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Production of the Monamycins, Novel Depsipeptide Antibiotics

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Methods are described for the production of the monamycins by *Streptomyces jamaicensis* in shake flasks and jar fermentors. The effects on the fermentation of variations in *p*H, temperature, medium composition, volume of inoculum, and strain of the organism are discussed. The methods employed for the extraction and for the microbiological assay of the antibiotics are outlined.

As a result of an extensive screening program, Meredith (6) isolated in 1943 a variety of soil microorganisms which were antagonistic to *Fusarium oxysporum* var. *cubense*, the causative agent of the Panama disease of bananas. Reexamination of the most active of Meredith's cultures led to the isolation of a new species, *Streptomyces jamaicensis*, with marked antibacterial activity. A small quantity of a crystalline antibacterial preparation, which was named monamycin, was isolated from both stationary and submerged cultures of this organism (2).

This paper describes the investigation of factors influencing the production of the monamycins in submerged culture.

MATERIALS AND METHODS

Culture. *Streptomyces jamaicensis* was isolated by Hassall and Wong (*unpublished data*) from a mixed culture supplied by Meredith. It was incubated at 30 C on agar slants [0.6% Neopeptone (Difco), 5% glucose monohydrate, 1.2% agar (Oxoid, no. 3) in tap water] for 10 days, during which time it produced a dark green-to-black soluble pigment. Taxonomic description of *S. jamaicensis* will be reported elsewhere (M. J. Hall, *in preparation*).

Preparation of inoculum. A slope was flooded with water (10 ml), and the mycelium was gently scraped off with a wire loop (4 mm) to give a turbid suspension. The latter was transferred to a 250-ml Erlenmeyer flask containing 100 ml of a seed medium, medium "S," consisting of 5% glucose monohydrate, 4% skim milk (Cadbury's Marvel), 0.2% Na_2SO_4, 0.2% $CaCO_3$, and 0.1% peptone P (Oxoid) in tap water. The flasks were incubated on a rotary shaker at 30 C for 4 days by which time the organism had undergone extensive mycelial fragmentation. Estimations of the fragment count were made with a Thoma hemocytometer (Cristalite, Hawksley Ltd., London, England). The inoculum used for the fermentation media (usually 1 to 4% by volume) was varied to give a final fragment count of 500,000 per ml in the fermentation flask or vessel.

Cultivation in shaken flasks. The fermentation medium, medium "F," which contained 0.2% Neopeptone (Difco), 5% glucose monohydrate, 0.2% $CaCO_3$, and 0.002% Na_2HPO_4, was dispensed into 250-ml Erlenmeyer flasks (55 ml/flask) which were inoculated and incubated on an orbital shaker (200 rev/min, 5-cm diameter throw) at 26 C. Optimal yields were achieved at 48 to 55 hr, the titer dropping to a low value by 80 to 90 hr.

Cultivation in 5-liter and 100-liter stirred jar fermentors. Five-liter vessels (model F-07; New Brunswick Scientific Co., New Brunswick, N.J.) with peripheral equipment for the control of temperature (±0.5 C), air flow, foaming, and agitation were employed. The optimal temperature, inoculum level, and medium composition were found to be the same as those for shake flasks. Low rates of agitation and aeration were favored (200 rev/min, 0.25 liter of air per liter of broth per min). Maximal yields of mycelium and of monamycin were obtained during 48 to 60 hr. Foaming was controlled with RD Emulsion (Midland Silicones) as a 30% aqueous suspension, added manually.

Production of monamycin in a 100-liter fermentor (New Brunswick "Fermacell") utilized similar conditions, but optimal yields were obtained with a lower agitation rate (150 rev/min) and by harvesting between 39 and 43 hr.

Antibiotic assay procedure. The titers of antibiotic were determined by a disc-plate agar diffusion assay. A 150-ml amount of sterility test agar [1% glucose, 0.5% Neopeptone, 0.3% laboratory Lemco (Oxoid), 0.5% NaCl, and 1.2% agar (Oxoid no. 3)] was melted, cooled to 48 C, and inoculated with 1 ml of a log-phase suspension of *Staphylococcus aureus* (NCTC 6571), containing 6 mg (dry weight) of cells/ml. This was poured into a carefully levelled glass plate (30 by 30 cm) to a depth of 0.2 cm. Samples (in ethyl alcohol, 0.075 ml) were applied to antibiotic assay discs (Whatman, 13 mm), dried, and placed on the agar surface in an 8 by 8 quasi-Latin square layout (5). A

high (50 μg/ml) and a low standard (10 μg/ml) were included on all plates. Zones of inhibition were measured after incubation at 37 C for 18 hr. The curve obtained by plotting micrograms per milliliter of crystalline monamycin against the difference between the square of the zone diameter and the square of the disc diameter was linear over the range 0 to 50 μg/ml.

Mutation studies. Mutation for higher-yielding strains was investigated by ultraviolet (UV) irradiation of both mycelia fragments and spore suspensions. The suspension in sterile water (10 ml) was placed in a 9-cm petri dish at a distance of 30 cm from a "Chromatolite" 25-w, UV lamp (Hanovia Ltd.), with filters to pass light at 253.7 nm. Samples were withdrawn at either 1 or 0.1% survival times (previously determined), and appropriate dilutions were plated on petri dishes containing medium "F" to which 1.2% agar had been added. After incubation at 30 C for 5 to 7 days, random colonies were selected and transferred to slants of the same medium. They were incubated until good growth developed, macerated in sterile water, and used as seed for shake-flask cultures as previously described. Antibiotic titers were determined at 36, 42, and 48 hr.

Isolation of monamycin. As a result of exploratory experiments involving a variety of alternative procedures, the following was adopted routinely for the isolation of monamycin (R. B. Morton, *personal communication*). In the case of small volumes (e.g., shake-flask cultures), the broth was adjusted to *p*H 6.5 to 7.5 and shaken with an equal volume of ether. The ether extract was dried (anhydrous sodium sulfate) and evaporated to give crude material. This material was redissolved for assay in ethyl alcohol. For larger-scale extraction (100 liters), it was more convenient to use light petroleum (boiling point 40 to 60 C) in a continuous liquid-liquid extractor (Q.V.F. Ltd.; three 33-liter batches for 12 hr each). The oil obtained by evaporating the solvent was dissolved in chloroform (20 ml), diluted with pentane (200 ml), and filtered to obtain crude crystalline monamycin.

Thin-layer chromatography. Glass plates (20 by 20 cm), coated with Kieselgel G (E. Merck A. G., Darmstadt), were activated before use by heating at 120 C for 1 hr. Samples applied in 10 to 30 μliters of solvent at 2-cm intervals were eluted by the ascending technique with the solvent system chloroform-methanol (20:1). The monamycin was located on the plates (R_F approximately 0.4 to 0.6) by spraying with a solution of iodine (2 g/100 ml of methanol-water, 50:1) or, alternatively, by scraping sections (0.5 by 2 cm) and applying the Kieselgel to small wells (7-mm diameter) in the agar medium of an assay plate seeded with *S. aureus*. The diameters of zones of inhibition were measured after incubating the plates at 37 C overnight.

RESULTS AND DISCUSSION

Since earlier experiments had led to the isolation of only a small amount of monamycin, it was necessary to increase the yields very substantially if significant quantities of the antibiotic were to become available for further studies of its chemical and biological properties. We, therefore, investigated the influence of the inoculum, the fermentation medium, the cultivation conditions (aeration, agitation, *p*H, and temperature), and the time of harvesting on the yield of monamycin.

The absence of abundant sporulation necessitated the use of a vegetative inoculum. Sparse sporulation does occur on a synthetic medium containing xylose as carbon source but is lost after subculturing on richer media which favor the growth of *S. jamaicensis*. Attempts to use a two-stage fermentation employing the medium "F" in both stages produced low antibiotic titers, and an alternative medium had to be developed. The extensive mycelial fragmentation observed in some complex media after 4 days of incubation was used as a basis for the development of medium "S," which produced from 5×10^7 to 12×10^7 fragments per ml with a viability of 90 to 95%. The volume of inoculum was found to have less effect on titer than variation in the total number of mycelial fragments added (Table 1). The optimum fragment count in medium "F" was approximately 500,000/ml.

Glucose was the most suitable carbon source for the fermentation medium, whereas all others tested (mannitol, D-galactose, sucrose, dextrin, and starch) produced much reduced yields. Likewise, Neopeptone could not be replaced by any similar product. Tryptone T, peptone P, and bacteriological peptone (all Oxoid products) and Evans bacteriological peptone produced low activity. Only oxoid peptone L6 approached 85% yields of neopeptone. The use of the complex ingredients, corn steep liquor, soybean meal, distillers solubles, and Pharmamedia, as substi-

TABLE 1. *Effect of fragment count of the inoculum (strain RG) on the maximum titer of monamycin in "F" medium in shake flasks*

Sterile seed medium added	Seed medium with mycelial fragments	Fragments[a]	Antibiotic at 48 hr[b]
ml	*ml*		
4	0.001	2.5×10^3	32
3.99	0.01	2.5×10^4	56
3.9	0.1	2.5×10^5	64
3.8	0.2	5×10^5	86
3.6	0.4	10^6	81
3.0	1.0	2.5×10^6	64
0	4.0	10×10^6	59

[a] Expressed per milliliter of fermentation medium.

[b] Titers are expressed in micrograms per milliliter.

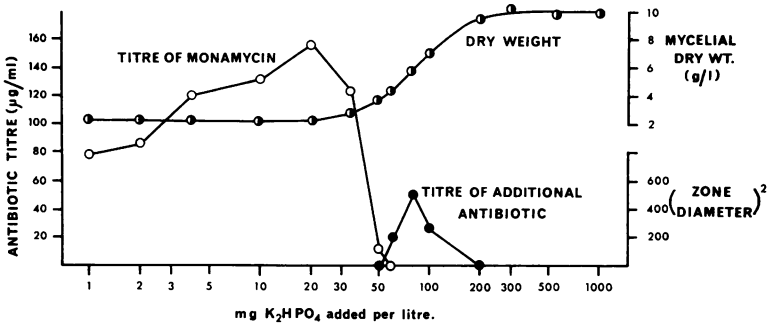

FIG. 1. *Effect of varying concentration of K_2HPO_4 in medium "F" on the titer of monamycin, the production of a second antibiotic activity, and on the mycelial dry weight of Streptomyces jamaicensis.*

tutes for or supplements to Neopeptone resulted in complete suppression of monamycin production.

The importance of the ratio of Neopeptone to glucose in morphological changes, such as the induction of sporulation (7), and in stimulating the production of secondary metabolites is well known (10, 11; A. Rhodes et al., British Patent 784618, 1955). The importance of this ratio to the production of monamycin is indicated in Table 2. A glucose concentration of 25 g/liter and a corresponding reduction of Neopeptone to 1 g/liter did produce a titer almost equal to that for the standard medium "F," but the fermentation was less reproducible. Further experiments, in which the favored ratio of Neopeptone to glucose was maintained but the concentration of glucose was varied, did not lead to improvements in the yield of monamycin.

Of the 22 common amino acids tested as sole nitrogen sources, lysine, proline, asparagine, alanine, and threonine gave the best yields of monamycin. A synthetic medium (DL-α-alanine, 0.9 g; urea, 0.3 g; glucose, 50 g; $CaCO_3$, 2 g; Na_2HPO_4, 20 mg; and tap water, 1 liter) was subsequently developed which gave titers approximately 60% of those obtained with medium "F."

The addition of trace metals and vitamins had no effect on antibiotic titer. The inclusion of calcium carbonate as a buffer maintained the optimal *p*H range, 7 to 8, increased the mean titer by 20 to 30%, and facilitated the reproducibility of the fermentations.

The yield of monamycin is related to the concentration of inorganic phosphate in the fermentation medium containing Neopeptone and glucose. The optimal concentration of added disodium hydrogen phosphate was 20 mg/liter, but monamycin biosynthesis was completely inhibited when levels of 400 to 500 mg/liter were reached. The beneficial effects of phosphates have been observed in various fermentations: 1 to 4 g/liter improved the yields of griseofulvin (D. J. D. Hockenhull, British Patent 868958, 1959), penicillin (3), erythromycin (11), and novobiocin (4). The production of streptomycin, under certain conditions, was also enhanced by additions of up to 0.2 g/liter of diammonium hydrogen phos-

TABLE 2. *Effect of ratio of Neopeptone to glucose (N:C) of "F" medium on the production of monamycin in shake-flask culture (strain RG)*

Time of harvesting	N:C ratio[a]				
	1:50	2:50	4:50	6:50	8:50
hr					
36	48[b]	66	48	15	0
48	64	80	66	16	0
72	52	64	46	17	0

[a] Ratios are expressed in grams per liter.
[b] Values are expressed in micrograms of monamycin per milliliter.

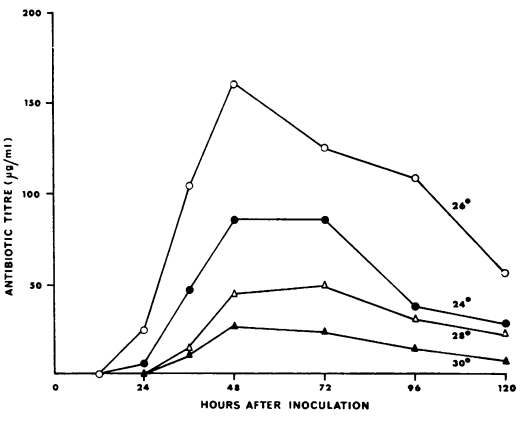

FIG. 2. *Production of monamycin by Streptomyces jamaicensis in medium "F" incubated at various temperatures.*

phate, but higher concentrations inhibited biosynthesis (1, 12). Evidently, monamycin production was exceptionally sensitive to phosphate ion concentration. The inhibition of monamycin production by increasing concentrations of phosphate was paralleled by an increase in the dry weight of mycelium and, at higher concentrations, by a co-production of a second antibiotic, which does not belong to the monamycin family (Fig. 1).

Another unusual feature became apparent in the course of these studies. Potassium salts had a marked effect on the antibiotic titers. The diameters of zones of inhibition were reduced when there were potassium (but not sodium) salts in the agar medium. The reduction, which was virtually complete at 5 g of potassium dihydrogen phosphate per liter for 350 μg of monamycin/ml, was proportional to the potassium salt concentration at lower levels. The addition of potassium salts to the fermentation media resulted in marked reduction in the antibiotic titers, but the effect was not so marked with sodium salts. We attribute this phenomenon to the formation of a complex between monamycin and the potassium ion, as has been observed for valinomycin (8) and other cyclodepsipeptides. This is supported by the observation that monamycin forms very insoluble complexes with potassium, rubidium, and cesium salts on admixture in aqueous ethyl alcohol (M. J. Hall, *in preparation*), but not with sodium or lithium.

A systematic study of other fermentation variables such as temperature (Fig. 2), duration of fermentation, and agitation and aeration rates resulted in the definition of optimal conditions.

A further substantial increase in antibiotic titer was achieved by isolating a strain obtained from a suspension of mycelial fragments from the parent strain irradiated with UV light. Of the 280 survivors tested, only two produced more monamycin, one of which, FLM-1, yielded an increase of 100% and was adopted for routine production of the antibiotic. It differed morphologically from the parent in that the slopes had a moderate covering of short aerial mycelium and produced considerably greater quantities of foam in the stirred fermentation vessels. This latter effect was easily controlled by using a silicone antifoam; the other antifoam agents that were investigated (groundnut oil, octadecanol, and various lard oil preparations) markedly depressed the yield.

At all stages during the development of the fermentation and extraction procedure, the antibiotic activity was due to monamycin, as determined by isolation and thin-layer chromatography, and not to the other, as yet, uncharacterized antibiotic which is formed on prolonged fermentation.

The result of the work reported above has been to increase the monamycin yield from 3 to 5 μg/ml at the outset, to 160 to 170 μg/ml at the conclusion. With these yields and the efficient extraction procedure, it was possible to obtain large quantities of these compounds for study of their chemical and biological properties. These chemical investigations have established that the crystalline monamycin which was obtained in this investigation consists of a complex mixture of novel cyclohexadepsipeptides (K. Bevan et al., *in press*).

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