NEW ANTITUMOR ANTIBIOTICS, ACLACINOMYCINS A AND B

Sir:

In the study of antitumor antibiotics new members of anthracycline group, aclacinomycins A and B, have been isolated from *Streptomyces* sp. No. MA144-M1 (ATCC 31133) which was classified as *S. galilaeus*.

Aclacinomycins were produced by the shaking culture of MA144-M1 strain at 28°C for 4 days in a medium containing 1 % glucose, 1 % potato starch, 1.5 % soybean meal ("Prorich", Ajinomoto Co.), 0.1 % KH_2PO_4, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.3 % NaCl, 0.007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MnCl_2 \cdot 4H_2O$ and 0.0002 % $ZnSO_4 \cdot 7H_2O$, pH 7.0. Total aclacinomycin was assayed by a disc plate method against *Bacillus subtilis* or *Micrococcus flavus*, and aclacinomycins A and B were separately determined by a silica gel thin-layer chromatography using chloroform - methanol (20 : 1) and by reading the optical density of spots with Rf 0.36 and 0.71 at 430 nm using a Shimazu dual-wave length TLC scanner, Model CS-900. In the case of testing the cultured broth, aclacinomycins were extracted with chloroform or a chloroform - methanol mixture (1 : 1) before the thin-layer chromatography and were developed with acetone or chloroform - methanol mixture (20 : 1).

In an example, the cultured broth (pH 7.4) which contained 32 mcg/ml of aclacinomycin A and 23 mcg/ml of aclacinomycin B was adjusted to pH 4.5 and filtered. Aclacinomycins were extracted from the mycelia and the filtrate at pH 6.8 with acetone and ethyl acetate, respectively, and the active extracts were concentrated *in vacuo*, and a crude aclacinomycin mixture was precipitated by addition of *n*-hexane. The crude orange-yellow powder thus obtained was dissolved in a small amount of chloroform, and subjected to the silica gel (Mallinckrodt silicic acid, 100 mesh) column chromatography. After discarding the initial eluates with 1 : 1 and 2 : 1 benzene - acetone mixtures, aclacinomycin B was eluted with 1 : 3 and 1 : 5 benzene - acetone mixtures, and aclacinomycin A was eluted with 1 : 5 : 0.5 and 1 : 5 : 1 benzene - acetone - methanol mixtures. The eluates containing aclacinomycin A or B were dried over anhydrous sodium sulfate and concentrated to dryness, yielding a relatively pure powder in 40~20 % recovery from the cultured broth. Further purification of A and B was accomplished by a column chromatography of Column-Lite (Fuji Chemical Ind. Co., 30~60 mesh) developed with a chloroform - methanol mixture (1 : 1). Red fractions containing aclacinomycins A and B were evaporated to dryness *in vacuo*, and dissolved in a small amount of chloroform. After adding the 0.01 M phosphate buffer (pH 7.2) containing 1 mM EDTA to each aclacinomycin A and B solution and shaking vigorously to remove the residual metal ions, the chloroform phase was washed twice by shaking with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. Thus, pure aclacinomycins A and B were obtained as yellow microcrystalline powders by addition of *n*-hexane to the concentrated solution. The yields were 10 to 20 % from the cultured broth.

Physicochemical properties of aclacinomycins A and B are as follows:

Aclacinomycin A: m.p. 129~135°C under decomposition; $[\alpha]_D^{24}$ +29° (*c* 1.0, $CHCl_3$); anal. calcd. for $C_{42}H_{54}O_{15}N$: C 62.05, H 6.69, O 29.52, N 1.72; found: C 62.37, H 6.67, O

Fig. 1. Ultraviolet and visible light absorption spectra of aclacinomycin A.

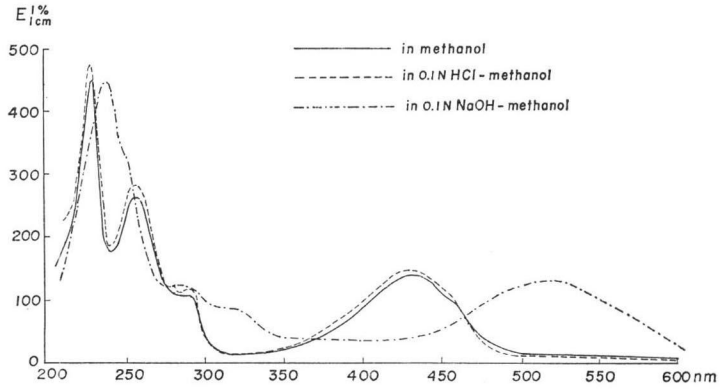
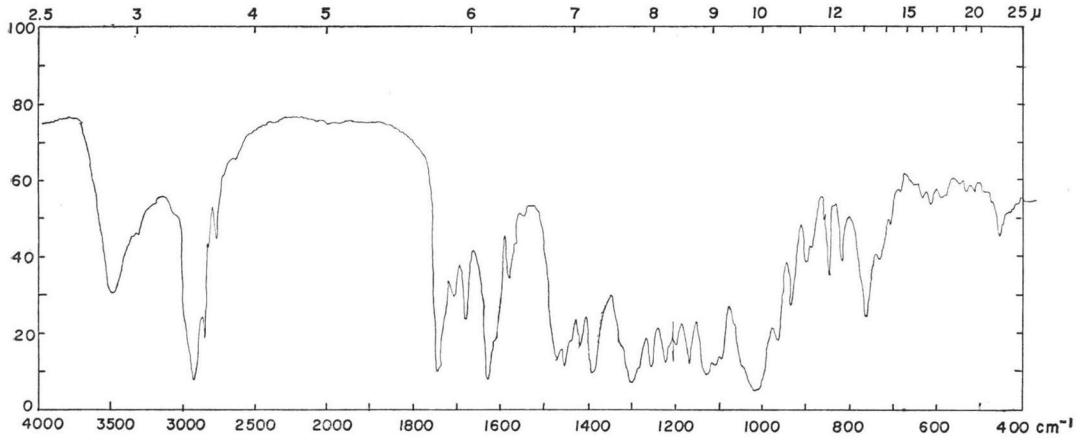


Fig. 2. Infrared absorption spectrum of aclacinomycin A (KBr).



29.38, N 1.82. The ultraviolet and visible light absorption spectra and the IR spectrum in KBr are shown in Figs. 1, 2.

Aclacinomycin B: m.p. 135~145°C under decomposition; $[\alpha]_D^{24}$ +3° (*c* 1.0, $CHCl_3$); anal. calcd. for $C_{42}H_{52}O_{15}N$: C 62.21, H 6.46, O 29.60, N 1.73; found: C 61.87, H 6.29, O 29.80, N 1.89. The ultraviolet and visible light absorption spectra and the IR spectrum in KBr are shown in Figs. 3, 4.

Fig. 3. Ultraviolet and visible light absorption spectra of aclacinomycin B.

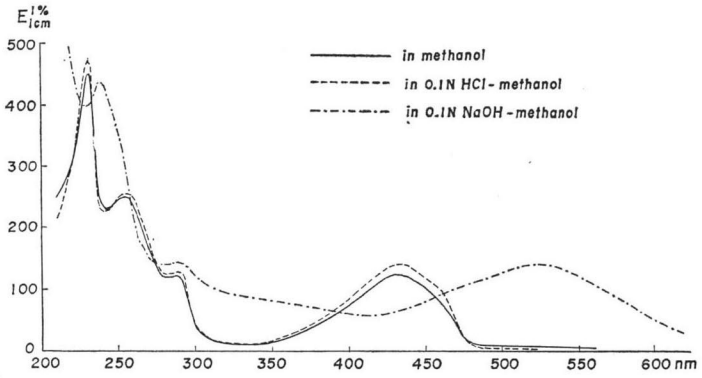
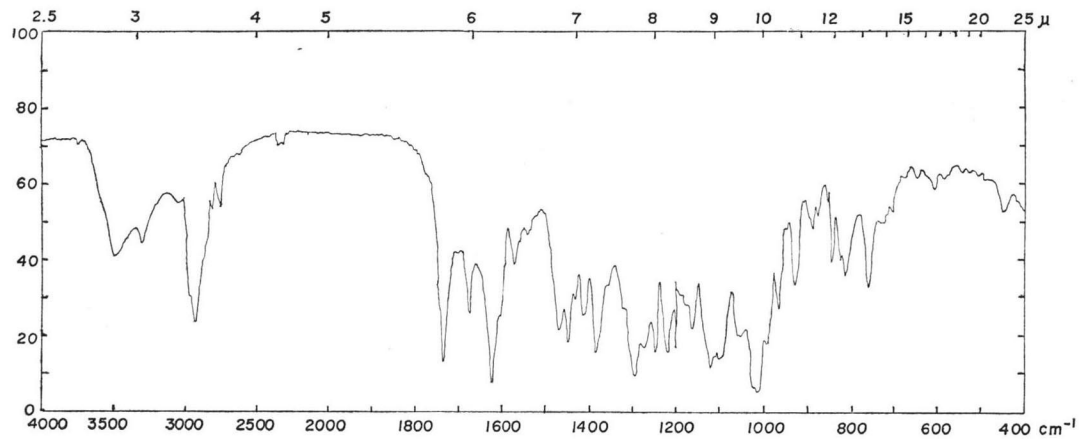
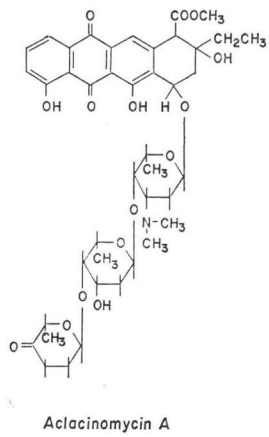


Fig. 4. Infrared absorption spectrum of aclacinomycin B (KBr).

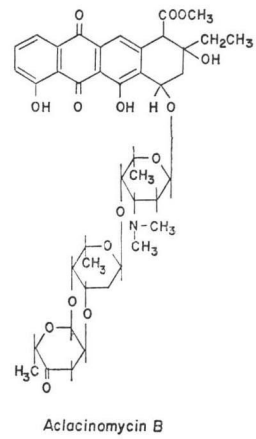


Aclacinomycins A and B are soluble in chloroform and ethyl acetate, and moderately soluble in methanol, ethanol, dioxane, benzene, acetone, acidic water and pyridine while sparingly soluble or insoluble in ethyl ether, *n*-hexane, cyclohexane and petroleum ether. The solution of aclacinomycins A and B in conc.HCl is yellow, that in conc.H_2SO_4 intensely reddish brown to reddish purple. Addition of alkali to aqueous solutions of the antibiotics gives an intense reddish purple color. Alcoholic magnesium acetate yields a purplish red color. On thin-layer chromatography using silica gel 60F-254 (E. Merck), the antibiotic gives a single spot at Rf 0.36 for A and 0.71 for B with chloroform - methanol (20 : 1), and at Rf 0.15 for A and 0.49 for B with acetone - *n*-hexane (1 : 1), respectively.

Acid hydrolysis of aclacinomycins A and B (25 mg each) with 2 ml of 0.3 N H_2SO_4 for 3 hours at 85°C yielded the aglycone, aklavinone, in the form of orange-yellow needles in good yield (16 mg and 14 mg): m.p. 171~174°C; anal. calcd. for $C_{22}H_{20}O_8$: C 64.08, H 4.86; found: C 64.19, H 5.11. *m/e* 412. The IR spectrum in KBr, the ultraviolet and visible light absorption spectra and the fragment ion peaks of the mass spectrum were identical with those of aklavinone.[1,2,3] Detection of sugars in the acid hydrolysates (0.3 N H_2SO_4, 85°C for 3 hours) on thin-layer chromatograms indicated that aclacinomycins A and B possess three sugars corresponding to rhodosamine, 2-deoxyfucose and cinerulose which were detected in the hydrolysate of an authentic cinerubin A. On partial hydrolysis of aclacinomycin A in methanol containing 5 % HCl for 2 hours at room temperature, the antibiotic gave the methylated disaccharide and 1-deoxypyrromycin: orange-yellow crystals $[\alpha]_D^{24}$ +216° (*c* 1.0, $CHCl_3$); anal. calcd. for $C_{30}H_{35}O_{11}N$: C 63.25, H 6.19, O 28.08, N 2.45; found: C 62.44, H 6.26, O 28.08, N 2.38. It solidified at 121~127°C and then melted at 230~235°C. The NMR and IR spectra and the ultraviolet and visible light absorption spectra showed that this compound is 1-deoxypyrromycin by the comparison with those of pyrromycin.[4,5] After neutralizing and evaporating the acid hydrolysate, the residue was extracted twice with chloroform and water. By evaporation and distillation of the aqueous layer, the methyl disaccharide of aclacinomycin A crystallized. The IR and NMR spectra and melting point of the sugar coincided with those of the methyl disaccharide obtained from cinerubin A.[5] On the other hand, when aclacinomycin B was hydrolyzed under the same condition, 1-deoxypyrromycin and a methylated disaccharide were obtained. This methyl disaccharide, which was crystallized from acetone-cyclohexane following the chloroform extraction of the acid hydrolysate and the silica gel column chromatography, was identified with the sugar moiety of cinerubin B[6] by the IR, NMR spectra and melting point. Thus, it was determined that aclacinomycins belong to the group of aklavinone glycosides including aklavin,[7] requinomycin[8] and galirubins,[9,10,11] and have the structures shown below.



Aclacinomycin A



Aclacinomycin B

Aklavin and requinomycin are different from aclacinomycin in the sugar moiety, molecular formula and optical rotation. The most similar to aclacinomycins are galirubins. ECKARDT[7] reported the isolation of galirubins and galirubinones from the mycelia of *S. galilaeus*: galirubin A (ε-pyrromycinone glycoside), galirubin B (aklavinone glycoside), galirubinone C (ζ-pyrromycinone), and galirubinone D (7-deoxyaklavinone). A sample of a galirubin mixture supplied by ECKARDT gave several spots on silica gel thin-layer chromatography using acetone or chloroform - methanol (20 : 1). The main component, an orange-red spot, was identified as cirerubin A[5] by co-

Table 1. Antimicrobial spectrum of aclacinomycin and 1-deoxypyrromycin

Organisms	Minimum inhibitory concentrations (mcg/ml)		
	Aclacinomycin A	Aclacinomycin B	1-Deoxypyrromycin
Bacillus subtilis ATCC 6633	<0.2	<0.2	1.25
Bacillus cereus ATCC 9634	<0.2	<0.2	1.25
Bacillus megaterium	0.63	<0.2	—
Staphylococcus aureus FDA 209P	0.63	<0.2	5.0
S. aureus Smith	<0.2	<0.2	2.5
Sarcina lutea ATCC 9341	<0.2	<0.2	1.25
Micrococcus flavus	<0.2	<0.2	1.25
Corynebacterium bovis 1810	<0.2	<0.2	0.63
Mycobacterium smegmatis ATCC 607	2.5	5	—
Streptococcus faecalis	2.5	2.5	—
Streptococcus pyogenes NY 5	1.25	1.25	—
Diplococcus pneumoniae Type 1	0.63	0.63	—
D. pneumoniae Type 2	0.63	0.63	—
Serratia marcescens A 20019	>100	>100	>100
Escherichia coli K 12	>100	>100	>100
Klebsiella pneumoniae ATCC 10031	>100	>100	>100
Pseudomonas aeruginosa A 20229	>100	>100	>100
Candida albicans IAM 4905	10	10	40
Candida tropicalis IAM 4942	20	20	40

Broth dilution method.

chromatography with an authentic cinerubin A. A faint yellow spot which was detected just above the orange-red spot in acetone showed the same Rf value as aclacinomycin A. However, it was not certain whether this minor component is galirubin B, since direct comparison with an authentic galirubin B was not possible and two or three other yellow spots were detected in the galirubin mixture. In respect of the sugar moiety, moreover, ECKARDT[11] reported the presence of two sugar spots in the acid hydrolysate of galirubin B by paper chromatography. Therefore, aclacinomycins A and B having three sugar moieties should be new members of the anthracyclic group.

Aclacinomycins exhibited inhibition against L 1210 leukemia in BDF_1 mice, and A was stronger. When 1.5 mg/kg/day of aclacinomycin A was injected intraperitoneally once daily for 10 days, the survival period was 300 % of the control. A slight decrease of body weight appeared in a dose of 4 mg/kg/day. Aclacinomycins inhibited the growth of cultured L 1210 cells (ID_{50} at 0.12 mcg/ml of A, and at 0.24 mcg/ml of B) and vaccinia virus in HeLa cells. Fifty per cent inhibition of RNA synthesis was caused at 0.1 mcg/ml of A and 0.2 mcg/ml of B and at 0.5 mcg/ml of 1-deoxypyrromycin. The LD_{50}'s of aclacinomycins A and B in mice were 22.6 mg/kg and 13.7 mg/kg by a single intraperitoneal injection, and 33.7 mg/kg and 16.4 mg/kg by a single intravenous injection, respectively. In addition, ECG change in hamster produced by adriamycin at 3 mg/kg, single i.p., was not exhibited by aclacinomycin A at 50 mg/kg, single i.p. The antimicrobial spectra of the antibiotics tested by the broth dilution method are shown in Table 1.

Acknowledgement

The authors are grateful to Dr. J. GELZER, CIBA-GEIGY Ltd., for a supply of cinerubin A, and to Dr. K. ECKARDT, Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Akademie der Wissenshaften der DDR., for a supply of galirubin mixture.

Toshikazu Oki
Yasue Matsuzawa
Akihiro Yoshimoto
Kenji Numata
Iwao Kitamura
Senji Hori
Akira Takamatsu

Central Research Laboratory of Sanraku-Ocean Co., Ltd., Jonan, Fujisawa, Kanagawa, Japan

Hamao Umezawa
Masaaki Ishizuka
Hiroshi Naganawa
Hiroyuki Suda
Masa Hamada
Tomio Takeuchi

Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo, Japan

(Received June 4, 1975)

References

1) Gordon, J. J.: Aklavinone. Tetrahedron Lett. 1960-8: 28～34, 1960
2) Brockmann, Jr., H.; H. Budzikiewicz, C. Djerssi, H. Brockmann & J. Niemeyer: Das massenspektroskopische Fragmentierungsverhalten der Anthracyclinone. Chem. Ber. 98: 1260～1269, 1965
3) Eckardt, K.; D. Tresselt & J. Tax: Antibiotika aus Actinomyceten. Isolierung und Konstitution von Aklavinon-I und Aklavinon-II, zwei neuen Anthracyclinonen aus Fermentation-sansätzen von *Streptomyces galilaeus*. Tetrahedron 30: 3787～3791, 1974.
4) Brockmann, H. & W. Lenk: Actinomycetenfarbstoffe. VII. Pyrromycin. Chem. Ber. 92: 1904～1909, 1959
5) Keller-Schierlein, W. & W. Richle: Metabolic products of microorganisms. LXXXVI. Structure of cinerubine A. Antimicr. Agents & Chemoth. -1970: 68～70, 1971.
6) Richle, W.; E. K. Winkler, D. M. Hawley, M. Dobler & W. Keller-Schierlein: Stoffwechselprodukte von Mikroorganismen. 49. Die Struktur des Cinerubins B. Helv. Chim. Acta 55: 467～480, 1972
7) Strelitz, F.; H. Flon, U. Weiss & I. N. Ashesov: Aklavin, an antibiotic substance with antiphage activity. J. Bact. 72: 90～94, 1956
8) Hori, M.; T. Takeuchi, S. Kondo, M. Hamada, T. Okazaki, Y. Okami & H. Umezawa: Requinomycin, an inhibitor of R-factor transfer: Isolation, characterization and properties. J. Antibiotics 25: 393～399, 1972
9) Eckardt, K. & G. Bradler: Zur Kenntnis einiger roter Hydroxychinonfarbstoffe aus einem *Streptomyces*-Stamm: Galirubinonen und Galirubin. Naturwiss. 52: 539～540, 1965
10) Bradler, G.; K. Eckardt & R. Fügner: Zur Charakterisierung des *Streptomyces*-Stammes JA 3043 und Gewinnung von Galirubin und Galirubinonen. Zeit. Allg. Mikrobiol. 5: 361～365, 1966
11) Eckardt, K.: Rote Antibiotica aus Actinomyceten. Zur Identifizierung einer Reihe von Anthracyclinonpigmenten aus *Streptomyces galilaeus* Stamm JA 3043 (Galirubine und Galirubinone). Chem. Ber. 100: 2561～2568, 1967