

ANTITUMOR ANTHRACYCLINE ANTIBIOTICS, ACLACINOMYCIN A AND ANALOGUES

I. TAXONOMY, PRODUCTION, ISOLATION AND PHYSICOCHEMICAL PROPERTIES

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Aclacinomycin A and B, two major components of a new antitumor antibiotic complex, and their 19 analogues were produced by a culture of strain No. MA144-M1, which was identified as *Streptomyces galilaeus*. They were isolated by chelation with copper ion and silicic acid chromatography, and characterized by physicochemical methods in the anthracycline group of antibiotics.

During the course of screening for new antitumor antibiotics, the cultured broth of an organism MA144-M1, which was isolated from a soil sample collected in Kamiosaki, Shinagawa-ku, Tokyo, Japan, showed marked inhibitory effect on L1210 leukemia in mice. The organism produced a new water-insoluble pigment antibiotic complex named aclacinomycin, which was extracted from the cultured broth and separated into thirteen yellow and seven red-colored components. They belong to the aklavinone and ϵ -pyrromycinone group of anthracyclines, and aclacinomycins A and B¹⁾ were produced preferentially. The results of structural studies²⁻⁴⁾ indicated that twelve components; A1 (aclacinomycin A), B1 (aclacinomycin B), Y1 (aclacinomycin Y), L1 (N-monodemethyl aclacinomycin A), G1 (MA144 G1), M1 (MA144 M1), N1 (MA144 N1), S1 (MA144 S1), T1 (1-deoxypyrrromycin), E1 (7,7'-dideoxy-7,7'-biaklavinone), M2 (MA144 M2), and S2 (MA144 S2) were confirmed to be novel compounds. Three yellow components, C1, D1 and F1, and five red-colored components, A2, B2, T2, C2 and D2 were identical with 7-deoxyaklavinone⁵⁾, aklavinone⁵⁻⁷⁾, bisanhydroaklavinone⁷⁾, cinerubin A⁹⁾, cinerubin B⁹⁾, pyrromycin¹⁰⁾, 7-deoxypyrrromycinone (ζ -pyrromycinone)⁵⁾, and ϵ -pyrromycinone¹⁰⁾, respectively.

This paper deals with the taxonomy of the producing organism, the production, isolation and physicochemical properties of aclacinomycin A and its analogues.

Taxonomy

(1) The Strain MA144-M1 has the following morphological characteristics: Open spirals were observed to develop well from branched substrate mycelia. There were no whorls, and mature spore

chain was moderately long with more than ten spores. The spores were ellipsoidal and measured $0.4 \sim 0.8 \mu \times 0.8 \sim 1.6 \mu$, and with a smooth surface.

(2) Properties on various media. The description in parenthesis follows the color standard "Color Harmony Manual" published by Container Corporation of America, U.S.A.

(a) On glucose-asparagine agar, incubated at 27°C, pale yellowish brown (3 gc, Lt Tan) to dull yellow green (24 lg, Moss Green) growth; no aerial mycelium; no soluble pigment.

(b) On sucrose-nitrate agar, incubated at 27°C, colorless to pale yellowish brown growth (3 gc, Lt Tan); no aerial mycelium; no soluble pigment.

(c) On glycerol-asparagine agar (ISP medium No. 5), incubated at 27°C, yellowish orange (4 ic, Suntan) to brown (5 lg, Cocoa Brown) growth; white to light gray (2 fe, Covert Gray) aerial mycelium; brownish soluble pigment.

(d) On inorganic salts-starch agar (ISP medium No. 4), incubated at 27°C, pale orange (3 ea, Lt Melon Yellow) to pale yellowish brown (3 ie, Camel) growth; light gray (2 fe, Covert Gray) to gray (e, Gray) aerial mycelium; brownish soluble pigment.

(e) On tyrosine agar (ISP medium No. 7), incubated at 27°C, brownish gray (3 li, Beaver) to brown (4 lg, Toast Tan) growth; scant, white aerial mycelium; black soluble pigment.

(f) On nutrient agar, incubated at 27°C, colorless to grayish brown growth; no aerial mycelium; brown soluble pigment.

(g) On yeast extract-malt extract agar (ISP medium No. 2), incubated at 27°C. Light brown (4 le, Maple) to brown (4 ng, Lt Brown) growth; light gray (3 fe, Silver Gray to 3 ih, Beige Gray) aerial mycelium; brown soluble pigment.

(h) On oatmeal agar (ISP medium No. 3), incubated at 27°C, colorless to pale yellowish brown (2 gc, Bamboo) growth; light gray (3 fe, Silver Gray) aerial mycelium; brownish soluble pigment.

(i) On glycerol-nitrate agar, incubated at 27°C. Colorless to pale yellowish brown (3 gc, Lt Tan) or light olive gray (1 ½ db, Parchment) growth; no aerial mycelium; no soluble pigment.

(j) On starch agar, incubated at 27°C, pale yellowish brown (3 gc, Lt Tan) growth; gray (e, Gray) aerial mycelium; slight brown soluble pigment.

(k) On calcium malate agar, incubated at 27°C, colorless growth; grayish white (b, Oyster White) to light brownish gray (3 dc, Natural) aerial mycelium; no soluble pigment.

(l) On gelatin stab, incubated at 20°C, pale brown to pale yellowish brown growth; white aerial mycelium; brown soluble pigment.

(m) On glucose-peptone-gelatin stab, incubated at 27°C, pale brown to brown growth; no aerial mycelium; brown soluble pigment.

(n) On skimmed milk, incubated at 37°C, pale brown to brown growth; no aerial mycelium; brown soluble pigment.

(3) Physiological properties:

(a) The effect of temperature on growth was examined on maltose-yeast extract agar (maltose 1.0%, yeast extract 0.4%, agar 3.5%, pH 6.0) at 20, 24, 27, 30, 37 and 50°C. Optimal temperature for the growth was 27~37°C with no growth at 50°C.

(b) Gelatin liquefaction on 15% gelatin stab, incubated at 20°C; and on glucose-peptone-gelatin stab, incubated at 27°C; on the former medium, gelatin liquefaction was observed weakly on the 14-day incubation, but on the latter weak or moderate liquefaction after 7-day incubation.

(c) Starch hydrolysis on inorganic salts-starch agar, incubated at 27°C; weak hydrolysis was observed after 5-day incubation.

(d) Peptonization and coagulation of skimmed milk, incubated at 37°C; moderate or strong peptonization began after 5-day incubation and finished on around 17 days. No coagulation.

(e) Melanin formation on tyrosine agar (ISP medium No. 7), tryptone-yeast extract broth (ISP medium No. 1) and peptone-yeast extract-iron agar (ISP medium No. 6), incubated at 27°C; positive on all media.

(f) Liquefaction of calcium malate on calcium malate agar, incubated at 27°C; strongly positive.

Fermentation

A well-grown agar slant of *Streptomyces galilaeus* MA144-M1 was used to inoculate vegetative medium containing 15 g "Prorich" (soybean meal, Ajinomoto Co.), 10 g glucose, 10 g potato starch, 1 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, 3 g NaCl, 0.008 g $MnCl_2 \cdot 4H_2O$, 0.002 g $ZnSO_4 \cdot 5H_2O$, 0.07 g $CuSO_4 \cdot 5H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$ in 1 liter distilled water. The seed culture was incubated at 28°C for 48 hours on a rotary shaker (230 r.p.m.), and the vegetative culture was transferred to a 20-liter stir jar fermentor containing 10 liters of production medium composed of 25 g "meat" (soybean meal, Ajinomoto Co.), 30 g glucose, 15 g potato starch, 1 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, 3 g NaCl, 0.008 g $MnCl_2 \cdot 4H_2O$, 0.002 g $ZnSO_4 \cdot 5H_2O$, 0.07 g $CuSO_4 \cdot 5H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$ in 1 liter tap water, the pH being adjusted to 7.2 before sterilization. The fermentation was carried out at 28°C at 325 r.p.m. with an aeration rate of 5 liters/minute. The cultured broth at 36 hours exhibited the maximum accumulation of 35 mcg/ml and 15 mcg/ml of aclinomycins A and B, respectively. A typical time course of fermentation in a 20-liter jar fermentor is shown in Fig. 1.

The amount of aclinomycin A and its analogues in the cultured broth was determined by spectrophotometry after separating the components on silica gel thin-layer plate as described previously¹⁾, and by paper disc agar-diffusion assay using *Bacillus subtilis* ATCC 6633 as test organism.

A mutant strain No. 6UV-21 which was obtained by ultraviolet treatment of strain MA144-M1 pro-

Fig. 1. Time course of fermentation of aclinomycins by *Streptomyces galilaeus* MA144-M1.

Cultivation was carried out at 28°C in 20-liter stir jar fermentor (325 r.p.m., aeration, 5 liters/min). Medium is shown in the text.

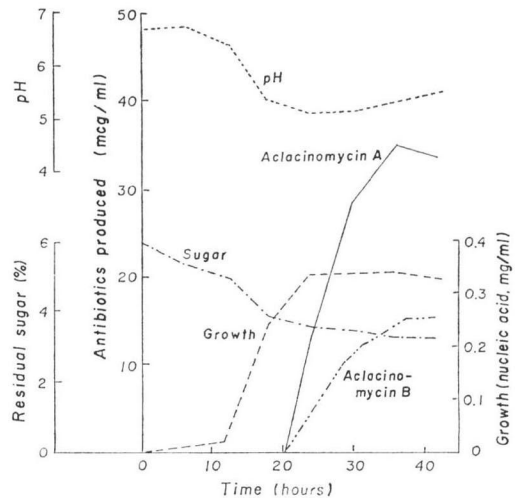
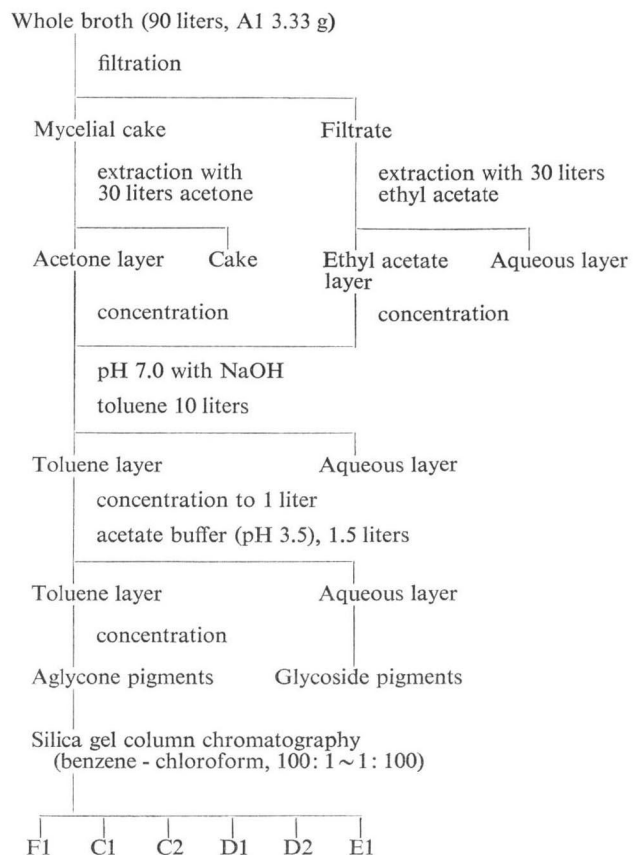


Fig. 2. Isolation and purification of aclinomycins.



duced only yellow pigments and showed improved productivity over the original strain.

Isolation of the Antibiotics

Aclacinomycin A and its analogues were purified to crystals or microcrystalline powders by the procedures illustrated in Figs. 2 and 3. The harvested broth was filtered with filter aid and the pigments were extracted separately from the mycelium with acetone and from the filtrate with ethyl acetate. After evaporating the solvents, the extracts were combined and the pigments were re-extracted with toluene. Then, the glycosidic components having weakly basic properties were separated from the aglycones by transferring to pH 3.5 acetate buffer. The aglycone-type pigments remaining in the toluene layer were concentrated to dryness and subjected to silicic acid column chromatography. By gradient elution with a chloroform-benzene mixture with mixing ratio of 1:100~100:1, the components F1, C1, C2, D1, D2 and E1 were successively eluted. The components E1 and D1 were crystallized as yellow needles from chloroform and ethyl acetate, and the components F1, C1 and C2 were crystallized from benzene as orange, yellow and red needles, respectively.

On the other hand, the purification of glycosidic components was fairly troublesome, because there were yellow and red pigments with the same sugar moiety and similar chromatographic behavior, for example aclacinomycin A and cinerubin A. It was very difficult to separate yellow pigments from red ones. Among several methods tested, the best one was to use their chelating properties. That is, red pigments were found to form a precipitate of chelate complex with metals such as copper, iron, aluminium *etc.* in an aqueous solution, and chelating activity was stronger for red pigments than for yellow ones. As shown in Fig. 3, cupric sulfate solution was added to the acetate buffer layer containing red and yellow pigments at pH 5.0, and the resulted precipitate of the red pigment-copper complex was

Fig. 3. Purification of glycosidic components.

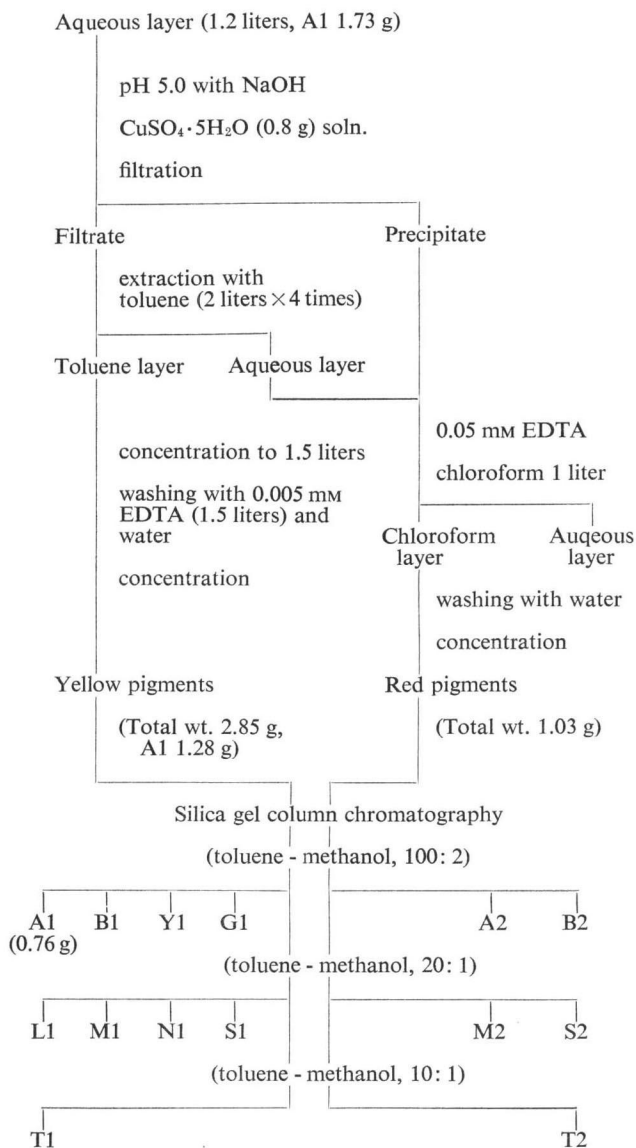


Table 2. Physicochemical properties of aclacinomycins.

Compound designation	Molecular formula	Elemental analysis						
		Calculated				Found		
		C	H	N	O	C	H	N
A1 Aclacinomycin A	C ₄₂ H ₅₃ NO ₁₅	62.14	6.58	1.73	29.56	62.37	6.67	1.82
B1 Aclacinomycin B	C ₄₂ H ₅₁ NO ₁₅	62.29	6.35	1.73	29.63	61.87	6.29	1.89
G1 MA144 G1	C ₄₂ H ₅₃ NO ₁₅	62.14	6.58	1.73	29.56	61.45	6.31	1.24
M1 MA144 M1	C ₄₂ H ₅₅ NO ₁₅	61.98	6.81	1.72	29.49	62.37	7.08	2.07
N1 MA144 N1	C ₄₂ H ₅₅ NO ₁₅	61.98	6.81	1.72	29.49	61.43	6.71	1.71
S1 MA144 S1	C ₃₆ H ₄₅ NO ₁₃	61.79	6.84	2.00	29.72	61.29	6.42	2.05
T1 1-Deoxypyrrromycin	C ₃₀ H ₃₅ NO ₁₀	63.25	6.19	2.45	28.08	62.44	6.26	2.38
Y1 Aclacinomycin Y	C ₄₂ H ₅₁ NO ₁₅	62.29	6.35	1.73	29.63	61.98	6.30	1.70
L1 N-Monodemethyl-aclacinomycin A	C ₄₁ H ₅₁ NO ₁₅	61.72	6.44	1.76	30.08	60.88	6.31	1.54
E1 MA144 E1	C ₄₄ H ₃₈ O ₁₄	66.83	4.84	—	28.32	66.12	4.90	—
M2 1-Hydroxy-MA144 M1	C ₄₂ H ₅₅ NO ₁₆	60.79	6.68	1.69	30.85	60.43	6.74	1.75
S2 1-Hydroxy-MA144 S1	C ₃₆ H ₄₅ NO ₁₄	60.41	6.34	1.96	31.29	60.36	6.41	1.91

Table 3. UV and IR absorption spectra of aclacinomycins.

Compound		λ_{mrx} (nm) ($E_{1cm}^{1\%}$)
A1	90% MeOH	229.5(550), 259(326), 289.5(135), 431(161)
	0.1 N HCl	229.5(571), 258.5(338), 290(130), 431(161)
	0.1 N NaOH	239(450), 287(113), 523(127)
B1	90% MeOH	229.5(498), 259(313), 289.5(137), 432(159)
	0.1 N HCl	229(586), 259(355), 289.5(156), 431(162)
	0.1 N NaOH	239(510), 284(160), 315s(101), 522(145)
G1	90% MeOH	230(537), 259(330), 290(140), 432(165)
	0.1 N HCl	229.5(610), 259(372), 289(159), 430(169)
	0.1 N NaOH	239(521), 285(151), 317(96), 522(144)
M1	90% MeOH	230(515), 258.5(310), 290(125), 433(146)
	0.1 N HCl	230(500), 259(309), 290(122), 433(158)
	0.1 N NaOH	238.5(457), 285(121), 318s(77), 525(133)
N1	90% MeOH	229.5(482), 259(298), 290(121), 433(144)
	0.1 N HCl	229.5(488), 259(304), 290(123), 433(151)
	0.1 N NaOH	239(450), 287(121), 318s(76), 525(133)
S1	90% MeOH	230(638), 258.5(371), 289.5(160), 432(177)
	0.1 N HCl	229.5(652), 258.5(380), 289.5(163), 431(192)
	0.1 N NaOH	237.5(553), 286(141), 320(90), 524(161)
T1	90% MeOH	229.5(666), 258(395), 290(153), 432(188)
	0.1 N HCl	230(735), 259.5(465), 290(197), 433(201)
	0.1 N NaOH	238(951), 254s(690), 285s(400), 522(252)
Y1	90% MeOH	229.5(580), 259(320), 290(126), 432(158)
	0.1 N HCl	229.5(590), 259(334), 290.5(130), 433(160)
	0.1 N NaOH	239(497), 287(133), 320s(82), 524(138)
L1	90% MeOH	230(480), 259(298), 290(118), 433(151)
	0.1 N HCl	230(530), 259(324), 290(128), 433(156)
	0.1 N NaOH	238(412), 287(100), 318s(68), 525(140)
E1	90% MeOH	232(576), 262.5(507), 292s(179), 439(252)
	0.1 N HCl	232(712), 262(634), 292s(220), 438(310)
	0.1 N NaOH	241.5(683), 250s(664), 290s(220), 525(230)
M2	90% MeOH	235(600), 259(310), 269(170), 291(105), 492(165)
	0.1 N HCl	235(615), 259(325), 269(185), 291(115), 492(170)
	0.1 N NaOH	237(505), 269(145), 292(95), 330(55), 554(175), 597(145)
S2	90% MeOH	234.5(607), 258.5(306), 293(110), 491(189)
	0.1 N HCl	234.5(629), 258.5(318), 293(114), 491(197)
	0.1 N NaOH	242(606), 566(244), 606(210)

O	Melting point (°C)	$[\alpha]_D^{25}$
29.83	151~153	-11.5° (c 1.0, CHCl ₃)
28.80	163~167	+ 3° (c 1.0, CHCl ₃)
28.93	141~145	+46° (c 0.5, CHCl ₃)
28.81	149~150	+36.7° (c 1.0, CHCl ₃)
	146~147	+38.6° (c 1.0, CHCl ₃)
	145~146	+90.8° (c 1.0, CHCl ₃)
28.08	121~125	+217° (c 1.0, CHCl ₃)
	153~155	+66° (c 1.0, CHCl ₃)
30.13	134~136	- 5.2° (c 1.0, CHCl ₃)
	266~272	
29.70	151~152	+120° (c 1.0, CHCl ₃)
	154~158	

ν_{\max} (cm ⁻¹)
3450, 2980, 2945, 2820, 2760, 1735, 1675, 1620, 1600s, 1010
3450, 2980, 2945, 2820, 2760, 1730, 1675, 1620, 1600s, 1010
3450, 2975, 2945, 2820, 2760, 1735, 1675, 1620, 1600, 1010,
3440, 2975, 2945, 2830, 2760, 1740, 1680, 1625, 1600, 1010, 1000
3420, 2975, 2945, 2820, 2750, 1735, 1675, 1620, 1600, 1000
3450, 2970, 2945, 2820, 2755, 1735, 1675, 1620, 1600, 1010
3475, 2970, 2940, 2820, 2770, 1735, 1675, 1620, 1600, 1010
3475, 2970, 2930, 2800, 2750, 1730, 1700, 1672, 1620, 1600, 1005
3475, 2980, 2945, 2800, 1740, 1735, 1680, 1625, 1610, 1010
3550, 2960, 2880, 1745, 1680, 1625, 1600
3430, 2975, 2950, 2820, 2770, 1740, 1620s, 1600, 1010, 1000
3470, 2970, 2940, 2820, 2755, 1740, 1640, 1610s, 1600, 1005

separated by filtration. The yellow pigments in the solution were extracted into toluene, washed with EDTA solution and precipitated by addition of *n*-hexane. The red pigments precipitated as a copper complex were shaken with EDTA solution at pH 3.0 to break the chelation, and extracted into chloroform. The chloroform extract was washed with water, concentrated under reduced pressure, and poured into *n*-hexane to precipitate the red pigments. The mixture of yellow pigments obtained was fractionated by silicic acid column chromatography with the toluene-methanol mixture. Each component was further purified by preparative silica gel thin-layer chromatography. As shown in Fig. 3, the yellow pigments consisted of nine glycosidic components; A1, B1, Y1, G1, L1, M1, N1, S1 and T1. In spite of intense effort, none of the yellow glycosidic components could be crystallized, and they were precipitated as free base form by the addition of *n*-hexane. The red pigments were also purified by the same procedure as the yellow pigments, and five components; A2, B2, M2, S2 and T2 were obtained. As reported previously²³, components A2, B2 and T2 were found to be identical with cinerubins A and B and pyrromycin, respectively. The novel components M2 and S2 crystallized as red needles from ethyl acetate - *n*-hexane, and as red platelets from chloroform - *n*-hexane.

Physicochemical Properties

As reported previously²³ and in a companion paper⁴³, the aglycone-type components C1, D1, F1, C2 and D2 were identified by NMR and mass spectroscopic analyses as 7-deoxyaklavinone, aklavinone, bis-anhydroaklavinone, 7-deoxypyrromycinone (ζ -pyrromycinone) and ϵ -pyrromycinone, respectively. MA144 E1 crystallized from chloroform was as yellow needles, insoluble or sparingly soluble in most of organic solvents. The glycosidic components were gen-

erally soluble in methanol, ethanol, chloroform, ethyl acetate, acetone, benzene, dimethyl sulfoxide, methyl cellosolve and acidic water, but were sparingly soluble or insoluble in water, ethyl ether, *n*-hexane, cyclohexane, and petroleum ether. These glycosidic components gave negative ninhydrin and FEHLING reactions. Elementary analyses, empirical formulae, melting points and $[\alpha]_D$ of these components are summarized in Table 2. The specific rotation of aclacinomycin A is revised to -11.5° from $+29^\circ$. The value in our preliminary report¹⁾ was affected by a small contamination with the metal chelate complex and analogues such as 1-deoxypyrrromycin and aclacinomycin Y. The yellow glycosidic components in dilute hydrochloric acid and neutral solutions gave a reddish brown color in concentrated sulfuric acid and turned intense reddish purple upon addition of alkali. On the other

Fig. 4. PMR spectrum of aclacinomycin A (100 MHz, CDCl_3).

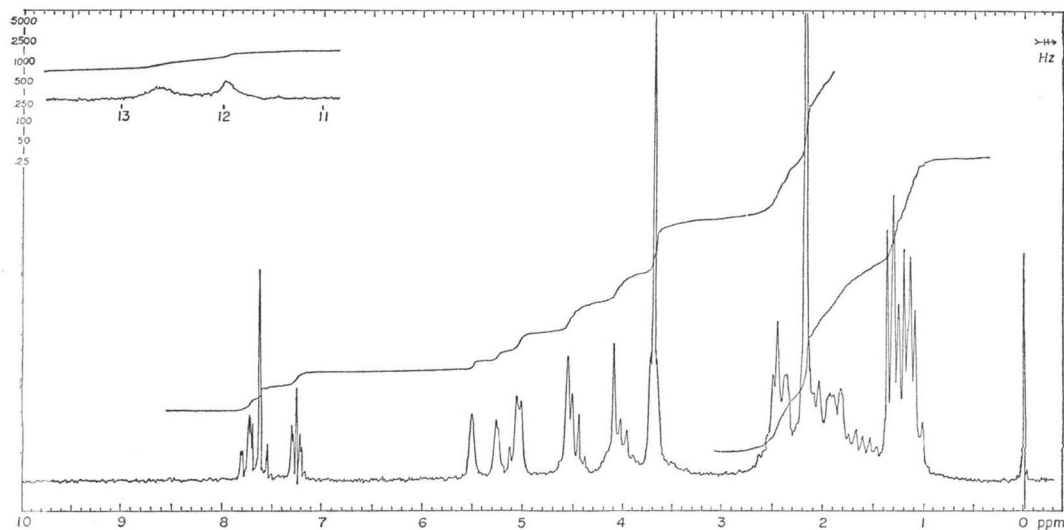


Fig. 5. PMR spectrum of MA144 M1 (100 MHz, CDCl_3).

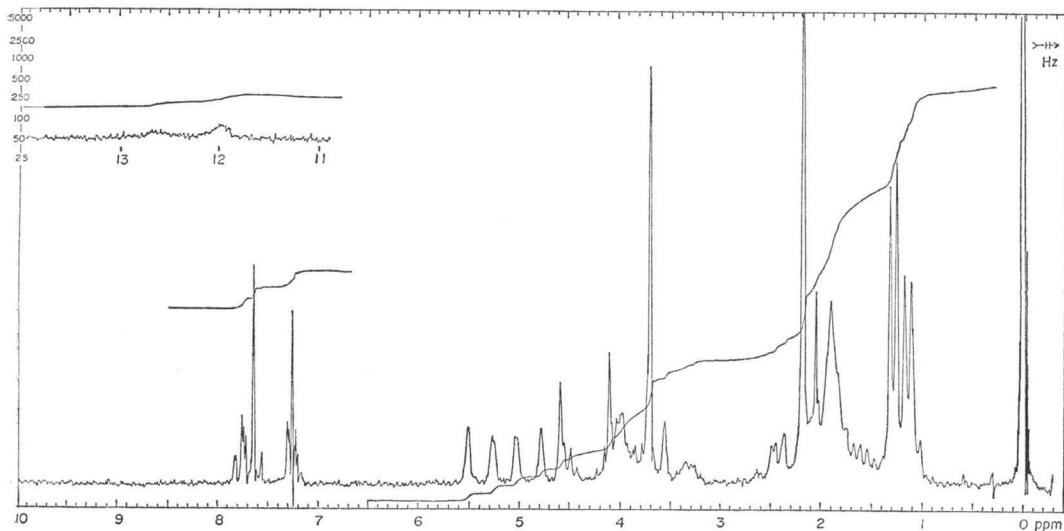
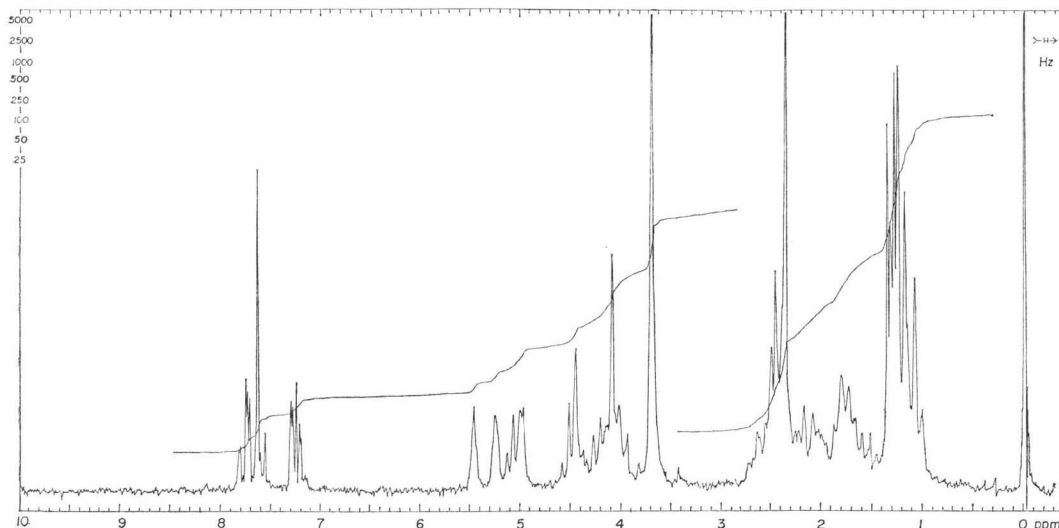


Fig. 6. PMR spectrum of MA144 L1 (100 MHz, CDCl_3).

hand, the red glycosidic components in dilute hydrochloric acid solution turned to purple and blue violet by the addition of sulfuric acid and alkali, respectively. The ultraviolet and visible light absorption spectra of these glycosidic components showed maxima in neutral and acidic solutions around 230, 259, 290 and 432 nm for the yellow components and 230, 258, 292 and 492 nm for the red ones, as shown in Table 3, and the absorption maxima at 432 and 492 nm in neutral and acidic solutions are shifted to 523 and around 600 nm, respectively in alkaline solution. The infrared absorption spectra (KBr) of the yellow components indicated the presence of ester carbonyl (1740 cm^{-1}), non-chelated carbonyl (1670 cm^{-1}) and chelated carbonyl (1620 cm^{-1}), as shown in Table 3. From these results, the anthracyclonones aklavinone, 7-deoxyaklavinone and ϵ -pyrromycinone, were demonstrated in the culture broth and acid hydrolyzate, and thus, it is deduced that the yellow glycosidic components are aklavinone glycosides, and the red ones are ϵ -pyrromycinone glycosides because of the absence of non-chelated carbonyl in the IR spectra. Representative PMR spectra of aclacinomycin A, MA144 M1 and MA144 L1 determined with a Varian XL-100 spectrometer operating at 100 MHz in CDCl_3 using tetramethylsilane (TMS) as the internal reference are shown in Figs. 4~6. The characteristic chemical shift, around δ 2.2, in the PMR spectra of yellow and red glycosides shows the dimethyl or monomethyl amino group, suggesting the presence of an amino sugar moiety in their structure. Further structural elucidation will be reported in the accompanying paper.

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