PRODUCTION OF NINETEEN ANTHRACYCLIC COMPOUNDS BY *STREPTOMYCES GALILAEUS* MA144-M1

Sir:

As reported in a previous paper,[1] aclacinomycins A and B have been isolated from fermentation broths of strain MA144-M1 (ATCC 31133) which has been classified as *Streptomyces galilaeus*. In a study of the yellow and red-colored pigments produced by this strain, we found 17 other anthracyclic compounds and elucidated their structures. The isolation and structures of these compounds are reported in this paper.

Streptomyces galilaeus MA144-M1 was cultivated at 28°C for 2 days on a rotary shaker or in a jar fermentor with aeration and agitation in a medium consisting of 1% glucose, 1.5% soluble starch, 3% soybean meal, 0.1% K_2HPO_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. Pigments in the culture medium were extracted with toluene at pH 7.5, separated by silicic acid thin-layer chromatography using chloroform - methanol (20 : 1 in volume) and detected with a Shimazu dual wave length TLC scanner. The antibacterial activity of culture filtrates was determined by a disc diffusion method using *B. subtilis* ATCC 6633 as the test organism.

The broth was adjusted to pH 4.0 and filtered to separate the mycelium. Pigments in the filtrate were extracted with toluene after adjustment of the pH to 7.0, whereas those in the mycelial cake were extracted with acetone first, dried and then re-extracted with toluene. Toluene extracts were combined and concentrated to a small volume which was extracted with 0.02 M acetate buffer at pH 3.0. Some of the pigments remained in the toluene layer; others were extracted into the aqueous layer. The organic layer was concentrated to dryness, dissolved in a small amount of benzene and subjected to silicic acid column chromatography. The pigments F, C1, C2, D1, D2 and E1 were successively eluted with a gradient of chloroform in benzene from 1/100 to 100/1.

The acidic buffer layer described above was adjusted to pH 5.0. The red pigments were precipitated as their copper complex by addition of cupric sulfate and separated by filtration. After removal of the red pigments, the yellow pigments in solution were extracted into ethyl acetate, which was then washed with EDTA solution and concentrated under reduced pressure. The yellow pigments were precipitated by addition of *n*-hexane.

The mixture of yellow pigments was dissolved in a small amount of toluene and subjected to silicic acid column chromatography. The first fraction, containing A1 and B1, was eluted with the 100: 2 mixture of toluene - methanol, the second fraction, containing L1, G1, M1, S1 and N1 was eluted with the 100 : 5 solvent mixture whereas the third fraction, containing T1, was eluted with the 100 : 10 mixture.

The red pigments (precipitated as the copper complex) were extracted with chloroform which was then shaken vigorously with EDTA solution at pH 3.0 to break the chelation. The chloroform extract was concentrated under reduced pressure and the concentrate was poured into *n*-hexane to precipitate the red pigments. The mixture of the red pigments thus obtained was fractionated by the same procedure (silicic acid chromatography with toluene - methanol mixtures) as described for the yellow pigments.

Each of the yellow or red pigments in the fractions described above was further purified

Table 1. Some properties of pigments produced by *Streptomyces galilaeus* MA144-M1

Components	Color	Melting point (°C)	Rf value*
A1	Yellow	144~146.5	0.50
B1	〃	163~167	0.77
L1	〃	134~136	0.31
G1	〃	146~148	0.38
M1	〃	149~150	0.25
N1	〃	146~147	0.21
S1	〃	144~147	0.14
T1	〃	121~125	0.03
F	〃	233~234	0.95
D1	〃	167~171	0.86
C1	〃	229~231	0.90
E1	〃	266~272	0.82
A2	Red	126~134	0.50
B2	〃	145~152	0.77
M2	〃	151~152	0.25
S2	〃	154~158	0.14
T2	〃	160~162	0.03
C2	〃	214~219	0.90
D2	〃	225~232	0.86

* Rf value: Thin-layer chromatography on silica gel using chloroform-methanol (20: 1, v/v)

Table 2. Proposed structures of aclacinomycin analogues

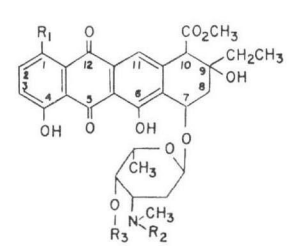
Components	Products by Hydrolysis*1 Aglycones	Products by Hydrolysis*1 Sugars	Products by Methanolysis*2	Designation	Structure*4 R_1	Structure*4 R_2	Structure*4 R_3
A1	Aklavinone	Ra, dF, Cin	Dp, methyl-dF-Cin A	Aclacinomycin A	H	CH_3	dF-L-Cin
B1	〃	〃 〃 〃	〃, methyl-dF-Cin B	Aclacinomycin B	〃	〃	dF=L-Cin
L1	〃	mRa 〃 〃	mDp, methyl-dF-Cin A	N-Monodemethylaclacinomycin A	〃	H	dF-L-Cin
G1	〃	Ra 〃 D-Cin	Dp, methyl-dF-D-Cin A	MA144 G1	〃	CH_3	dF-D-Cin
M1	〃	〃 〃 Ami	〃, methyl-dF-Ami	MA144 M1	〃	〃	dF-Ami
N1	〃	〃 〃 Rho	Dp*3,	MA144 N1	〃	〃	dF-Rho
S1	〃	〃 〃		MA144 S1	〃	〃	dF
T1	〃	〃		1-Deoxypyrromycin	〃	〃	H
F	Bisanhydroaklavinone				〃		
D1	Aklavinone				〃		
C1	7-Deoxyaklavinone				〃		
E1	MA144 E1				〃		
A2	ε-Pyrromycinone	Ra, dF, Cin	Py, methyl-dF-Cin A	1-Hydroxyaclacinomycin A (Cinerubin A)	OH	CH_3	dF-L-Cin
B2	〃	〃 〃 〃	Py, methyl-dF-Cin B	1-Hydroxyaclacinomycin B (Cinerubin B)	〃	〃	dF=L-Cin
M2	〃	〃 〃 Ami	Py, methyl-dF-Ami	1-Hydroxy-MA144 M1	〃	〃	dF-Ami
S2	〃	〃 〃		1-Hydroxy-MA144 S1	〃	〃	dF
T2	〃	〃		Pyrromycin	〃	〃	H
C2	7-Deoxypyrromycinone (ζ-Pyrromycinone)				〃		
D2	ε-Pyrromycinone				〃		

*1: 0.1 N HCl for 30~60 minutes at 85~110°C

*2: Methanol containing 0.01~0.04 N HCl, for 30~60 minutes at room temperature. Dp: 1-Deoxypyrromycin, mDp: N-Monodemethyl 1-deoxypyrromycin, Py: Pyrromycin.

*3: Hydrolysis of N1 in 0.5% HCl for 10 minutes at room temperature gave S1 and Rho.

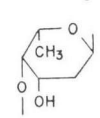
*4:



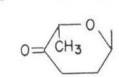
Ra (L-Rhodosamine, $R_2 = CH_3$)

mRa (N-Monodemethylrhodosamine, $R_2 = H$)

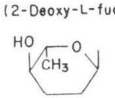
R_3 :



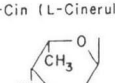
dF (2-Deoxy-L-fucose)



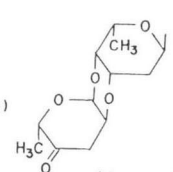
L-Cin (L-Cinerulose)



Ami (L-Amicetose)

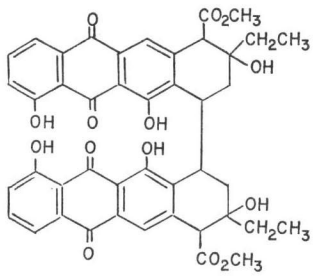


Rho (L-Rhodinose)



dF = L-Cin

Fig. 1. The structure of MA144 E1.



by preparative thin-layer chromatography using chloroform - methanol mixtures (30 : 1 to 5 : 1). Thus, 12 yellow and 7 red pigments were obtained in a pure form. Their melting points and Rf values (silicic acid thin-layer chromatography Kieselgel $60F_{254}$, Merck Co., developed with chloroform - methanol 20 : 1) are shown in Table 1.

On acid hydrolysis in 0.1 N HCl for 30~60 minutes at 85°~110°C, pigments A1, B1, L1, G1, M1, N1, S1 and T1 yielded aklavinone (yellow-colored)[2,3,4], and A2, B2, M2, S2 and T2 gave ε-pyrromycinone (red-colored),[5] the yellow pigments F, D1, C1 and E1 and the red pigments, C2 and D2, were resistant to acid hydrolysis. Pigments C1, D1, F, C2 and D2 were confirmed to be identical with 7-deoxyaklavinone,[3] aklavinone,[2,3,4] bisanhydroaklavinone,[4] 7-deoxypyrromycinone (ζ-pyrromycinone)[3] and ε-pyrromycinone,[5] respectively. MA144 E1 (in Table 2) which was resistant to acid hydrolysis showed M^+ 396, but the molecular weight determined by the vapor pressure osmometer method in dioxane was 799. The reductive condensation of aklavinone to a dimer could account for the discrepancy between the molecular ion peak and the molecular weight determination, and its structure is proposed by PMR and ^{13}C-NMR as shown in Fig. 1.

Thin-layer chromatography of the acid hydrolysates indicated that: components A1, B1, A2 and B2 possess three sugars corresponding to rhodosamine, 2-deoxyfucose and cinerulose; that M1 and M2 contain rhodosamine, 2-deoxyfucose and amicetose; that N1 possess rhodosamine, 2-deoxyfucose and rhodinose; and L1 possesses N-methyldaunosamine (N-monodemethyl rhodosamine), 2-deoxyfucose and cinerulose. For the identification of sugars, authentic samples of cinerubin A[6], cinerubin B[7], streptolydigin[8] and amicetin[8] were hydrolyzed and hydrolysate sugars were employed for comparison.

Table 3. Antimicrobial spectrum of anthracyclic glycosides

Compounds	Minimum inhibitory concentrations (μg/ml)									
	(1)*	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Aclacinomycin A	3.12	0.78	0.78	3.12	0.2	0.2	0.4	1.56	12.5	50
Aclacinomycin B	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	5.0	10	20
N-Monodemethyl aclacinomycin A	25	6.25	3.12	12.5	0.78	1.56	1.56	12.5	100	100
MA144 G1	6.25	3.12	1.56	6.25	0.78	0.2	1.56	3.12	100	100
MA144 M1	3.12	1.56	0.78	3.12	0.2	0.4	0.4	3.12	50	100
MA144 N1	6.25	3.12	0.78	3.12	0.78	0.4	0.78	>100	>100	>100
MA144 S1	6.25	3.12	1.56	3.12	1.56	1.56	0.78	>100	>100	>100
1-Deoxypyrromycin	5.0	1.25	1.25	—	1.25	1.25	0.63	—	40	40
1-Hydroxyaclacinomycin A	0.31	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	1.25	2.5	5
1-Hydroxyaclacinomycin B	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	1.25	5	10
1-Hydroxy-MA144 M1	0.78	<0.1	<0.1	0.78	<0.1	—	0.1	1.25	50	100
1-Hydroxy-MA144 S1	6.25	3.1	0.78	3.1	1.56	0.78	0.78	>100	>100	>100

* Organisms: (1) *Staphylococcus aureus* FDA209P, (2) *Bacillus subtilis* ATCC6633, (3) *Bacillus cereus* ATCC9634, (4) *Bacillus megaterium* NRRLB-938, (5) *Sarcina lutea* ATCC9341, (6) *Micrococcus flavus*, (7) *Corynebacterium bovis* 1810, (8) *Mycobacterium smegmatis* ATCC607, (9) *Candida albicans* IAM4905, (10) *Candida tropicalis* IMA4942

T1 and T2 hydrolysates contained a simple sugar corresponding to rhodosamine; however, S1 and S2 hydrolysates gave two spots corresponding to rhodosamine and 2-deoxyfucose. A1, B1 and T1 were found to be identical with aclacinomycins A and B and 1-deoxypyrromycin, respectively. A2, B2 and T2, on the other hand, were identical with cinerubins A and B and pyrromycin,[5] respectively.

Methanolysis of the components A1, A2, L1, B1, B2, G1 and M1 (treating with methanol containing 0.01~0.04 N HCl for 30~60 minutes at room temperature) gave 1-deoxypyrromycin, N-monodemethyl-1-deoxypyrromycin or pyrromycin and their methylated disaccharides: 1-O-methyl-2-deoxy-L-fucose-L-cinerulose A from A1, A2 and L1, 1-O-methyl-2-deoxy-L-fucose-L-cinerulose B from B1 and B2, 1-O-methyl-2-deoxy-L-fucose-D-cinerulose A from G1 and 1-O-methyl-2-deoxy-L-fucose-L-amicetose from M1 and M2. On acid hydrolysis in 0.5% HCl for 10 minutes at room temperature, N1 gave S1 and L-rhodinose.

The results of acid hydrolyses of the components and their proposed structures are summarized in Table 2. Details of the structural determination of these compounds will be reported elsewhere.

It is of interest to point out that the red pigments produced by strain MA144-M1 are anthracyclic compounds containing the 1-hydroxyl group, and that the anthracyclic glycosides of this group and their aglycones can be precipitated by addition of cupric sulfate as described above. By contrast the yellow pigments elaborated by this strain lack the 1-hydroxyl group, and the anthracyclic glycosides of this group and their aglycones are not precipitated by cupric sulfate. Among 19 anthracyclic compounds described above, aclacinomycins A and B, N-monodemethylaclacinomycin A, MA144 G1, MA144 M1, MA144 N1, MA144 S1, MA144 E1, 1-hydroxy-MA144 M1 and 1-hydroxy-MA144 S1 have been found for the first time in the culture broth of the strain MA144-M1.

The antimicrobial activities of the anthracyclic compounds containing sugar moieties are shown in Table 3. These anthracyclic glycosides exhibit marked activity against Gram-positive bacteria and *Mycobacterium smegmatis*. Moreover, aclacinomycins A and B, cinerubins A and B and 1-deoxypyrromycin showed moderate activity against *Candida*. The anthracyclic glycosides except for N-monodemethylaclacinomycin A possess marked activity against L1210 leukemia in BDF_1 mice at the dose of 0.5~2 mg/kg once daily for days 1~9.

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References

1) Oki, T.; Y. Matsuzawa, A. Yoshimoto, K. Numata, I. Kitamura, S. Hori, A. Takamatsu, H. Umezawa, M. Ishizuka, H. Naganawa, H. Suda, M. Hamada & T. Takeuchi: New antitumor antibiotics, aclacinomycins A and B. J. Antibiotics 28: 830~834, 1975
2) Gordon, J. J.: Aklavinone. Tetrahedron Lett. 1960: 28~34, 1960
3) Brockmann, Jr., H.; H. Budzikiewicz, C. Djerssi, H. Brockmann & J. Niemeyer: Das massenspektroskopische Fragmentierungsverhalten der Anthracyclinone. Chem. Ber. 98: 1260~1269, 1965
4) Eckardt, K.; D. Tresselt & J. Tax: Antibiotika aus Actinomyceten. Isolierung und Konstitution von Aklavinon-I und Aklavinon-II, zwei neuen Anthracyclinonen aus Fermentationsansätzen von *Streptomyces galilaeus*. Tetrahedron 30: 3787~3791, 1974
5) Brockmann, H. & W. Lenk: Actinomycetenfarbstoffe. VII. Pyrromycin. Chem. Ber. 92: 1904~1909, 1959
6) Keller-Schierlein, W. & W. Richle: Metabolic products of microorganisms. LXXXVI.

Structure of cinerubine A. Antimicr. Agents & Chemoth.-1970: 68~77, 1971

7) RICHLE, W.; E. K. WINKLER, D. M. HAWLEY, M. DOBLER & W. KELLER-SCHIERLEIN: Stoffwechselprodukte von Microorganismen. 49. Die Struktur des Cinerubins B. Helv. Chim. Acta 55: 48~49, 1972

8) RINEHART, Jr., K. L.; J. R. BECK, D. B. BORDERS & D. KRAUSS: Streptolydigin. III. Chromophore and structure. J. Amer. Chem. Soc. 85: 4038~4039, 1963

9) STEVENS, C. L.; K. NAGARAJAN & T. H. HANSKELL: The structure of amicetin. J. Org. Chem. 27: 2991~3005, 1962