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A New Actinomycin Complex Produced by a *Micromonospora* Species: Fermentation, Isolation, and Characterization

G. H WAGMAN,* J. A. MARQUEZ, P. D. WATKINS,[1] F. GENTILE, A. MURAWSKI, M. PATEL, AND M. J. WEINSTEIN

Microbiological Sciences, Schering Corporation, Bloomfield, New Jersey 07003

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A species of *Micromonospora, Micromonospora floridensis* NRRL 8020, has been found to produce an actinomycin complex consisting of at least 25 active components. After solvent extraction of the complex, separation of the individual components was carried out by preparative thin-layer chromatography. Hydrolysis and subsequent electrophoretic and chromatographic identification of the amino acid content of each of the isolated components have shown differences from known actinomycins, and the probability exists that these contain a number of amino or imino acids not previously found in other members of this group of antibiotics.

Numerous actinomycins have been reported to be produced by various strains of *Streptomyces* since the initial discovery by Waksman and Woodruff of the production of actinomycin A 35 years ago (10). Since that time the only account of an actinomycin-producing *Micromonospora* was that in 1951 by Fisher et al. (3) who reported on an organism that closely resembled *Micromonospora globosa* and produced actinomycin A; however, the strain is no longer available for comparative purposes.

This report concerns the production of a novel actinomycin complex that is produced by a species of *Micromonospora, Micromonospora floridensis*. Details of the fermentation procedure, isolation of the components and their preliminary characterization, and some biological characteristics are described.

MATERIALS AND METHODS

Producing organism. The organism that produces the antibiotic complex was isolated from a soil sample obtained in Fort Lauderdale, Fla., belongs to the genus *Micromonospora* and was named *M. floridensis*. A culture of *M. floridensis* has been deposited at the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill., where it has been assigned the designation NRRL 8020. Detailed taxonomic studies relating to this organism will be published elsewhere. *M. floridensis* can be distinguished from the actinomycin-producing culture reported to be similar to *M. globosa* by spore shape and size. Furthermore, *M. globosa* is reported to be colorless; *M. floridensis* produces a yellow-orange diffusible pigment. *M. globosa* has slender mycelia 0.7 μm in diameter with spherical conidia 1.4 μm in diameter borne singly on short condiophores in grapelike clusters. *M. floridensis* has much thinner mycelia ranging from 0.4 to 0.6 μm in diameter, and the spores are borne predominately on sympodially branched sporophores. Also, it will be demonstrated that the actinomycins produced by *M. floridensis* are novel.

[1] Present address: Hoffman-La Roche, Inc., Nutley, N.J.

Fermentation conditions. *M. floridensis* produces substantial quantities of an antibiotic complex when cultivated under submerged aerobic conditions. The fermentation is carried out with one or more germination stages followed by a fermentation stage. As a general rule, flask fermentations utilize a single germination stage, whereas tank fermentations utilize two germination stages.

For shake-flask antibiotic production by *M. floridensis* a 3-ml portion of frozen whole broth is added to 70 ml of a germination medium (Table 1) contained in a 300-ml Erlenmeyer flask and incubated at 28 C on a rotary shaker at 250 to 300 rpm for 72 h. A 5% transfer of the germination stage is made to another 300-ml flask containing the same medium and incubateed under the same conditions as for the germination; this is the inoculum stage.

Five milliliters of inoculum is transferred to 100 ml of the fermentation medium shown in Table 1

TABLE 1. *Media for growth of M. floridensis for production of actinomycin complex*

Germination medium[a]		Fermentation medium	
Component	Quantity (g)	Component	Quantity (g)
Potato starch	24	Soluble starch	20
Tryptone	5	Dextrose	10
Yeast extract	5	Yeast extract	8.75
Beef extract	3	NZ-amine	5
$CaCO_3$	2	$CaCO_3$	4
Dextrose	1	Water (tap)	1,000[b]
Water (tap)	1,000[b]		

[a] Adjust to pH 7.5 with NaOH before sterilizing.
[b] Given in milliliters.

contained in a 500-ml Erlenmeyer flask. The culture is incubated on a rotary shaker at 28 C for 96 h.

For tank fermentations of *M. floridensis* the germination stage is carried out as for the flask fermentations. Inoculum is prepared by transferring 25 ml of the germination stage to 500 ml of germination medium in a 2-liter Erlenmeyer flask and incubated on a rotary shaker at 28 C for 72 h.

Inoculum (500 ml) is added to 9.5 liters of fermentation medium contained in 14-liter New Brunswick fermenters. The tanks are agitated at 400 rpm, and air is introduced at 4.0 liters/min. The fermentation is allowed to proceed for 72 to 96 h at 28 to 30 C.

Microbiological assay. The relative antibiotic activities were determined by tube dilution assays against *Staphylococcus aureus* ATCC 6538P, the test organism, in Mueller-Hinton broth at pH 7.4 for 18 to 24 h.

Antibiotic isolation. To obtain the antibiotic complex, the harvested fermentation broth was adjusted to about pH 6.5 and extracted with 2 volumes of ethyl acetate. The spent broth was again extracted with 2 volumes of ethyl acetate, and the extracts were combined and concentrated to dryness under vacuum. This extract was redissolved in methylene chloride, dried over anhydrous sodium sulfate, and finally precipitated with hexane. An orange-red precipitate formed which was washed with hexane and dried under vacuum.

Separation of components. The antibiotic complex was separated into 11 fractions by preparative thin-layer chromatography on Silica Gel GF plates (500 μm in thickness), using the lower phase of a solvent system composed of chloroform-methanol-water in a ratio of 2:1:1. The fractions were detected by observation of the color of the spots or bands, which are orange-yellow to orange, or by bioautography against *S. aureus* ATCC 6358P. The fractions were designated 1 to 11 based upon their respective R_f values (Table 2), with fraction 1 being most dis-

TABLE 2. *R_f values of actinomycin fractions*[a]

Fraction	R_f
1	0.70
2	0.68
3	0.65
4	0.64
5	0.62
6	0.48
7	0.44
8	0.42
9	0.40
10	0.37
11	0.10

[a] The medium used was Silica Gel G. The solvent system consisted of chloroform-methanol-water (2:1:1).

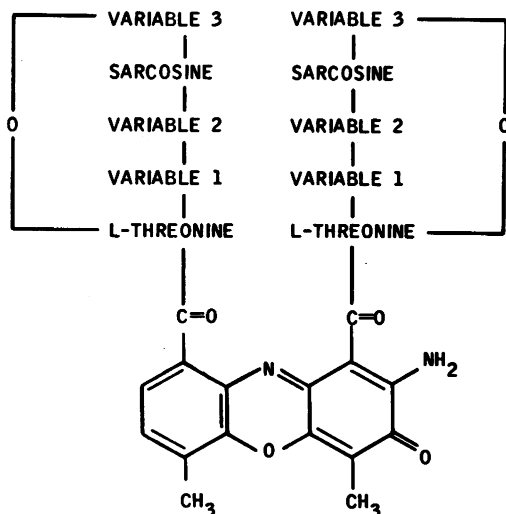

FIG. 1. *General structure of actinomycin.*

TABLE 3. *Known amino acids contained in actinomycin components*

Component	Sarcosine, valine, and threonine	N-methyl alanine	Proline	N-methyl valine
1				
A	+	+	−	+
B	+	−	−	+
C	+	−	−	+
D	+	−	−	+
2				
A	+	+	−	+
B	+	+	−	+
C	+	+	+	+
D	+	+	+	+
3				
A	+	+	+	+
B	+	+	+	+
C	+	+	+	+
4				
A	+	+	+	+
B	+	−	+	+
C	+	−	−	+
5				
A	+	+	+	−
B	+	+	+	+
C	+	+	−	−
6				
A	+	+	−	+
B	+	+	−	+
C	+	+	−	+
7	+	+	+	+
8	+	−	−	+
9	+	−	−	+
10	+	−	−	+
11	+	−	−	+

TABLE 4. *High-voltage electrophoresis and paper chromatographic analysis of hydrolyzed actinomycin components*[a]

Zone identification	HVE (R_{sarc})	PC (R_f)	1A		1B		1C		1D		2A		2B		2C		2D		3A		3B		3C		4A		4B		4C		5A		5B		5C		6A		6B		6C		7	
			HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC	HVE	PC
Purple spot	1.62	0.10																	+	+																								
Purple spot	1.42	0.37															+	+																										
Purple spot	1.24	0.24																									+	+																
Purple spot	1.08	0.30																									+	+													+	+	+	+
Sarcosine	1.00	0.26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Yellow spot	0.91																								+																			
Valine	0.88	0.46	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Purple spot	0.89	0.21																	+	+							+	+																
N-methyl alanine	0.85	0.31			+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pink spot	0.84	0.63							+	+					+	+	+	+					+	+							+	+	+	+					+	+			+	+
Threonine	0.82	0.24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Proline	0.80	0.34													+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+									+	+
Purple spot	0.74	0.21													+	+												+(0.31)																
Yellow spot[b]	0.74	0.44							+	+															+	+	+	+	+	+							+	+	+	+	+	+	+	+
Yellow spot[b]	0.71	0.28	+	+	+	+					+	+	+	+					+	+	+	+							+	+									+	+	+	+	+	+
Purple spot	0.73	0.59															+	+									+	+	+	+	+	+		+	+	+								
N-methyl valine	0.70	0.56	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+			+	+	+	+	+	+	+	+
Purple spot	0.69	0.23																																			+	+						
Yellow spot	0.65	0.23																																									+	+
Pink spot		0.29																																								+		
Pink spot	0.60	0.38									+	+																																
Pink spot	0.58	0.31																											+	+														
Yellow spot[b]	0.48	0.34					+	+	+	+	+				+	+	+	+					+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Abbreviations: HVE, high-voltage electrophoresis; PC, paper chromatography.
[b] Imino acids.

tant from the origin and fraction 11 being closest to the origin. The fractions were physically separated from each other by scraping the bands from the plates, extracting with acetone, concentrating the extracts, and again subjecting each concentrate to chromatography on silica gel using the same solvent system, thereby obtaining each fraction in substantially pure form.

The fractions were further separated into the individual antibiotic components by preparative thin-layer chromatography on silica gel using a solvent mixture composed of methylene chloride-acetone (7:3) as the developer. The individual components of each fraction were detected by the appearance of colored bands, and the antibiotics were isolated by extracting the individual bands from the plates with acetone.

Determination of hydrolysis products of antibiotics. A sample of each of the antibiotic components was subjected to hydrolysis in screw-capped tubes containing 6 N hydrochloric acid for 3 h at 120 C. After hydrolysis, the individual components were subjected to high-voltage electrophoresis at 4,800 V for 3 h in 4% sodium formate buffered at pH 1.9 with formic acid, using Whatman 3MM paper. This procedure was followed by ascending paper chromatography in a direction 90° from that of the electrophoresis, using a solvent mixture consisting of butanol-acetic acid-water (4:1:2). Detection for both procedures was effected by spraying with 0.2% ninhydrin in acetone. The location of the ninhydrin-positive spots after the electrophoresis procedure was determined relative to that of sarcosine (1.00), and the location of the spots after chromatography was determined relative to the solvent front (R_f). Thus, the hydrolysis products from each actinomycin could be mapped.

In vitro studies. For determination of in vitro susceptibility, all test organisms were incubated in Mueller-Hinton broth (pH 7.4) at 37 C for 18 to 24 h, except when otherwise indicated. The volume in each tube was 3 ml, and the inoculum was 0.05 ml of a 1:1,000 dilution of an 18-h broth culture.

In vivo studies. Animal studies were carried out in CF-1 male albino mice weighing approximately 20 g each. Mean-lethal-dose values were determined by probit procedures. Generally, groups of seven mice per dosage level were used.

RESULTS AND DISCUSSION

By means of preparative thin-layer chromatography it was found that the major fractions could be further subdivided into 25 components. Fractions 1 and 2 contained four components each, designated A through D. Fractions 3 through 6 contained three components each, which were designated A through C. Fractions 7 through 11 appeared to contain only single components.

Based on chromatography and ultraviolet and infrared analysis and further confirmed by amino acid analysis, all components were shown to be members of the actinomycin family of antibiotics.

The actinomycins represent a comprehensive class of antibiotics; they share a common phenoxazine nucleus to which are attached a number of amino or imino acids (Fig. 1). The designations 1, 2, and 3 are amino or imino acids such as *N*-methyl valine, proline, valine, or isoleucine, for example.

The antibiotics in this complex have been distinguished from the previously known actinomycins by comparison of their hydrolysis products with those of the known compounds (1, 2, 4-9). The components have as constituent parts both known amino and imino acids as well as amino and imino acids that have not previously been shown to be constituents of actinomycin antibiotics. The acids whose identities have not been unequivocally established were designated by their respective locations after high-voltage electrophoresis and paper chromatography of hydrolysates of the respec-

TABLE 5. *Relative activities of actinomycin components*

Component	Activity[a]
1	
A	+++
B	+++
C	+++
D	+++
2	
A	+++
B	+++
C	+++
D	+++
3	
A	+++
B	+++
C	+++
4	
A	+++
B	+++
C	+
5	
A	+
B	+++
C	+++
6	
A	+++
B	+++
C	+
7	++
8	++
9	++
10	++
11	++

[a] Tested against *S. aureus* 6538P.

TABLE 6. *In vitro activity of the actinomycin complex and fractions 5 and 6 in comparison with actinomycin D*

Organism	Complex	MIC (μg/ml)[a]		
		Fraction 5	Fraction 6	Actinomycin D
Staphylococcus aureus				
209P	0.3	0.1	0.3	0.1
12	0.3	0.1	0.3	0.1
Streptococcus pyogenes C	0.1	0.1	0.3	0.1
Escherichia coli Sc	>10	>10	>10	>10
Pseudomonas aeruginosa Sc	3.0	3.0	3.0	3.0
Candida albicans	0.1	0.1	0.1	0.1
Trichophyton mentagrophytes	>10	>10	>10	>10

[a] Mueller-Hinton broth at pH 7.4. MIC, Minimal inhibitory concentration.

TABLE 7. *Acute toxicity of actinomycin complex and fractions 5 and 6 in comparison to actinomycin D in mice*

Route	LD_{50} (mg/kg)[a]			
	Complex	Fraction 5	Fraction 6	Actinomycin D
Intraperitoneal	3.5	0.5	2.8	1.2
Subcutaneous	6.5	1.2	2.8	1.8

[a] LD_{50}, Mean lethal dose.

tive components and are presently undergoing identification.

The preliminary data indicate the known amino acids to be found (Table 3). All of the components contain sarcosine, valine, and threonine. Components 1A through 4A and 5A through 7 also have *N*-methyl alanine as a constituent. Components 2C through 4B, 5A, 5B, and 7 contain proline. *N*-methyl valine may be isolated from all components, except components 5A and 5C. a detailed analysis of electrophoretic and chromatographic data on the hydrolysis products of components 1 to 7 is presented in Table 4. Data for components 8 to 11 are not complete at this time.

The relative activities of the individual components against *S. aureus* are shown in Table 5. It can be seen that all components are active and that those in groups 1 through 3 as well as several others had the highest level of activity. On the basis of limited assay data available on two fractions (5 and 6) and the complex, they appear to have the same potency and antibacterial spectrum as does actinomycin D. This comparison is illustrated in Table 6.

The acute toxicities of the same compounds are illustrated in Table 7 by both the intraperitoneal and subcutaneous routes. These appear to have a potential of lowered toxicity, as seen with the complex and one of the two fractions (6) tested, as compared to actinomycin D. Should this hold true for some of the individual components, and if the antitumor activities are of a nature comparable to or better than actinomycin D, further studies are certainly warranted.

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