C_{36}H_{29}NO_{13}$. Its NMR spectrum revealed the presence of two aromatic protons, one methoxyl group, one aromatic C-methyl group and five acetyl groups. In its mass spectrum, acetyl groups were broken down to a large extent by the sequence M−n×42 (M=683 ($C_{36}H_{29}NO_{13}$), n=1 to 5 in turn). This sequence was supported by each corresponding appropriate metastable

peak. The IR spectrum of antibiotic AB-64-pentaacetate lactone exhibited the lactone and ester carbonyl bands at 1765 and 1720 cm^{-1}.

On the other hand, antibiotic AB-64 was methylated with dimethyl sulfate followed with diazomethane to give antibiotic AB-64-permethyl ether, yellow prisms, m. p. 268~272°C (dec.). Antibiotic AB-64-permethyl ether was analysed for $C_{34}H_{38}NO_9^+ \cdot CH_3SO_4^- \cdot H_2O$, the molecular formula of which was confirmed by mass spectrometry showed a molecular ion peak at *m/e* 589 corresponding to $C_{33}H_{35}NO_9$ ($C_{34}H_{38}NO_9 - CH_3$). The presence of monomethylsulfate ion in antibiotic AB-64-permethyl ether was reduced from its IR (1220~1260 and 740 cm^{-1}) and NMR spectrum (δ 3.75, sharp singlet, 3H). Further bands in the IR spectrum at 1740 cm^{-1} suggested the presence of carbomethoxyl group. Its NMR spectrum showed two singlet aromatic protons, one aromatic C-methyl proton, one N^+-methyl signal and eight methoxyl protons besides monomethyl sulfate protons.

Therefore, these spectral evidences led to the partial formulation of the structures I, II and III for antibiotic AB-64 and its acetylated and methylated products, respectively. Although we have no evidence on the residual part ($C_{23}H_6$) of the AB-64 except the UV spectra shown in Fig. 2, it may be considered that $C_{23}H_6$ part comprises mainly a polycyclic aromatic ring.

$C_{23}H_6$ {
- two aromatic protons
- (phenolic) $(OH)_6$
- COOH
- (aromatic) OCH_3
- (aromatic) CH_3
- (aromatic) =N-

} I AB-64

$C_{23}H_6$ {
- two aromatic protons
- (aromatic) $(OCOCH_3)_5$
- lactone CO—O
- (aromatic) OCH_3
- (aromatic) CH_3
- (aramatic) =N-

} II AB-64-pentaacetate

$C_{23}H_6$ {
- two aromatic protons
- (aromatic) $(OCH_3)_7$
- $COOCH_3$
- (aromatic) CH_3
- (aromatic) $=N^+-$ $CH_3SO_4^-$

} III AB-64-permethyl ether

Experimental

AB-64 (I)

Red powder, m. p. 275~277°C (dec.). Mass; *m/e* 474 (M(491)—OH), *m/e* 473 (M—H_2O). UV; $\lambda_{max}^{0.02N\ NaOH}$ nm (log *e*) 508 (4.14), 310 (4.41), 258 (4.40). $\lambda_{min}^{0.02N\ NaOH}$ 400 (3.70), 286 (4.29) see Fig. 2. Anal. Calcd. for $C_{26}H_{21}NO_9$: C 61.29, H 4.55, N 2.75. Found : C 60.53, H 4.55, N 2.75. $[\alpha]_D$ could not be measured for deep red color.

AB-64-pentaacetate (II)

AB-64 (46 mg) was dissolved in 7% NH_4OH (10 ml) and the solution was cooled in an ice-bath.

Table 5. Antimicrobial spectrum of antibiotic AB-64.

Test organisms	MIC (mcg/ml)
Staphylococcus aureus Terajima	1*
S. aureus ATCC 6538	1
S. aureus sp. S-21	0.3
S. aureus sp. S-23	1
S. aureus No. 5	1
S. aureus No. 6	0.3
S. aureus Miyamoto	1
S. aureus P-7 C (SM, CP, TC-R)	0.3
S. aureus P-32 (SM, PC, TC-R)	0.3
S. aureus P-213 (ABPC-R)	0.3
S. aureus FDA 209 P (SM, STH-R)	10
S. aureus FDA 209 P JC-1 (KM, STH-R)	1
S. aureus FDA 209 P JC-1 (SM, STH-R)	1
S. epidermidis No. 8	1
S. albus AKM	1
Escherichia coli K-12	>100*
Shigella flexneri 2a EW 10	100*
Mycobacterium tuberculosis $H_{37}Rv$	>30*
Candida albicans ATCC 10257	>30*
Trichophyton mentagrophytes	>30*
Trichomonas vaginalis 4 F	30*

* MIC: dilution method.
No mark MIC: streaking method.

Acetic anhydride (10 ml) was added and the mixture was stirred vigorously at 60~70°C for 5 minutes. After cooling, the reaction mixture was extracted with $CHCl_3$. The organic layer was washed with H_2O and evaporated. The red residue (45 mg) was acetylated with acetic anhydride and pyridine in usual way. The crude acetate was recrystallized from CH_2Cl_2EtOH to give pale yellow prisms (29 mg), m.p. 285~290°C. UV; λ_{max}^{EtOH} nm (log e) 368 (3.89), 283 (4.66), 255(4.62). λ_{min}^{EtOH} 328(3.84), 270(4.61), NMR; δ in $CDCl_3$; 7.85^s (1H, aromatic proton), 7.38^s(1H, aromatic proton), 4.83^{bs}(2H), 3.92^s (3H, OMe), 2.65^s, 2.52^s, 2.37^s, 2.33^s (each 3H, OAc×5), 2.16^s(3H, Me). Mass; (m/e), 683(M^+), 641 (M—42, m* 601.6), 599 (M—84, m* 558.8), 557 (M—126, m* 517.9), 515 (M—168, m* 476.2), 473 (M—210, m* 434.4). Anal. Calcd. for $C_{36}H_{29}NO_{13}$: C 63.41, H 4.25, N 2.05. Found C 63.25, H 4.28, N 2.05.

AB-64-permethyl ether (III)

To a solution of AB-64 (236 mg) in 10% NaOH (5 ml), Me_2SO_4 (4.5 ml) and 10% NaOH (17 ml) were separately added dropwise with stirring at room temperature. After 8 hours, the reaction mixture was acidified with conc. HCl and extracted with $CHCl_3$. The red residue obtained after the evaporation of solvent was methylated with diazomethane as usual and the crude product was chromatographed over silica gel with 10% MeOH-$CHCl_3$ to give a yellow solid (0.15 g). Recrystallization from EtOH-ether afforded yellow prisms, m.p. 268~272°C (dec.). IR: 1740 cm^{-1}($\nu_{C=O}$), 1670 cm^{-1}($\nu_{C=N^+}$), 1220-1260 and 740 cm^{-1} ($CH_3SO_4^-$). UV; λ_{max}^{EtOH} nm (log e) 382 (3.96), 292 (4.68), 224(4.54). λ_{min}^{EtOH} 344 (3.79), 260 (4.25). NMR (δ in $CDCl_3$); 7.62^s, 7.48^s (each 1H, aromatic proton), 4.02^s, 4.00^s, 3.98^s, 3.95^s, 3.60^s, 3.49^s, 3.37^s and 3.37^s (each 3H, OMe ×8), 3.37^s (3H, N^+-Me), 3.75^s(3H, $MeSO_4$), and 2.26^s(3H, Me). Anal. Calcd. for $C_{34}H_{38}NO_9 \cdot CH_3SO_4 \cdot H_2O$: C 57.29, H 5.90, N 1.91, S 4.37. Found: C 57.39, H 5.54, N 1.91, S 4.40.

Biological Properties of Antibiotic AB-64

The antimicrobial spectrum of antibiotic AB-64 determined by the dilution method and the streaking method is shown in Table 5. From these results it is evident that antibiotic AB-64 is active against gram-positive bacteria and resistant staphylococci.

Discussion

Antibiotic AB-64 is a phenolic indicator substance, being orange in acid and neutral solutions and red in alkaline solution, and seems to be a heteroaromatic compound as shown by its UV, IR and NMR spectra. Due to similarities in UV and visible spectra and elemental analyses, antibiotic AB-64 was compared with known nitrogen-containing antibiotics having acid-base-indicating properties: cyanomycin[10], cinerubin[10], daunomycin[10], 1, 6-dihydroxyphenazine[10], iyomycin B_1[10], luteomycin[10], pluramycin[10], ractinomycin[10], rubiflavin[10], rubradirin[10], rhodomycin[10], ruticulo-

mycin[10], streptorubin[10], streptovaricins[10], trypanomycin[7], violarins[10], and xanthomycin[10]. But, the UV maxima characteristic, melting point and molecular formula of antibiotic AB-64 were not accorded with that obtained in these antibiotics. Red pigments with the methoxytripyrrole nucleus of prodigiosin (prodiginine)[8,9] have been isolated from *Actinomadura madura* and *A. pelletieri*. These pigments are soluble in benzene, chloroform and hexane, but antibiotic AB-64 is not soluble, and the UV maxima of these pigments are not observed in antibiotic AB-64. Therefore, antibiotic AB-64 is a new antibiotic which differs from other known antibiotics.

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